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Review

Nrf2 Pathway in Age-Related Neurological Disorders: Insights into MicroRNAs

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Abstract
A general hallmark of neurological diseases is the loss of redox homeostasis that triggers oxidative damages to biomolecules compromising neuronal function. Under physiological conditions the steady-state concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are finely regulated for proper cellular functions. Reduced surveillance of endogenous antioxidant defenses and/or increased ROS/RNS production leads to oxidative stress with consequent alteration of physiological processes. Neuronal cells are particularly susceptible to ROS/RNS due to their biochemical composition. Overwhelming evidences indicate that nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-linked pathways are involved in protective mechanisms against oxidative stress by regulating antioxidant and phase II detoxifying genes. As such, Nrf2 deregulation has been linked to both aging and pathogenesis of many human chronic diseases, including neurodegenerative ones such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. Nrf2 activity is tightly regulated by a fine balance between positive and negative modulators. A better understanding of the regulatory mechanisms underlying Nrf2 activity could help to develop novel therapeutic interventions to prevent, slow down or possibly reverse various pathological states. To this end, microRNAs (miRs) are attractive candidates because they are linked to intracellular redox status being regulated and, post-transcriptionally, regulating key components of ROS/RNS pathways, including Nrf2.

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) pervade a wide spectrum of biological processes; therefore, their levels are tightly regulated under normal

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physiological conditions. At low concentrations, some ROS/RNS function as important components of numerous signalling pathways. Conversely, increased ROS/RNS levels can generate damage to nucleic acids, lipids and proteins, thereby contributing to cellular and tissue dysfunctions [1-3]. This latter condition, known as oxidative stress, may be created in a number of ways involving both intrinsic cellular factors (i.e., enhanced activity of ROS/RNS-generating enzymes and/or reduced capacity of the antioxidant/detoxifying systems) and extrinsic insults (i.e., exposure to environmental agents such as xenobiotics, radiations, drugs) [3, 4]. To avoid harmful effects of ROS/RNS, cells must promptly respond to their deleterious increase. Functionally, the main driver of the cellular antioxidant/detoxifying responses is the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that induces the expression of a wide range of cytoprotective genes [5-7]. Several works indicate that an optimal activity of Nrf2 is essential to protect cells against different stressors, and that its dysfunction is correlated with decreased tolerance to oxidative/chemical insults [8]. Therefore, alterations of the Nrf2 activity has been linked to the natural aging process [9, 10] and to the pathogenesis of many human chronic diseases, including neurodegenerative [reviewed in 11-13] and cardiovascular diseases [14], fibrosis [15], inflammatory states [16], as well as with abnormalities in the susceptibility of tumor cells to chemo- and radio-therapy [reviewed in 17, 18]. For these reasons, pharmacological/natural strategies that potentiate Nrf2 activity could be beneficial for preventing diseases in which oxidative stress exerts a pivotal role [7, 19-21].

Here, alongside a general description of Nrf2 regulation and interplay of redox-related signaling, we will review the recent evidences of the functional interaction between microRNAs (miRs) and Nrf2 redox-related pathways in the pathogenesis of several neurodegenerative diseases. This analysis points out how miRs could represent a promising tool to rescue Nrf2 activity and counteract oxidative stress in neuronal cells.

Nrf2 as regulator of redox homeostasis

Oxidative stress and antioxidant defenses

As a result of the aerobic metabolism, living organisms continuously produce ROS and RNS, which represent both free radicals and non-radical oxidant species generated in aerobic conditions. The main ROS include the superoxide anion radical ($O_2^{\bullet -}$), the hydroxyl radical ($HO^{\bullet}$), the hydroperoxyl radical ($HOO^{\bullet}$) and the non-radical hydrogen peroxide (H_2O_2), a source of $HO^{\bullet}$. The related class of RNS comprises nitric oxide ($NO^{\bullet}$), the nitrogen dioxide ($NO_2^{\bullet}$) radical, as well as the peroxynitrite anion ($ONOO^-$) derived from $O_2^{\bullet -}$ combined with $NO^{\bullet}$, among others [reviewed in 1, 2, 3].

As the ROS/RNS sources and their roles have been recently reviewed [2, 3], herein we will only very briefly describe the main aspects of ROS/RNS production and function. In biological systems, ROS/RNS can be generated in different cellular compartments, including mitochondria, recognized as the main source of ROS in eukaryotic cells, peroxisomes and lysosomes, as well as plasma membrane and endoplasmic reticulum. Endogenous ROS/RNS production arises from the mitochondrial-resident electron transport chain (ETC), from the action of different enzymes [i.e., NAD(P)H oxidases (NOXs), xanthine oxidase (XO), nitric oxide synthase (NOS), lipoxygenase, cyclooxygenase, cytochrome P450s] as well as from redox metal ion–catalyzed reactions involving iron or copper (i.e., Fenton reaction). ROS/RNS can have both positive and potentially damaging effects, depending on their nature, concentration and duration [2, 3, 22, 23]. At low/moderate levels, ROS/RNS possess highly specialized physiological functions with positive actions in redox-sensitive signaling that coordinate basic activities of cells. In this context, $O_2^{\bullet -}$ and H_2O_2 are considered second messengers for signaling pathways induced by growth factors (insulin, EGF, FGF, VEGF) or mediated by redox-sensitive kinases [e.g., protein kinase B (PKB/Akt), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK)] as well as for transcription factors (e.g., Nrf2, NF-κB, p53 and HIF-1α). At elevated concentrations, ROS/RNS can take part

in uncontrolled "redox reactions" [reactions in which one molecule (electron acceptor) is reduced while the other (electron donor) is concurrently oxidized]. In particular, elevated unstable free radicals that bear one or more unpaired electrons in their outer orbital, are prone to react with virtually all the chemical components of the cells trying to reach stability by losing or capturing electron/s. These processes potentially result in oxidative damages to the biomolecules, such as proteins, nucleic acids, carbohydrates and lipids. In this latter context, the most reactive oxygen-centered radicals $HO^{\bullet}$ and $HOO^{\bullet}$ can promote the initiation step of lipid peroxidation that generates final highly-reactive degradation products, such as 4-hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein, if not 'quenched' by endogenous antioxidants.

Under physiological conditions the steady-state concentrations of ROS/RNS are finely regulated for proper cellular function/redox control. The equilibrium between the ROS/RNS production and their cellular clearance/inactivation (often indicated as "Redox Homeostasis") is maintained by sophisticate antioxidant mechanisms [24, 25]. The condition of oxidative stress occurs when the production of ROS/RNS exceeds the capability of the cells to antagonize their possible deleterious effects (damages of biomolecules). In addition, this imbalance leads to a disruption of redox signaling, the outcome of which is coupled to the intensity and to the specific molecular targets [26].

Excessive amounts of ROS/RNS can be generated by enhanced activity of the ETC system or increased function of enzymes involved in ROS/RNS production as aforementioned. In addition, numerous extrinsic factors, such as exposure to environmental agents/conditions, can induce production of ROS/RNS. Most of the drugs and xenobiotics, during their biotransformation, are converted into polar compounds through the activity of the cytochrome P450s, key enzymes of phase I, and this is coupled to ROS/RNS production [27]. Moreover, there is expanding evidence that ionizing radiations (gamma and X-rays), cigarette smoking and air pollution also induce ROS/RNS formation [28, 29].

Reduced activity of the endogenous antioxidant/detoxifying systems could be another cause of oxidative stress. These antioxidant defenses are composed of several enzymes and many non-enzymatic compounds that neutralize the harmful effects of ROS/RNS [7, 25]. The antioxidant enzymes include the superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\bullet -}$ into H_2O_2 and O_2 and exists in three isoforms in mammals; the catalase (CAT), which catalyzes the conversion of H_2O_2 into H_2O and O_2 and protects cells from deleterious effects of H_2O_2; the glutathione peroxidase (GPX), which reduces H_2O_2 to H_2O and lipid peroxides to lipid alcohols, and in turn oxidizes glutathione (GSH), generating glutathione disulfide (GSSG); the glutathione reductase (GR), an enzyme critical for restoring GSH from GSSG; the thioredoxin reductase (TRXR), which reduces the oxidized thioredoxins (TRX), which are proteins implicated in the reduction of disulfide bond of peroxiredoxins (PRX) that are also involved in reduction of H_2O_2; the class of GSH S-transferases (GSTs), which mediates elimination of a wide range of toxic molecules, including ROS/RNS, xenobiotics and drugs; NAD(P)H:quinone oxidoreductase 1 (NQO1), NRH:quinone oxidoreductase 2 (NQO2), involved in detoxification of quinones, thus preventing reactive semiquinone, as well as $O_2^{\bullet -}$ and H_2O_2 formation. In particular, the PRX system includes a large family of peroxidases (PRX1-6) that are considered the most abundant antioxidant cytosolic enzymes. They possess very high specificity for H_2O_2, $ONOO^-$ and other organic hydroperoxides. With the exception of PRX6, the mammalian PRXs contain two reactive cysteines: one located in the active site, "peroxidatic" Cys, and the other, "resolving" Cys. First, the peroxidatic Cys is oxidized to a sulphenic acid (Cys-S-OH) that forms a disulfide bond with the resolving Cys, and then this disulfide is reduced via the TRX and TRXR system at the expense of NADPH. The related class of GPX enzymes acts with a similar mechanism [7, 25].

The nonenzymatic antioxidant defenses comprise low molecular weight molecules, such as GSH, uric acid, vitamins A and E, bilirubin and lipoic acid. GSH is an endogenous antioxidant tripeptide, with a thiol group that can directly interact with ROS/RNS or contribute indirectly as a cofactor for various antioxidant enzymes such as GPX and GSH S-transferases [30, 31].

In the context of antioxidant response mechanisms, a transcriptional reprogram for ROS/RNS defense/detoxification is activated by the transcription factor Nrf2 that mediates the induction of most of the aforementioned cytoprotective genes, thus restoring redox homeostasis. The Nrf2 signaling, as described in the next paragraph, is also involved in other cellular processes, including metabolism and cell proliferation/differentiation [5-8, 25].

Oxidative stress is generally recognized as a common feature of both acute diseases (like ischemia-induced damages) [25, 32] and chronic degenerative diseases, including neurodegeneration [33], diabetes [34] and cardiovascular diseases [35], as well as cancer [18, 36] and normal aging [37]. Importantly, since Nrf2 activity plays a pivotal role in redox homeostasis, its fine regulation is fundamental to counteract redox related damages.

Nrf2 and its regulated genes

Nrf2 is considered the most important transcription factor for antioxidant responses across the spectrum of organisms; from worms to humans [5-8]. It is a member of the family of conserved transcription factors called Cap'n'Collar (CNC), characterized by the presence of the CNC motif of 43-amino acid, along with a basic leucine zipper (bZIP) domain [5, 7]. This family includes the *Caenorhabditis elegans* SKN-1 (Skinhead family member 1), the *Drosophila* CncC and four vertebrate homologs: p45 NF-E2 (nuclear factor erythroid-derived 2), Nrf1, Nrf2 and Nrf3 [37, 38]. Nrf2 protein contains seven NRF2-ECH homology (Neh 1-7) domains, each with a specific function [7, 18, 39, 40]. Under basal conditions, newly synthesized Nrf2 is retained in the cytosol by binding two Keap1 (Kelch-like ECH-associated protein 1) molecules through its N-terminus domain (Neh2). Keap1 facilitates Nrf2 ubiquitination, thus driving its degradation. Oxidative and electrophilic stresses inactivate Keap1, thus *de novo* synthesized Nrf2 translocates into the nucleus where it dimerizes with other transcription factors through its Neh1 domain, in particular with a member of the small musculoaponeurotic fibrosarcoma oncogene homolog (sMAFs) protein family. Indeed, Nrf2 binds DNA only as a heterodimer with sMAFs, thus regulating several genes containing a cis-acting sequence, often present in multiple copies, called ARE (Antioxidant Response Element) or EpRE (Electrophile Response Element) (core ARE/EpRE sequence: 5'-A/GTGAC/GNNNGCA/G-3') [5, 7, 41, 42] discovered in the rat GSTA2 promoter [43]. On the other hand, Nrf2 stability could also be regulated through the phosphorylation of some serine residues in its Neh6 domain by different kinases [7, 18, 39, 41]. The C-terminal Neh3 domain is involved in transcriptional activation by recruiting the chromo-ATPase/helicase DNA-binding protein (CHD); similarly the Neh4 and Neh5 regions cooperatively interact with CBP (CREB-binding protein)/p300 or BRG1 (Brahma-related gene 1)/SMARCA4 that transactivate Nrf2-dependent transcription. Finally, the Neh7 domain binds to RXRα, thus preventing recruitment of coactivators [7, 18, 39, 40].

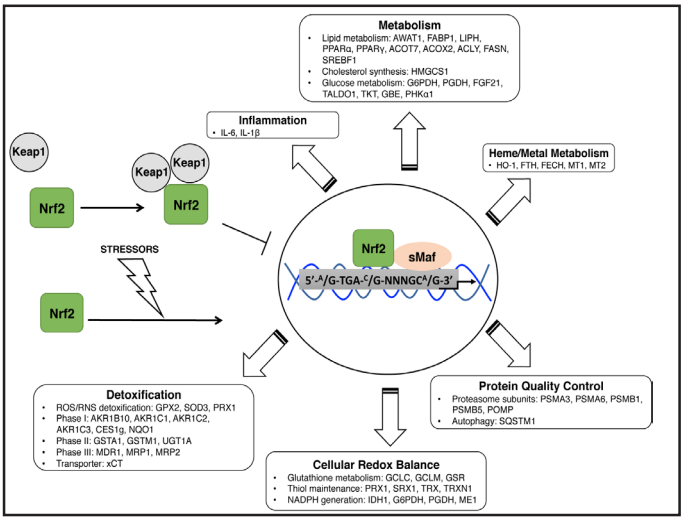

Fig. 1. Nrf2 activation and its regulated genes. Upon several types of stress, inactivated Keap1 allows newly synthesized Nrf2 to migrate into the nucleus. Here, together with a heterodimeric partner, in particular with members of the small Maf (sMaf) protein family (here depicted), Nrf2 regulates the expression of numerous ARE-dependent genes that can be grouped into several categories as indicated.

Nrf2 controls redox homeostasis either by regulating basal expression of a range of genes involved in antioxidant defenses or by inducing their expression under stress conditions (Fig. 1). In particular, Nrf2 activates the transcription of several antioxidant genes and major phase II detoxifying enzymes [5-7, 41]**.** However, the Nrf2-responsive genes are not limited to these functions and can be grouped into several categories [5-7, 18, 44]. A first group includes Nrf2-dependent genes encoding proteins/enzymes that contribute to cellular redox balance mainly through endogenous thiol resources. These include glutathione-synthesizing or -regenerating enzymes, as well as small components of antioxidant systems (PRX, TRX sulfiredoxins-SRX,) and enzymes producing NADPH, an essential cofactor to recover both GSH and TRX from their disulfide forms. Thus, these Nrf2-regulated genes play a critical function in thiol–based redox homeostasis by sustaining glutathione levels, a major antioxidant system with additive functions in the brain (see below) and ensuring a reduced state of cysteine thiols [45].

In a second group are included Nrf2-responsive genes encoding numerous enzymes of Phase I-III, that detoxify oxidative stress products such as HNE, MDA (AKR isoenzymes) and xenobiotics/drugs ((NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferases (GSTs)), as well as ROS/RNS. This group also includes molecules implicated in the transport of metabolites like multidrug resistance- and multidrug-related proteins (MDR, MRPs) as well as of amino acids (x-CT). The members of the GST family catalyze detoxification reactions through conjugation with GSH, thereby facilitating elimination of toxic molecules [46]. Another group includes enzymes implicated in heme metabolism (heme oxygenase-1 (HO-1) and ferrochetalase) and in defense against metals, like metallothioneins. In particular, HO-1 indirectly engages a strong antioxidant program mediated by ferritin, carbon monoxide and bilirubin, three molecules originated from the conversion of its metabolic products that also protect from apoptosis and inflammation [47]. Two other groups of Nrf2-related genes comprise proteins involved in protein quality control, proteasome or autophagy pathway (proteasome subunits, sequestosome1-p62/SQSTM1, nuclear dot protein 52-NDP52) and pro-inflammatory citokines (IL-6 and IL-1β), recently correlated to Nrf2 activity [48].

Finally, an expanding group of Nrf2-regulated genes considers many enzymes governing different metabolic pathways. The subgroup of lipid metabolism includes genes involved in *de novo* lipogenesis and lipid degradation, as well as genes involved in lipid transport and uptake. The subgroup of glucose homeostasis includes genes encoding enzymes of the pentose phosphate pathway, as well as some enzymes of glycogen metabolism, like glycogen branching enzyme (GBE), phosphorylase b kinase subunit A1 (PHKA1), recently linked to Nrf2 signaling in muscle [49].

Negative and positive regulators of Nrf2 protein

Nrf2 protein level is tightly regulated by several mechanisms, most of which act at the post-translational level to allow its activation upon specific stimuli.

Negative regulators

The best characterized negative regulator of Nrf2 is Keap1, which, in absence of stress, binds to the neosynthesized Nrf2, targeting it for polyubiquitination and degradation by the 26S proteasome [7, 8]. In particular, Keap1 is a cysteine-rich protein that acts as a key sensor for oxidative/electrophilic stresses. In detail, Keap1 homodimer binds a single Nrf2 molecule: one monomer associates with the ETGE motif and the other one interacts with the DLG motif (at lower affinity), both present in the Neh2 domain of Nrf2. In this conformation, Keap1 constantly targets Nrf2 to the Cullin3/Rbx1 system for polyubiquitination of seven lysines in the Neh2 domain and subsequent degradation. The most accepted model proposes that under stress conditions specific cysteine residues of Keap1 are modified, perturbing the interaction between Keap1 and Nrf2. In particular, the DLG motif of Nrf2 detaches from Keap1, whereas the binding with ETGE motif remains. Thus, inactivation of Keap1 allows

newly synthesized Nrf2 to translocate into the nucleus [7, 8, 39, 50]. Nrf2 stabilization is also regulated by SCF/β-TrCP complex, involving the S-phase kinase-associated protein 1 (Skp1), Cullin-1 (Cul1) and F-box protein E3 ubiquitin ligase [39, 51]. This regulation is unrelated to redox-sensitive modifications via reactive cysteine residues of Keap1. Indeed, Nrf2 is first phosphorylated by the enzyme glycogen synthase kinase 3 (GSK-3β) at specific serine residues located in the Neh6 domain of Nrf2, generating a phosphorylated destruction motif called "phosphodegron." Hence, Nrf2 is recognized by the adapter protein β-TrCP that targets it for ubiquitination and subsequent 26S-mediated degradation [13, 52]. Nrf2 can also be regulated through the protein synoviolin (Hdr1) E3 ubiquitin ligase [39], recently associated to the endoplasmic reticulum (ER) stress produced by misfolded protein accumulation. Noteworthy, unfolded protein response (UPR) by ER stress has been recently implicated in neurodegeneration [53].

Other negative modulators of Nrf2 are its direct interactors: seven in absentia homolog 2 (SIAH2) [54], CR6-interacting Factor 1 (CRIF1) [55], and the scaffold protein of membrane caveolae caveolin-1 (Cav-1) [56]. Under hypoxia, Nrf2 is down-regulated by SIAH2, a regulator of the hypoxic responses belonging to RING finger E3 ubiquitin ligase classes that interacts with non-phosphorylated Nrf2, favoring its degradation. CRIF1, a mitochondrial protein implicated in the production of the oxidative phosphorylation complex, can bind Nrf2 and promotes its degradation in a redox-independent manner, affecting Nrf2 activity to coordinate the cell cycle in stress-response.

Positive regulators

Regulators of Nrf2 stability. Nrf2 can be positively regulated by several proteins [reviewed in 7, 18, 44; Fig. 2]. Some of them, such as cyclin-dependent kinase (CDK) inhibitor $p21^{waf1}$, breast cancer susceptibility gene 1 (BRCA1) and IQ motif-containing GTPase-activating protein 1 (IQGAP1), interact directly with Nrf2, whereas others, like p62/SQSTM1, interact indirectly with it; for example, by binding Keap1. Furthermore, there are other factors, such as protein/nucleic acid deglycase DJ-1/Parkinson's disease protein 7 (DJ-1/PARK7), whose direct association with Keap1or Nrf2 has not been demonstrated yet.

In particular, $p21^{waf1}$ protein competes with Keap1 for the binding to Nrf2, thus preventing its ubiquitination and subsequent degradation [57, 58]. Interestingly, $p21^{waf1}$ and Nrf2 can control each other with a feedback mechanism, which implies that the increase of $p21^{waf1}$ enhances Nrf2 activity, and vice versa. BRCA1 promotes Nrf2-dependent transcription via direct interaction with Nrf2 protein or indirectly interacting with CREB-binding protein (CEBP) or with c-Myc [59]. IQGAP1, a scaffold protein interacting with actin, E-cadherin, β-cadherin and calmodulin, enhances Nrf2 stability probably by acting as a platform to recruit MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK pathway [60]. p62/SQSTM1 regulates Nrf2 by preventing the association of Nrf2 with Keap1, thereby promoting stability and nuclear function of Nrf2 [50, 61]. p62/SQSTM1 acts as a cargo receptor for the

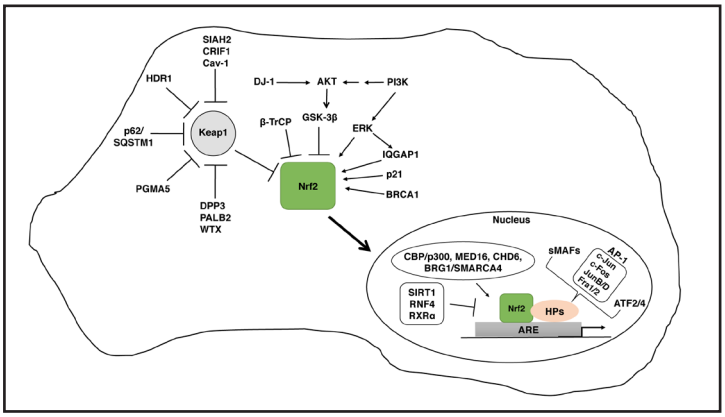

Fig. 2. Positive and negative regulators of Nrf2. Nrf2 can be directly or indirectly regulated by different proteins acting both in the cytoplasm and/or in the nucleus.

degradation of ubiquitinated proteins by the catabolic process of autophagy, thus preserving organisms from proteotoxic/oxidative stresses. p62/SQSTM1 sequesters Keap1 into the aggregates/autophagosomes, thus increasing activity of Nrf2. Furthermore, p62/SQSTM1 also affects the half-life of Keap1, suggesting that the interplay of p62/SQSTM1-Nrf2-Keap1 makes the response to redox-signalling more robust [reviewed in 62, 63].

DJ-1/PARK7 inhibits the interaction between Nrf2 and Keap1, thus activating Nrf2 against a variety of oxidative stresses. DJ-1/PARK7 is a well-established multifunctional protein associated with both Parkinson's disease and cancer [64]. Besides Nrf2, DJ-1 acts as an activator of various other antioxidant factors/pathways involved in both ROS/RNS inactivation and the recycle of oxidized molecules [reviewed in 65].

Nuclear regulators of Nrf2-transcriptional activity. Nrf2 regulates gene expression while also interacting with other transcriptional factors and various regulators that serve as activators or repressors of transcription of several genes. Indeed, several proteins heterodimerize with Nrf2 and promote its transcriptional activity. In particular, Nrf2 forms heterodimers with sMAFs proteins such as MAFF, MAFG and MAFK [5-7, 66]. The heterodimer Nrf2/sMAF activates the transcription of genes containing antioxidant/electrophile response element (ARE/EpRE) binding motif.

Nrf2 can also heterodimerize with several members belonging to the AP-1 family [25, 67], effectors of signal transduction cascades involving MAPK/ERK, MAPK/JNK, or with ATF2/4 [68], and effectors of PERK signaling. In these cases, ARE sequences recognized by Nrf2 overlap with those employed by these factors.

Another mechanism by which Nrf2 can bind to ARE/s and activate gene expression implicates the removal of the factor Bach1 from the ARE sequence/s [69]. In particular, Bach1 is related to the basal repression of the HO-1 gene [70]. Notably, Bach1 itself is a target gene of Nrf2, thus generating a negative feedback circuit for ARE-dependent gene transcription. This cross-talk could be essential to produce the correct level of antioxidant genes under stress [71]. Very recently, it has been reported that Nrf2 directly interacts with the subunit MED16 of the Mediator complex. Given that the association Nrf2-MED16 does not affect Nrf2 binding to the ARE, and that MED16 can recruit and/or activate RNA Polymerase II, the interaction Nrf2-MED16 is crucial for connecting stress signals to the transcriptional apparatus [72].

Nrf2 cross-talks with other cellular pathways

The cellular regulatory circuits that define outcomes of Nrf2 activity are particularly intriguing and correlate with other signal transduction cascades that can either promote or inhibit the Nrf2 pathway. Indeed, ROS/RNS activate MAPK, phosphoinositide 3-kinase (PI3K) and PKC pathways, which converge on different redox transcriptional factors [reviewed in 7, 39, 44, 57, 73]. In particular, post-translational modifications of Nrf2, such as phosphorylation and acetylation, are critical for its transcriptional activation and/or subcellular localization. In particular, PKC- or casein kinase 2- (CK2) phosphorylation of Nrf2 at Ser-40, as well as the MAPK/PERK-mediated phosphorylation at Thr-80, allow Nrf2 nuclear accumulation. CBP/p300-mediated acetylation of lysines within the Neh3/Neh1 domain increases Nrf2-dependent transcription, whereas their SIRT1-mediated deacetylation antagonizes Nrf2 signal. In addition, all these pathways also regulate the activity of Nrf2 interacting proteins, like the members of the AP-1 family, as well as that of nutrient-sensitive proteins, like AMPK (a downstream effector of Akt), that further promotes phosphorylation of Nrf2 blocking its nuclear export or increasing stability. Conversely, p38/MAPK, GSK-3β, or PTEN cascade signals result in the inhibition of Nrf2 activity [7, 39, 44, 57, 73].

On the other hand, the data about the relationship between Nrf2 and ROS-dependent activation of HIF-1α/2α are complex, and in some cases, controversial. Indeed, Nrf2 signal can either sustain HIF-1α pathway or decrease HIF-1α activity [reviewed in 74]. Cross-talk between Nrf2 and heat-shock factor 1 (HSF1) has been reported in adaptation/survival mechanisms against redox changes/electrophiles, as well as heat shock, heavy metals,

hypoxia or pH changes. HSF1 is the most important transcriptional regulator of the heat shock response, and, in particular, it prevents protein misfolding. Moreover, Nrf2 and HSF1 have opposing roles during autophagy induced by bis(2-hydoxybenzylidene)acetone (HBB2) [75]. Thus, Nrf2 and HSF1 pathways share overlapping target genes, including heat shock protein 70 (Hsp70), p62/SQSTM1, activating transcription factor 3 (ATF3) and heme oxygenase-1 (HO-1).

Furthermore, Nrf2 is also involved in anti-inflammatory pathways by a network with NF-κB signaling. NF-kB is activated through the proteasome-mediated degradation of its inhibitor IkB that allows nuclear translocation and induction of inflammatory factors i.e., TNFα, IL-6, IL-1 and IL-8, as well as genes related to antioxidant response. Several studies on Nrf2-null mice/cells demonstrated an increased sensitivity to inflammation and reported a role of HO-1, as well as its metabolites (iron, CO and bilirubin) in the anti-inflammatory effects mediated by Nrf2 [reviewed in 47]. Recently, it has been described a direct transcriptional involvement of Nrf2 in the suppression of LPS-induced pro-inflammatory genes, such as IL-6 and IL-1β [48] *in vivo*. This activity is independent from ROS-induced response mediated by ARE sequences.

The functional relevance on Notch-Nrf2 cross-talk has been reported *in vivo* for liver development/protection. The Notch1 gene is a direct target of Nrf2, which regulates its expression through a canonical ARE sequence. In turn, mammalian Nrf2 genes contain a functional binding sequence for RBPjk, a key transcriptional regulator of Notch1 [reviewed in 76]. However, further studies are necessary to evaluate whether this cross-talk affects the brain, where balanced Notch/Nrf2 axis could be potentially cytoprotective for neurons.

The cross-talk between Nrf2 and p53 is essential to preserve cellular homeostasis and involves both positive and negative interactions. Wild-type p53 can act either as an antioxidant or pro-oxidant factor to regulate intracellular ROS levels. This dual activity is correlated to the extent of numerous signals that converge on p53. On one hand, at low level of stress, basal p53 decreases ROS production by inducing the expression of many antioxidant genes, such as SESN1 and SESN2 of the sestrin family, GPX1 of the glutathione peroxidase family, and metabolic genes, such as ALDH4, TIGAR (TP53-induced glycol sis and apoptosis regulator) and GLS2 (phosphate-activated glutaminase). On the other hand, under severe stress, p53 is increased/activated, thus inducing pro-oxidant/proapoptotic genes such as tumor protein p53-inducible protein 3 (TP53I3/PIG3), proline dehydrogenase 1 (PRODH/PIG6), ferredoxin reductase (FDXR), BCL2 binding component 3 (BBC3/Puma) and BCL2 associated X (BAX). In this way, wild-type p53 contributes to preserve the integrity of the genome and to inhibit cancer initiation/propagation [44]. Functional interactions between Nrf2, p53, p53-regulated proteins and p53 regulators have been described at several levels. Tung et al. reported that Nrf2 mRNA levels were higher in p53-mutant tumors than in p53-wild-type non-small cell lung cancer (NSCLC) [77]. This might confer resistance to cisplatin-based chemotherapy in NSCLC patients [77]. Accordingly, p53 missense mutants cooperate with Nrf2 to activate proteasome gene transcription, resulting in resistance to the proteasome inhibitor carfilzomib in triple-negative breast cancer [78]. Furthermore, as reported above, $p21^{waf1}$, a downstream target of p53, directly interacts with Nrf2 and positively affects the Nrf2 signaling. In addition, a downstream target of Nrf2, the NAD(P)H dehydrogenase quinone 1 (NQO1) prevents p53 degradation [79]. Conversely, Nrf2 is essential for the basal transcription of MDM2, thus promoting p53 degradation [80], and we have demonstrated that p53, in condition of high oxidative stress, negatively regulates some Nrf2-dependent genes [81]. The interplay between Nrf2, MDM2 and p53 may involve also the Ser/Thr homeodomain-interacting protein kinase 2 (HIPK2), a modulator of several cellular functions, which acts as a signal integrator of a wide variety of stress signals, and as a regulator of transcription factors and cofactors. Indeed, HIPK2 is a well-characterized p53 regulator, promoting the expression of apoptosis-related p53-target genes [82]. Recently, it has demonstrated that HIPK2 ablation induces cerebellar dysfunction compatible with an ataxic-like phenotype [83] and, being a redox-sensitive kinase [84], HIPK2 shares several fields of interaction with Nrf2. Interestingly, HIPK2 gene is a direct transcriptional target of

Nrf2, which positively regulates its expression. HIPK2, in turn, supports Nrf2 cytoprotective activity, promoting its nuclear accumulation and activity [84]. These data suggest that HIPK2 may be an important modulator of Nrf2 in the regulation of oxidative stress-related genes and correlated diseases.

Nrf2 and redox homeostasis in the brain

The brain is an organ with elevated consumption of oxygen, and neuronal cells rely on oxidative metabolism to supply the high demand of energy [85]. Thus, neurons are consistently exposed to oxidative stress injuries, being $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, and $ONOO^-$ molecules generated in neurons from a variety of sources including mitochondrial respiratory chain, iron/copper mediated Fenton reaction and NOS enzymes. Indeed, protein carbonyls, HNE and 3-nitrotyrosine (3-NT) represent the most frequent products derived from oxidative/nitrosative damage to biomolecules that are considered early hallmarks of degeneration in different pathologies of the neuronal tissue [86, 87]. Proteins with carbonyl groups originate from direct oxidation of specific amino acid lateral groups, as well as from Michaelis addition reactions, in particular with HNE. These processes can induce protein misfolding and aggregation that is a feature of neurodegenerative diseases. HNE also reacts with sulfhydryl groups of low molecular weight molecules, such as glutathione, to yield stable thioether derivatives. In addition to HNE, MDA and acrolein also are produced from decomposition of lipid peroxidation, thus affecting the integrity/functions of biological membranes of neurons [86].

Irreversible 3-NT and thiol nitrosylation of cysteine (S-nitrosylation) represent the major protein modifications induced by increased $ONOO^-$ and $NO^{\bullet}$, respectively. At physiological levels, $NO^{\bullet}$ is implicated as a signaling molecule in many important processes within the central nervous system, and is generated by the neuronal NOS (nNOS) isoform. Its production can be stimulated by neuroinflammatory mediators and/or toxins that upregulate the expression of the inducible NOS (iNOS) or hyperstimulate nNOS. Increased $NO^{\bullet}$ levels can lead to $ONOO^-$ overproduction that is rapidly decomposed into reactive $HO^{\bullet}$ and $NO_2^{\bullet}$, triggering further oxidative stress. Furthermore, activated glia induces NOX2 enzyme, producing high amounts of $O_2^{\bullet -}$. The radical superoxide can dismutate either to H_2O_2 by SODs, or react with $NO^{\bullet}$ to produce peroxynitrite that fuel in a vicious cycle of radical production [87]. Another feature of brain degenerative diseases is mitochondrial dysfunction. Indeed, both nitration and nitrosation of proteins affect activity of several mitochondrial enzymes such as succinate dehydrogenase, creatine kinase and Mn-SOD [88].

Moreover, astrocytes could play a crucial role in the physiological function of the brain, exerting various functions to support neurons through glycogen storage and lactate release, uptake and release of transmitters, synapse formation/remodelling and activity, tissue repair and defense against oxidative stress [89, 90]. Indeed, astrocytes sustain redox homeostasis by producing and secreting high amounts of GSH that is essential to prevent oxidative damages in nearby neurons. The elevated production of GSH in astrocytes is sustained by both elevated biosynthesis and regeneration under the control of cell-autonomous Nrf2 signaling. Accordingly, astrocytes express basal levels of Nrf2 that can be further increased by several stresses, whereas Nrf2 expression is low in neurons due to epigenetic inactivation of its promoter and/or continuous destabilization of Nrf2 protein [91, 92]. Therefore, in astrocytes, Nrf2 regulates the basal expression of genes such as solute carrier family 7 member 11/xCT (SLC7A11/xCT), glutamate-cysteine ligase modifier subunit/gamma-glutamylcysteine synthetase (GCLM/γGCS) and glutathione-disulfide reductase (GSR) required for synthesis and metabolism of GSH, which is essential for redox homeostasis in the brain [45, 93]. Nrf2 also regulates the expression of MRP1-favoring GSH export that is broken by neurons that reuse amino acids to synthesize their own GSH. In fact, neurons in which the GSH biosynthetic enzymes are deleted are more prone to damage/death [94]. Thus, neurons rely on non-cell autonomous antioxidant protection. Recently, it

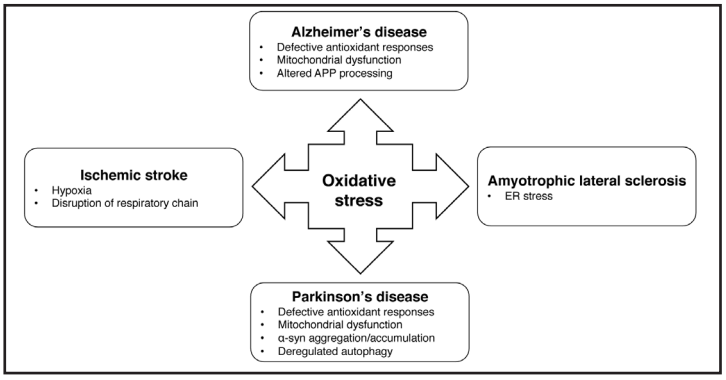

Fig. 3. Oxidative stress in neurological disorders. For each neurological disorder, the impairment of different pathways might lead to the oxidative stress.

has been demonstrated that Nrf2 signal can be activated by chronic stimulation of N-methyl-D-aspartate receptors (NMDAR) in astrocytes, thus protecting neurons against ROS/RNS damages due to high mitochondrial Ca^{2+} uptake [92].

In the brain, Nrf2 signaling also coordinates the activity of TRXs and/or PRXs necessary for the removal of ROS in the mitochondria [45, 92]. Among the many substrates of TRX-reduced disulfides are PRXs, glutaredoxin 2, HO-1, cytochrome c, and also the protein DJ-1, a peroxiredoxin-like peroxidase that scavenges H_2O_2 and is an upstream regulator of Nrf2-mediated induction of TRX [64].

In summary, Nrf2 signaling is essential to prevent/counteract pathological ROS/RNS insults in the brain, thus opposing the onset and progression of neurological disorders (Fig. 3). Recent findings based on animal models studies have shown the neuroprotective effects of many compounds, such as the triterpene Ginsenoside Rb1 [95] and the alkaloid Glaucocalyxin B [96] as well as endogenous metabolites like alpha-lipoic acid [97] and estradiol [98], all involved in the activation of the Nrf2/ARE signaling. Aging is the main risk factor for neurodegeneration and is associated with enhanced ROS/RNS levels and lower antioxidant capacity. Consistently, deregulation of Nrf2 pathway has been linked with aging, as well as with an enhanced susceptibility to and/or accelerated progression of neurodegenerative diseases [99-101].

Oxidative stress and microRNAs

MicroRNAs (miRNAs or miRs) are small non-coding RNAs (about 18-25 nucleotides in length) that regulate a vast array of physiological and pathological processes, ranging from development, homeostasis, metabolism and immunity, to aging and age-related diseases [102-104]. miRs bind mainly to the 3' untranslated region (UTR) of target mRNAs, and suppress their expression by inducing translational repression and/or degradation, depending on the complementarity

Table 1. microRNAs implicated in neurological disorders regulating Nrf2 response. N.E., not evaluated

miRNA	Nrf2 target	Other members of Nrf2-pathway targets	Nrf2/ response	Physiopathological Conditions/implicated diseases
miR-27a miR-142-5p	√		<	Parkinson's disease
miR-34a	√		<	Parkinson's disease
miR-34b miR-34c	N.E.		>	Parkinson's disease
miR-93	√		<	Ischemic Stroke
miR-144	√		<	Alzheimer's disease Parkinson's disease
miR-153	√		<	Alzheimer's disease Parkinson's disease Ischemic Stroke
miR-494			>	Parkinson's disease
miR-7		Keap1	>	Parkinson's disease
miR-128b		MAFG	<	Ischemic Stroke

of the duplex [105, 106]. Numerous findings indicate a strict correlation between miRs and redox homeostasis. In this context, miRs can act at various levels and their activities could contribute directly or indirectly to the regulation of ROS/RNS levels. In particular, miRs can directly affect the expression of Nrf2 and/or proteins controlling Nrf2 activity, as well as enzymes implicated in ROS/RNS production and detoxification. Moreover, Nrf2 and redox-related pathways can regulate miRs transcription. Noteworthy, the intriguing cross-talk between ROS/RNS, miRs and Nrf2 is crucial in orchestrating antioxidant defences as well as maintaining redox homeostasis [9, 25, 107, 108].

Several studies revealed that the biogenesis of miRs could be self-regulated by cellular redox status, thus suggesting an additional intricate interplay between miRs production and ROS/RNS levels [109]. Primary transcripts of miRs (pri-miRs) are recognized in the nucleus and cleaved by the RNA-binding protein Di George critical region-8 (DGCR8) to produce intermediate precursor miRs (pre-miRs) that are then exported into cytoplasm for complete maturation. DGCR8 activity is modulated by intracellular redox conditions in dependence on heme levels. Thus, HO-1 activity, which is the main degrading enzyme of heme, could influence the expression levels of miRs. Indeed, a recent paper of Lin et al. correlates HO-1 overexpression with altered miR profile in astrocytes [110]. Because HO-1 is induced by many stressors and has been found overexpressed in neural tissues of Alzheimer's disease

Table 2. microRNAs implicated in neurological disorders regulated by Nrf2. N.E., not evaluated

miRNA	Regulation By Nrf2	Physiopathological Conditions/implicated diseases
miR-29a miR-29b miR-29c	ARE-mediated	Alzheimer's disease Ischemic Stroke
miR-125b	ARE-mediated	Alzheimer's disease
miR-206	Not ARE-mediated	Alzheimer's disease
miR-106b-25	N.E.	Amyotrophic lateral sclerosis

Table 3. microRNAs implicated in neurological disorders affecting cell redox homeostasis

miRNA	Other targets	Physiopathological Conditions/implicated diseases
miR-7	alpha-synuclein	Parkinson's disease
miR-27a miR-142-5p	alpha-synuclein	Parkinson's disease
miR-29a	VDAC1	Alzheimer's disease Ischemic Stroke
miR-29b	BH3-only proteins	Alzheimer's disease Ischemic Stroke
miR-29c	BACE1	Alzheimer's disease
miR-34b miR-34c	alpha-synuclein	Parkinson's disease
miR-125b	Bak1 p53	Alzheimer's disease
miR-144	ADAM10 alpha-synuclein	Alzheimer's disease Parkinson's disease
miR-153	alpha-synuclein APP APLP2	Parkinson's disease Alzheimer's disease Ischemic Stroke
miR-206	BDNF	Alzheimer's disease
miR-494	DJ1	Parkinson's disease

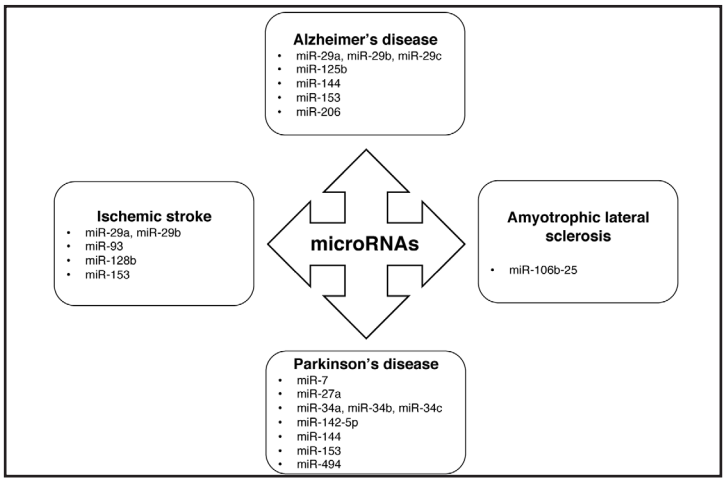

Fig. 4. Involvement of microRNAs-related to Nrf2 in neurological disorders. miRs involved in Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis and ischemic stroke are depicted. So far, several miRs directly downregulating Nrf2 levels have been identified. Furthermore, other miRs could contribute to the increase of oxidative stress by regulating proteins that have a crucial role in the pathogenesis of each disease.

(AD) and Parkinson's disease (PD), this could contribute to the pathogenesis of chronic brain disorders. In addition, the DGCR8/Drosha complex is regulated by the redox-sensitive enzyme GSK-3β that stabilizes Drosha into the nucleus. Conversely, GSK-3β regulates negatively Nrf2, thus adding further complexity in the modulation of miRs biogenesis and redox status.

ROS/RNS can also regulate the activity of Dicer, a key cytosolic endonuclease implicated in the synthesis of mature functional miRs. This enzyme is downregulated in various cellular models after H_2O_2-induced oxidative stress [109, 110] as well as in aging/senescence, which results in deregulation of mature miR levels. In addition, human and mouse Dicer genes contain ARE sequences in their promoters, suggesting that Nrf2 could participate indirectly to miRs maturation [107-109]. Consistently, antioxidant compounds, such as sulforaphane and resveratrol, prevent Dicer downregulation by increasing Nrf2 activity. Furthermore, Dicer suppression by the miR-let7 family, which is induced upon oxidative stress, could represent another mechanism to modulate miR levels, potentially related to redox status [107].

It is notable that Nrf2 plays a critical role in redox homeostasis and that several miRs have emerged to be important in its post-transcriptional regulation, as well as in antioxidant defenses. miRs could contribute to the modulation of the stress response mediated by Nrf2 at various levels in different physiopathological contexts including neurological disorders [9, 40, 107]. Firstly, several miRs are directly implicated in Nrf2 post-transcriptional regulation by targeting its mRNA, thus reducing Nrf2 protein levels and, consequentially, stress defenses (Table 1). Another group of miRs can indirectly contribute to the activation of Nrf2-dependent pathway by interacting with the 3'-UTRs of negative interactors/regulators of Nrf2 such as Keap1 (Table 1). Some miRs are transcriptionally regulated by Nrf2 and their activities can modulate apoptotic pathway and generation of β-amyloid peptide (Table 2). This leads to stabilization and/or activation of Nrf2 with consequent upregulation of the antioxidant response. Interestingly, some of these miRs are also involved in the regulation of proteins that can influence the Nrf2 activity (Table 3). Overall, it is likely that Nrf2-related miRs could play a crucial role in the pathogenesis of brain disorders (Fig. 4). Here, we discuss the role of miRs implicated in brain disorders.

Nrf2 and microRNAs in neurological disorders

Nrf2 and miRNAs in Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disease and represents the most common form of dementia in older people. At the cellular level, the pathogenesis of AD is characterized by the accumulation of hyperphosphorylated Tau protein, which forms neurofibrillary tangles, and of amyloid beta (Ab) peptides, produced by sequential proteolytic

cleavages of the amyloid precursor protein (APP) by β- and γ-secretases. The accumulation of these aberrantly-modified proteins results in oxidative damage, inflammation and increased intracellular calcium levels [11]. Because of its antioxidant potential, Nrf2 pathway has been shown to counteract AD progression, and Nrf2 activators, such as tert-butylhydroquinone (tBHQ), sulforaphane (SFN) and 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid–methyl amide (CDDO-MA), have been reported to reduce oxidative stress in cultured neurons, ameliorate cognitive dysfunction and spatial memory retention, and reduce plaque burden, antibodies levels, microgliosis and oxidative stress in AD mouse models [11]. Moreover, recent evidences suggest the involvement of miRs in AD through several mechanisms. Specifically, miR-153 and miR-144 directly target Nrf2 mRNA. Nrf2-regulating miR-153, whose expression is downregulated in APPswe/PSΔE9 AD murine model, directly targets APP and amyloid precursor-like protein 2 (APLP2) mRNAs binding their 3'-UTR [111]. APLP2 is a homologue of APP significantly upregulated in AD brains, and its C-terminal fragments contribute to the progression of the disease, decreasing cell survival [112]. Consistently, APP and APLP2 are downregulated in the brain of miR-153-transgenic mice with respect to wild type. The ability of miR-153 to regulate APP and APLP2 expression has been confirmed by Long et al., who showed also that miR-153 levels are inversely correlated to those of APP in AD patients. The authors reported that miR-153 levels were significantly decreased in a small cohort representative of advanced AD post-mortem brain specimens with neocortical neurofibrillary tangle (NFT) pathology (Braak stage III–VI), as compared with specimens lacking neocortical NFT pathology (control and Braak stage I/II specimens) and this is associated with a concomitant increase of APP levels in Braak stage III–VI specimens [113]. Moreover, miR-153 could represent an important regulator of cell survival in the development of AD, and is important for its ability to regulate the expression of the apoptotic proteins Bcl-2 and Mcl-1 [114].

miR-144 is another Nrf2-regulating miR, and it is downregulated in elder primate brains and AD patients. miR-144 directly targets the α-secretase ADAM10 (A Disintegrin and Metalloprotease 10) [115]. This protein is decreased in the platelets and neurons of AD patients, and is responsible for non-amyloidogenic APP processing that generates the neuroprotective and neurotrophic soluble APPs α-ectodomain, thus protecting the brain from the production of the Ab [116]. Intriguingly, the involvement of miR-144 in insulin signaling regulation could suggest a more complex role of this miR in AD, which is starting to be considered a metabolic disease with derangements in brain glucose utilization and responsiveness to insulin and insulin-like growth factor stimulation [117]. Deficiencies of insulin signaling have been consistently found in the brains of AD patients (in particular of the insulin receptor substrate-1, IRS-1), similar to those responsible for dementia symptoms of type 2 diabetes. miR-302 connects Nrf2 and insulin signaling by increasing Akt pathway attenuating the expression of phosphatase and tensin homolog (PTEN). AKT stimulates Nrf2 inhibiting Ab-induced neurotoxicity and suppresses Ab-induced inhibitory phosphorylation of IRS-1 at Ser307, which blocks the interaction with the insulin receptor and inhibits insulin action [118, 119].

Another important player in AD pathogenesis is brain-derived neurotrophic factor (BDNF), a pleiotropic regulator of synaptic plasticity, which affects cognitive function, memory and neurogenesis and is strongly downregulated in AD patient brains [120]. miR-206, which is indirectly regulated by Nrf2, and is upregulated in the brain of Tg2576 mice and in the hippocampal tissue, cerebrospinal fluid, and plasma of embryonic APP/PS1 transgenic mice, downregulates BDNF, thus affecting cognitive functions and memory of AD mice [121, 122].

Importantly, Moon et al. have evaluated miR-206 levels by real-time PCR in a small set of intranasal biopsies of the olfactory epithelia of early dementia patients compared to healthy controls and depression patients. miR-206 levels increased selectively in dementia patients, and its upregulation levels were inversely correlated to the cognitive assessment scores. If these data could be confirmed in adequate clinical trials, miR-206 could be utilized as a biomarker for the early diagnosis of AD [123].

miR-125b, which is directly regulated by Nrf2, has a controversial role in AD because it is upregulated in the brain of human AD patients and downregulated in APPswe/PSDE9 AD-mouse models and in murine primary hippocampal neurons treated with soluble Ab peptide [124, 125]. The authors suggest the neuroprotective role of this miR, at least in mice, reporting that miR-125b decreases the expression of the pro-apoptotic proteins Bak1 and p53, which are upregulated in some AD patients and in mouse primary cortical neurons. Interestingly, miR-125b levels are downregulated by Ab peptide treatment, with a consequent increase of Bak1 and p53 [125].

Loss of other Nrf2-regulated miRs, belonging to miR-29 cluster, has been found in sporadic AD associated with increased BACE1/beta-secretase expression, suggesting a potential relationship between the expression of these miRs and the accumulation of Ab peptide [126]. On this basis, Pereira and colleagues have started the development of a therapeutic approach aimed to restore miR-29b expression in AD patients lacking miR-29a/b-1. Firstly, they described a new arginine-affinity chromatography-based strategy to purify the recombinant pre-miR-29b. Then, they tested different strategies to deliver this recombinant pre-miR into cells. Interestingly, Chitosan/pre-miR-29b and Polyethylenimine/pre-miR-29b polyplexes efficiently delivered pre-miR-29b to mouse neuroblastoma N2a695 cells (stably transfected with cDNA encoding human APP isoform 695), thus reducing BACE1 expression and Aβ42 levels [127]. However, the members of the miR-29 cluster could also represent useful peripheral markers for AD diagnosis. In fact, the expression of miR-29c is downregulated in the peripheral blood of AD patients with respect to age-matched controls, and this downregulation is associated with a concomitant increase in BACE1 blood levels. Moreover, the authors reported that upregulation of miR-29c promotes learning and memory behaviors in SAMP8 mice, decreasing BACE1 expression and activating the neuroprotective function of the PKA/CREB pathway. Furthermore, Zong et al. found that miR-29c is aberrantly expressed in the frontal cortex and in the hippocampus of APPswe/PSΔE9 mice, where it downregulates the expression of neurone navigator 3 (NAV3), a regulator of axon guidance [128].

Nrf2 and miRNAs in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 1-2% of people over the age of 60 [129-132]. Clinically heterogeneous, PD is characterized by a wide spectrum of motor (such as resting tremor, bradykinesia, muscular rigidity and postural inability) and non-motor (sleep disorders, cognitive and behavioural manifestations) symptoms. The pathological hallmark of PD is the progressive loss of dopaminergic neurons (DA) in the substantia nigra pars compacta (SNpc) and the presence in surviving neurons of Lewy bodies, which are eosinophilic proteinaceous inclusions enriched in alpha-Synuclein (α-Syn) aggregates [129, 130]. Beyond the rare monogenic forms of the disease (accounting for 5-10% of PD), the majority of PD cases are sporadic and probably caused by a combination of genetic and environmental factors on a background of age-related changes. However, the exact underlying molecular mechanisms are still unknown, and it is still puzzling why dopamine (DA) neurons are the most affected in all forms of PD. The impairment of mitochondrial functions, as the dysfunction of protein quality-control machineries with subsequent accumulation of misfolded proteins, has been implicated in the PD pathogenesis [129, 131, 132]. Moreover, oxidative stress is also considered as one of the main causes of PD [3, 133, 134]. A number of findings indicate that oxidative stress concurs to PD not only in sporadic, but also in familial cases, where it might exacerbate the disease phenotype. Accumulation of ROS has been reported in PD patients [134, 135], and the oxidative damage is abundant in PD [134, 136-138]. From post-mortem studies, there is evidence for lipid peroxidation, protein nitration and nucleic acid oxidation in brain PD [139-141]. Moreover, the presence of protein adducts of HNE, as well as an increase in protein oxidation, has been observed in the SN of PD patients [142, 143].

Several findings indicate that the imbalance between ROS/RNS production and antioxidant systems leads to a stress oxidative in PD. The levels of GSH and coenzyme

Q10 are reduced in SNpc of PD patients [144-147], suggesting a loss of Nrf2 response. On the other hand, a recent study showed that Nrf2 has prevalently nuclear localization in the SN of PD brains in contrast to control brains [148], in agreement with activation of endogenous antioxidant response. The deficit in Nrf2 target genes expression might be due to the impairment of other mechanisms regulating Nrf2 activity or could simply reflect that the activity of Nrf2 is insufficient to protect neurons from the sustained oxidative stress. Notwithstanding, recent studies showed that Nrf2 has an important protective role against the neurotoxins, 6-hydroxydopamine and 1-methyl-4-phenyl-1, 2,3, 6-tetrahydropyridine (MPTP) *in vitro* and *in vivo* models of PD [149-153]. Hence, all these data indicate that Nrf2 plays a pivotal role in the antioxidant response in PD, and therefore there is a great interest in understanding how its activity can be modulated by emerging regulators as miRs to counteract PD progression. Indeed, recent studies showed that several miRs can regulate the redox state in *in vitro* and *in vivo* models of PD, suggesting that they might contribute to the pathogenesis of PD [154].

The brain-enriched miR-7 positively controls the Nrf2 activity. Kabaria and collaborators showed that this miR downregulates Keap1 expression by targeting its 3'-UTR in human neuroblastoma SH-SY5Y cells [155]. This induces the expression of Nrf2-dependent genes along with increase of total glutathione (from 2 to 8 fold). In addition, the overexpression of miR-7 protects neuronal cells against 1-methyl-4-phenylpyridinium (MPP)-induced death by decreasing the levels of hydroperoxides [155]. Recent findings show that the substantia nigra, striatum and olfactory bulb, which are the mainly affected regions in PD, have relatively higher miR-7 levels with respect to cerebral cortex and cerebellum [156-158], while miR-7 expression is reduced in brain of MPTP-triggered PD mice [158], suggesting that dysregulation of this miR might play a crucial role in PD pathogenesis.

Recently, four miRs, miR-27a, miR-142-5p, miR-144, and miR-153, have been identified by *in-silico* analysis by Narasimhan and co-workers, and validated as direct regulators of Nrf2 expression in neuronal SH-SY5Y cells [159]. They demonstrated that the overexpression of these miRs represses Nrf2 mRNA by targeting its 3'-UTR, thus mediating Keap1-independent repression of Nrf2 as previously reported for miR-28 in breast cancer [160]. Moreover, the abrogation of Nrf2-mediated transactivity leads to a strong alteration of GSH homeostasis, including the decrease of GSH/GSSG ratio as the reduction of GCLC and GR [159], indicating an important role of these miRs in the redox homeostasis of neuronal cells. However, endogenous levels of miR-142-5p and miR-144 are negligible in SH-SY5Y cells, while relatively higher than those of miR-27 and miR-153. Interestingly, miR-153 has been found enriched in the brain, pointing out a pivotal role of this miR in the redox homeostasis of the brain. Moreover, the same group demonstrated that miR-153 was increased after exposure to paraquat (PQ), an herbicide that causes irreversible damages to DA neurons and therefore is considered to be a potent risk factor of developing PD [161]. The neurotoxicity of PQ is attributed to the ROS generation and consequent induction of oxidative stress. Of interest, PQ significantly increases, through a mechanism H_2O_2-dependency, the expression of miR-153 in neuronal cells that, in turn, reduces both Nrf2 expression and activity. Conversely, the overexpression of an anti-miR-153 prevents ROS accumulation as H_2O_2-induced neuronal death [161]. Interestingly, both miR-7 and miR-153 are also implicated in the post-transcriptional regulation of α-Syn by binding specifically its 3'-UTR [158, 162]. In particular, α-Syn can promote mitochondrial dysfunction by inhibiting complex I activity with subsequent increase of ROS/RNS production [163]. In addition, it is well accepted that oxidative stress can induce α-Syn aggregation, thus possibly creating a very dangerous loop. Therefore, the reduction of miR-7 could play an important role in PD neurodegeneration through these two different mechanisms. However, a recent study has showed that miR-7 regulates the function of mitochondrial permeability transition pore by repressing VDAC1 expression [164], suggesting a potential role of this miR in preventing mitochondrial dysfunction. Hence, it will be important to determine the levels of miR-7 in PD patients in order to understanding how it can be dysregulated during the progression of the disease.

So far, miR-expression profiling of PD brains revealed that the expression of miR-34b and miR-34c is reduced in several brain areas, including the amygdala, SN, frontal cortex and cerebellum [165]. The depletion of both miRs through specific miR-inhibitors leads to the alteration of mitochondrial dynamics and functions with subsequent reduction of ATP synthesis and increased oxidative stress [165]. Moreover, all these events also result in a reduction of cell viability [165], thus suggesting the role of miR-34b and miR-34c in PD neurodegeneration. Interestingly, the fact that the inhibition of their expression produces moderate effects in undifferentiated SH-SY5Y with respect to differentiated cells suggests that these miRs might have a relevant role in the maintenance of proper functions of the brain in adults. Interestingly, another study showed that inhibition miR-34b and miR-34c enhances α-Syn expression in SH-SY5Y cells as inducing its aggregation in neuronal cells [166], further supporting that these miRs can contribute to PD pathogenesis.

The identification of DJ-1, a stabilizer of Nrf2, as a direct target of miR-494 is fundamental in this context [167]. While the overexpression of this miR renders neuroblastoma N2a cells more susceptible to oxidative stress and exacerbates neurodegeneration in MPTP treated mice [162], its reduction might protect neurons against oxidative insult by increasing SOD1 expression, as well as by attenuating mitochondrial dysfunctions [168].

Finally, a recent study by Ba and co-workers has highlighted the role of another miR, miR-34a, in regulating the Nrf2 activity [169]. They showed that Schisandrin B exhibits protective effects against 6-OHDA *in vitro* and *in vivo* through the inhibition of the negative modulation of miR-34a on Nrf2 activity. They also demonstrated that Nrf2 is a direct target of miR-34a, and that Schisandrin B prevents the 6-OHDA-induced miR-34a upregulation [169].

All these data provide clear evidence that miRs could play a role in PD pathogenesis by regulating redox homeostasis through different mechanisms. Thus, further studies will be crucial to understand whether specific miRs can directly induce and/or contribute to PD.

Nrf2 and miRNAs in amyotrophic lateral sclerosis

ER stress constitutes a physiological, as well as a pathological, stress stimulus activated by UPR, which can lead damaged cells to apoptotic death [170]. UPR-induced apoptosis is indeed a pathological feature of several diseases such as neurodegeneration, hypoxia, ischemia/reperfusion injury, atherosclerosis, and diabetes. Nrf2 has been involved in ER stress-induced apoptosis. In detail, Nrf2 is activated during UPR and, together with ATF4, causes the repression of miR-106b-25 cluster in a PKR-like endoplasmic reticulum kinase (Perk)-dependent manner in several cellular contexts. The repression of miR-106b-25 cluster causes in turn an increase of BIM expression and apoptosis [171]. Interestingly, the effects of Nrf2/ATF4/miR-106b-25 cluster may be relevant also for the pathogenesis of amyotrophic lateral sclerosis (ALS), in which ER stress has been strongly associated with motoneuron degeneration [172]. In fact, miR-106b-25 cluster is downregulated in the symptomatic SOD1G86R transgenic mice models of ALS, whereas sporadic ALS patients show upregulation of ATF4. Furthermore, it has also found BIM upregulation in a familial ALS (fALS) mouse model during the symptomatic stage and, *in vivo* BIM ablation reduced cellular apoptosis in the ventral horn of a transgenic mouse model of fALS, increasing lifespan [172].

Nrf2 and miRNAs in ischemic stroke

Stroke represents one of the most frequent causes of death and disability worldwide and is strongly linked to oxidative stress. In fact, it has been largely confirmed that cerebral ischemia/reperfusion rapidly triggers an excessive ROS/RNS production, mainly in endothelial and smooth muscle cells, but also in neurons, glial cells, and infiltrating neutrophils [173]. The main cause of this burst of ROS/RNS production is the disruption of mitochondrial respiratory chain followed by the activation of NOXs and XO enzymes, which generate large amounts of superoxide radical anion, the precursor of several ROS species [174]. However, after a stroke, besides excessive generation of ROS/RNS, there is also a decrease in ROS/RNS scavenging capacity due to consumption and subsequent insufficient replacement of endogenous antioxidants leading to impairment of antioxidant systems. The resultant ROS/RNS accumulation mediates the ischemia/reperfusion injury by

several mechanisms, such as blood-brain barrier (BBB) disruption, inflammation, apoptosis, and cellular necrosis [173, 175]. Interestingly, both miRs and Nrf2 seem to be involved in these mechanisms, and are involved in the pathogenesis of stroke [32]. In fact, the miRNA transcriptome largely changes after a stroke, and some ischemia-regulated miRs are implicated in BBB disruption (miR-15a), caspase-mediated cell death signaling (miR-497), neural cell survival (miR-200 family), and post-stroke inflammatory response (miR-125b, -26a, -34a, -145 and let-7b) [176]. Similarly, several studies report that Nrf2 expression and activity largely increase after ROS accumulation during the acute phase of stroke in different cell types, including neurons, astrocytes, microglia, and leukocytes, and that Nrf2 knockdown worsens ischemia-induced neurologic damages [32]. Nrf2 may play a protective role against ischemia/reperfusion injury potentiating the antioxidant defenses, via activation of HO-1, NQO1, SODs, CAT, GPX genes as well as induction of GSH metabolism genes, protecting BBB integrity and counteracting inflammation. For these reasons, there is a growing interest in the development of therapies aimed to activate the Nrf2 pathway, and several food antioxidants, especially tert-Butylhydroquinone (tBHQ) [177] and resveratrol [178], have been tested as Nrf2 activators in stroke. Intriguingly, some Nrf2-regulating or -regulated miRs are related to stroke, representing putative therapeutic targets for modulating Nrf2 expression and/or potentiating antioxidant defenses after ischemic damage. In particular, Nrf2 transcriptionally upregulates the expression of miR-29a and miR-29b belonging to the miR-29 cluster [179]. miR-29b is a recognized neurons survival factor, because of its ability to downregulate the expression of the pro-apoptotic proteins of the BH3-only family [180]. Furthermore, Khanna et al. have reported that miR-29b levels decrease at the infarct site after stroke and that this downregulation contributes to cell death in both cultured neurons and animal models [181]. In fact, the delivery of a miR-29b mimic is able to increase post-ischemia cell survival, whereas inhibition of miR-29b markedly potentiates cell death. Stroke-induced miR-29b downregulation is mediated by 12-lipoxygenase, an enzyme responsible for the production of reactive lipid metabolites, which induces mitochondrial damage, and, consistently, the 12-lipoxygenase inhibitor α-tocotrienol is able to reduce miR-29b downregulation, ameliorating stroke outcome [181]. The other member of the miR-29 cluster, miR-29a, seems to be responsible, at least in part, for the different sensitivity to cerebral ischemia of cornu ammonis 1 (CA1) and dentate gyrus (DG) hippocampal astrocytes. CA1 astrocytes are more sensitive to ischemic damage, and exhibit a greater decrease in miR-29a than DG astrocytes in response to glucose deprivation. The protective function of miR-29a is due to its ability to target the voltage-dependent cation channel-1 (VDAC1), a regulator of mitochondrial inter-compartmental transport, which can induce apoptosis favoring the release of pro-apoptotic proteins from mitochondria. Both overexpression of miR-29a and knockdown of VDAC1 improve post-ischemic survival of CA1 astrocytes, supporting the mechanism proposed by the authors [182].

Consistently with the protective role of Nrf2, Nrf2-targeting miR-93, already related to inflammation, oxidative stress, and cell apoptosis, increases in a time-dependent manner in the cerebral cortex following ischemia and reperfusion in mice, thus representing a potential therapeutic target for acute ischemic stroke [183]. As recently described, treatment with miR-93 antagomir reduces cerebral infarction volume, neural apoptosis and restores the neurological scores in mice in an Nrf2-dependent manner. In fact, these effects are associated with increased expression of Nrf2 and its target HO-1, and knockdown of Nrf2 or HO-1 prevents miR-93 antagomir-induced neuroprotection against oxidative stress in cultured neurons [184].

Finally, some of the Nrf2-associated miRs have been recently proposed as circulating non-invasive biomarkers for stroke diagnosis. In particular, the circulating levels of miR-107, miR-128b and miR-153 significantly increase in stroke patients in comparison to healthy volunteers, and their levels positively correlate with the severity of stroke [185]. Moreover, it has recently observed that miR-128 counteracts stress responses mediated by Nrf2 targeting MAFG and, as a consequence, it could mediate the adaptive response during hypoxia both *in vitro* and *in vivo* [186].

Conclusion

Redox homeostasis in the brain is under the control of Nrf2 and increasing interest has been drawn to identify natural or synthetic compounds to modify Nrf2 activity in those pathological conditions in which ROS/RNS have a recognized role. The best demonstration that Nrf2 induction could be beneficial for the treatment of neurological disorders is represented by BG-12, an oral formulation of the Nrf2-inducer dimethyl fumarate (DMF) for the treatment of remitting multiple sclerosis. Taking into account the involvement of miRs in numerous biological functions, their implication in Nrf2-driven antioxidant program is expected. Indeed, miRs directly or indirectly targeting Nrf2 activity as well as Nrf2-mediated transcription of miRs has emerged to play an important role in modulating the redox balance. Therefore, we can speculate that modulation of miR levels might be a promising and feasible strategy to enhance Nrf2 activity in conditions such as stroke, Alzheimer's and Parkinson's diseases, where Nrf2 down-regulation seems responsible, at least in part, for the pathological outcomes. Emerging data encourage miRs delivery (i.e., by exosomes) for the treatments of neurological pathologies. In this context, miRs that repress Keap1 as well as miR-inhibitors acting on Nrf2 could be useful to restore redox balance. However, the existence of mechanisms controlling Nrf2 activity different from Keap1, as well as the involvement of Nrf2 in several cellular processes, makes it difficult to design specific therapeutic agents based on miRs. Further studies are required to provide a complete picture of miRs functions in neurological disorders and to define whether they can be considered alone or in combination as valid means to positively increase Nrf2 activity.

Finally, current clinical research is trying to elucidate whether natural molecules recognized as Nrf2 activators (i.e., isothiocyanates and semi-synthetic triterpenoids) can prevent/ameliorate degenerative conditions. Hence, we speculate that cooperative action of miRs and natural compounds-based induction of Nrf2 could also be explored.

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Disclosure Statement

The authors declare to have no competing interests.

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