● **PERSPECTIVE**

Can we protect the brain *via* preconditioning? Role of microRNAs in neuroprotection

Preconditioning stimulus: Preconditioning is an adaptive response, whereby a small dose of a harmful substance protects the brain from a subsequent damaging insult (Dirnagl et al., 2009). The concept of preconditioning was first described in an ischemic heart model, it was observed that brief ischemic episodes protect against a subsequent ischemic insult (Murry et al., 1986). Consequently, several preconditioning treatment paradigms are used in the clinic to protect patients against an ischemic insult in heart pathologies (Jimenez-Mateos et al., 2015). This data shows the importance of understanding the underlying mechanism to preconditioning, and its translation in the clinic in brain disorders. In concordance, any injury to the brain applied below the threshold of cell damage, including seizures, will induce preconditioning and neuroprotection to the brain.

The mechanisms of preconditioning-induced tolerance are not well known, but *de novo* protein synthesis is required and is correlated with repressed gene expression. Furthermore, the preconditioning stimulus produces a transient effect, having an effect only for few days after administration (Stenzel-Poore et al., 2007). Preconditioning can induce neuroprotection over two phases: Phase one, rapid tolerance, this occurs in a short period of time and is independent of protein production and associated with synapse remodelling (Meller et al., 2008). Phase two, delayed (classical) tolerance, this evolves over 1–3 days and requires *de novo* protein production with a peak at 3 days and diminishes over the course of 1 week (Stenzel-Poore et al., 2007).

MicroRNAs biogenesis pathway: MicroRNAs are defined as small non-coding RNAs (~20–22 nucleotides) that regulate gene expression at a post-transcriptional level in a sequence-specific manner. Almost 50% of all identified miRNAs are expressed in the mammalian brain and there is significant cell- and region-specific distribution. This highlights its roles in gene expression directing the functional specialization of neurons and the morphological responses that are required to adapt to their continuously changing activity state (O'Carroll and Schaefer, 2013).

MiRNAs are abundantly expressed in the central nervous system, being involved in diverse functions, including neuronal migration and differentiation, synaptic plasticity and maintenance of functions.

MiRNAs regulate gene expression *via* translational inhibition, mRNA degradation or a combination of both mechanisms (O'Carroll and Schaefer, 2013). In the brain, miRNA targeting is associated with protein degradation without reduction in mRNA levels of the target genes (O'Carroll and Schaefer, 2013). MiRNAs and their biogenesis components display localization within neurons, with significant enrichment in dendrites, enabling local, activity-dependent miRNA regulation of protein levels (O'Carroll and Schaefer, 2013). Recent work demonstrated that certain pre-miRs, semi-processed nuclear miRNA, have localization signals which translocate them to synaptic sites, where final processing to mature miRNA occurs (Bicker et al., 2013).

Several proteins are involved in the biogenesis and mechanism of action of microRNAs, including the nuclear microprocessor, DGCR8 and Drosha; and the cytoplasmic proteins Dicer and Ago family proteins (Jimenez-Mateos, 2015).

Considerable evidence has shown the role of miRNAs in the brain, mainly by the use of genetic tools, including transgenic mice with constitutive and conditional deletion of biogenesis enzymes involved in the microRNA pathway. Deletion of DGCR8, a nuclear enzyme that regulates precursor miRNA production which affects the production of the precursor microRNA, results in a reduction in brain size and loss of inhibitory synaptic neurotransmission (Hsu et al., 2012). Conditional deletion of Drosha, a vital cytoplasmic mature miRNA processing enzyme, in neural progenitors did not affect neurogenesis in the developing brain, but did affect differentiation and migration of neurons (O'Carroll and Schaefer, 2013). Deletion of Dicer from neurons produces severe brain abnormalities, including microencephaly and defects in dendritic arborisation in the cortex and hippocampus (Jimenez-Mateos, 2015). Additionally mice lacking Dicer in astrocytes develop spontaneous seizures and many die prematurely (Jimenez-Mateos, 2015). This indicates that the miRNA biogenesis system, and subsequent miRNA, is essential for normal brain development and function. Surprisingly, one study reported that specific deletion of Dicer in the adult mouse forebrain transiently enhanced learning and memory, although these animals later displayed degeneration of neurons in the cortex and hippocampus (O'Carroll and Schaefer, 2013).

Analysis of argonaute (Ago1-4) proteins has given more controversial results, with Ago-2 being the most abundant form in the brain (Liu et al., 2004). It has been suggested that Ago-2 is critical for miRNA-mediated repression of mRNAs. Deficiency in Ago-2 results in death of mice during early embryogenesis or mid-gestation (Morita et al., 2007). This reflects not only the essential role of Ago2 in embryonic development but perhaps an effect of impaired microRNA generation. In contrast, studies in conditional mutants displaying individual deficiencies in Ago 1, 3 and 4 genes, do not produce obvious effects in mice, suggesting a redundancy among Ago family members (O'Carroll and Schaefer, 2013).

MicroRNAs and preconditioning: The role of microRNAs in preconditioning in brain has been analysed in several experimental models, including ischemic and epileptic murine models. In these studies several microRNAs have been identified as mediators of the neuro-protected effect of the preconditioning stimulus. In two different studies of ischemic preconditioning, miR-200 was found to be consistently up-regulated (Jimenez-Mateos, 2015). The neuro-protecting effects of miR-200b/c were associated with regulation of survival pathways in the neurons, including proteasome activation or hypoxia induced factor 1α (HIF1α) (Jimenez-Mateos, 2015). Another microRNA, miR-199a, has also been shown to induce preconditioning in rat brain. The effects of miR-199a in the brain have been associated with the regulation of the transcription factor SIRT1 (Jimenez-Mateos, 2015). Consistently, both microRNAs regulated pathways are associated with *de novo*-protein synthesis regulation, supporting the original findings of the association of preconditioning with the *de novo*-protein synthesis and the efficiency of the preconditioning treatment in the brain (Jimenez-Mateos, 2015).

Different results were found in epileptic preconditioning studies, when two different preconditioning models were addressed, a kainic acid model and an electrical stimulation murine model. In both models a general up-regulation of microRNAs was the main response in preconditioned mice compared to control mice. Two mainly microRNAs have shown to be neuroprotective in the epileptic preconditioning stimulus, miR-184 and miR-132 (Jimenez-Mateos, 2015).

MiR-184 has been shown to be neuroprotective in the brain, as silencing levels of miR-184, *via* antagomiRs, increased seizure damage after a preconditioned stimulus. The neuro-protected mechanisms of miR-184 are unknown, but two main pathways have been described. MiR-184 has been shown to regulate the survival factor Akt2 in neuroblastoma cells. Additionally, miR-184 promotes adult neural stem cell proliferation and interestingly, increased neurogenesis is required for tolerance. These results demonstrate the multi-targeting effects of microRNAs, and how they can contribute to the mechanisms underlying complex brain diseases (McKiernan et al., 2012).

A single microRNA has been commonly regulated in preconditioning experimental models of ischemia and epilepsy, miR-132. MiR-132 has been shown to have an important role in neural function, dendrite and neurite growth, synaptic plasticity and memory formation in wild type mice. In pathological conditions, inhibition of miR-132 protects the brain against neuronal damage (Jimenez-Mateos, 2015).

The regulation of mir-132 in brain has been related to the transcription factor CREB. After an insult to the brain, expression levels of CREB are elevated, and miR-132 will be expressed. Some of the targets of miR-132 include MeCP2 protein, a transcription factor which has been previously described as neuro-protected in a preconditioned ischemic mouse model (Jimenez-Mateos, 2015).

Future perspectives: MicroRNAs can potentially target hundreds of genes, which allows a multi-targeted net of neuroprotective proteins. However, the question still remains, could microRNAs be possible targets for new drugs therapeutics? In 2013, *New England Journal of Medicine* published the first microRNA targeted drug to enter clinical trial for the treatment of hepatitis C virus. The Phase2A study showed that microRNA-therapeutics were safe, well tolerated, with limited adverse effects and higher efficiency compared to previous traditional treatments (Janssen et al., 2013).

In contrast, the development of drugs to target microRNAs in neurodegenerative disorders needs more detailed analysis. One of the questions for microRNA therapy is the method of delivery. Presently, microRNA-targeting drugs have been used in experimental models *via* intra cerebral injection as these compounds do not cross the blood-brain barrier. Diverse strategies have been used to deliver these to the brain, mainly through viral vectors or nanoparticles or by modified oligonucleotides. Recent developments have overcome some of these problems using artificially synthesised exosomes, which are able to cross the blood brain barrier and can bind to specific cell types. Furthermore, inhibition of microRNAs has been the most effective manner to target microRNAs. Antagonists of microRNAs have shown long-lasting effects, in contrast, restoration of microRNAs function *via* mimics has not been profoundly studied, mainly because mimics of microRNAs have shown only a transient effect in the brain.

A more detailed analysis on microRNA regulators will be necessary; these include new microRNA-targeting drugs for systemic routes, toxicology analysis of inhibitors and mimics of microRNAs and long-lasting side effects of these regulators. Elucidation of these points will be absolutely necessary for the translation into the clinic.

A more suitable approach to microRNAs in the clinic will be their role as biomarkers. The main characteristics of microRNAs are easy accessibility, high specificity and sensitivity, low costs and requirement of standard laboratory equipment. MicroRNAs have been found in human body fluids, especially in plasma and serum, which requires minimally invasive sampling. These circulating microRNAs are stable and reliable, making them an optimal resource of biomarkers and translational approach from the bench-side to the clinic. Furthermore, the use of microRNAs as biomarkers in combination with traditional neuro-imaging could improve diagnostic reliability and determine preconditioning as the most adequate treatment and identify the success of the preconditioning treatment in ischemia (Jimenez-Mateos, 2015).

Some limitations are associated with the role of microRNA as biomarkers. One major question is how faithful is the peripheral profile to the original biological situation in the CNS. However, the same microRNA will not be used as a biomarker and therapeutics target. But still, more deep studies should be necessary to evaluate the correlation between the circulating microRNAs and the neuro-physiological condition (Jimenez-Mateos, 2015).

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