“FEDERICO II” UNIVERSITY OF NAPLES

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PhD PROGRAM IN NEUROSCIENCE

XXX CYCLE

IDENTIFICATION OF MICRORNAs REGULATING IONIC HOMEOSTASIS AS DIAGNOSTIC AND THERAPEUTIC TOOLS FOR CEREBRAL ISCHEMIA

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1. INTRODUCTION

1.1 Premise

Cerebral ischemia is a multifactorial disease accounting for 9% of global mortality, and it's estimated to be the second most common cause of death worldwide (Donnan, 2008; Khoshnam et al., 2017), causing 44 million physical disabilities annually, with 5.5 million deaths.

The triggering event of stroke results from cerebral artery occlusion obstructing blood flow to a portion of the brain, caused by two possible events: hemorrhagic or ischemic stroke. The first case is due to the rupture of a cerebral vessel and accounts for 15% of all strokes. On the other side, in the ischemic stroke a thrombus or embolus are responsible for the obstruction of the vessel that supplies the brain, accounting for 85% of cases. Neurological deficits which occur following stroke include balance problems, hemiplegia, loss of sensory and vibratory sensation, numbness, decreased reflexes, ptosis (of the eyelid), visual field defects, aphasia, and apraxia. Based on the origin of occlusion, cerebral ischemia can be classified into 2 different types: global or focal cerebral ischemia. In particular, in focal ischemia a blood clot causes the blockage of a cerebral vessel, with reduction of blood flow in a specific brain region, increasing the likelihood of brain cell death of that area; depending on the region of the brain that has been damaged, the presentation of symptoms will differ. Global cerebral ischemia is a condition in which the entire blood flow to the brain has been stopped or reduced; this may occur due to cardiovascular abnormalities that lead to significant periods of low blood pressure. According to the etiologies, ischemic stroke can be further classified into four main categories as follows: small vessel disease, athero-thrombotic, cardio-embolic, and undetermined causes (Amarenco et al., 2009).

In both cases (ischemic or hemorrhagic stroke), the loss of blood flow induces, within minutes of a focal ischemic stroke, rapid cell death in the "core" of the ischemic region (the brain area supplied by the occluded vessel and irreversibly damaged). On the other side, in the surrounding region, called "penumbra", where cells are metabolically active and potentially salvageable, are triggered mechanisms that induce changes of ionic concentrations and production of free radicals, such as

reactive oxygen and nitrogen species, leading to apoptosis-mediated injury (Broughton et al., 2009; Ferrer et al., 2003). In particular, the general idea up to twenty years ago was that energy failure leads to the inactivation of active ion pumps, such as Na^+/K^+-ATPase and Ca^{2+}-ATPase, inducing membrane depolarization of neurons, with ensuing glutamate release and causing a dramatic rise in intracellular calcium concentration during ischemia. (Dirnagl et al., 1999; Brouns et al., 2009). Moreover, intracellular Ca^{2+} overload is accompanied by Na^+ and Cl^- entry, while K^+ is released into the extracellular space. Ca^{2+} ions, functioning as intracellular messenger, overactivate numerous enzyme systems (proteases, lipases, endonucleases, etc.), causing cytoskeleton and membrane damage. In parallel, stroke pathophysiology involves also the production of free radicals, that play an important role in the mechanisms of mitochondrial damage (Niizuma et al, 2009), DNA damage and the activation of inflammatory processes (Khoshnam et al., 2017) (Figure 1.1). In this scenario, ischemic penumbra becomes therapeutic target for cerebral ischemia, and several works conducted in the last years aimed to arrest the apoptotic cascade occurring in this region.

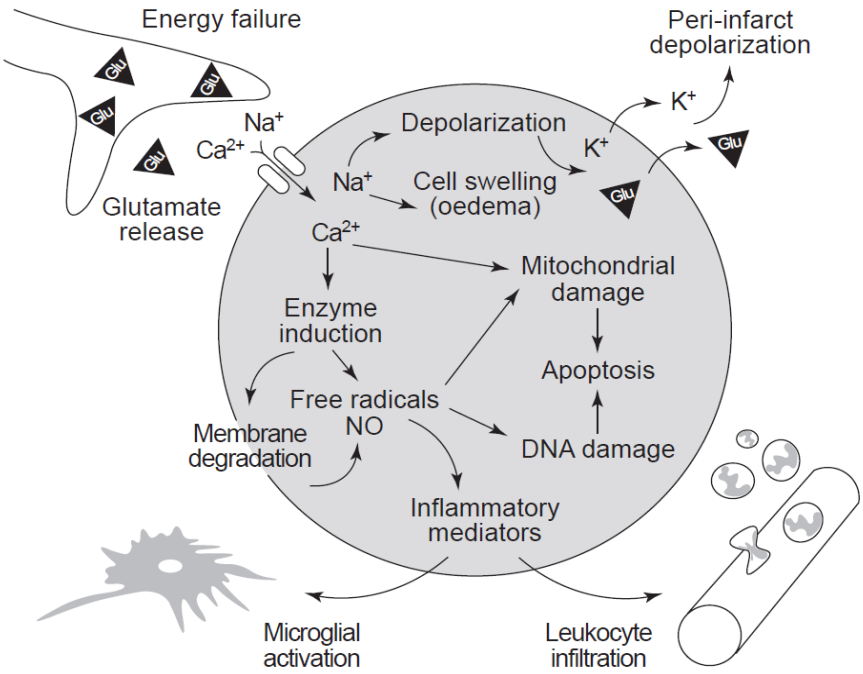

Figure 1.1: *Schematic representation of mechanisms activated in brain ischemia (by Dirnagl et al., 1999).*

Two basic therapeutic directions have emerged for acute treatment of ischemic stroke (Brouns et al., 2009). The first strategy targets the insult itself, trying to rapidly restore focal cerebral blood flow by mechanically removing an arterial thrombus or by thrombolytic drugs, and at present, the only effective treatment for stroke is limited to recombinant tissue plasminogen activator (tPA) (Bivard et al., 2013). Thrombolytic drugs dissolve (lyse) thrombi in the vascular bed by activating plasminogen to form plasmin, that is a proteolytic enzyme that breaks the crosslinks between fibrin molecules to destabilise the structural integrity of blood clots. In particular, tPA is a serine protease found on the endothelial cells lining blood vessels and is involved in the breakdown of blood clots. However, treatment benefit is time-dependent, because of the short therapeutic time window, related to stroke pathophysiology and penumbra restoring (Donnan, 2008), and for these reasons just a small portion of patients can receive this therapy. Currently, it's considered to be a good prospect of success acting within 4.5 hours after the ischemic event, as tested by the two National Institute of Neurological Disorders and Stroke (NINDS) trials (parts 1 and 2), the first two ECASS trials, two Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke (ATLANTIS) trials and the third ECASS trial (Hacke et al., 2017; Lees et al., 2010; ATLANTIS, ECASS, and NINDS rt-PA Study Group Investigators, Lancet, 2004).

The other aspect of experimental studies on stroke therapy concerns the phenomenon of neuroprotection, which aims to reduce the intrinsic vulnerability of penumbra. Many therapies have been developed in order to stimulate neuroprotection, acting on several factors, such as free radicals, glutamate-mediated excitotoxicity, immune response, hypothermia, however all clinical trials, based upon these factors, for the treatment of acute stroke have so far failed (Rajah et al., 2017; Patel et al., 2017).

In the last twenty years, the paradigm of glutamate excitotoxicity as unique cause of ischemia-mediated cell death has been re-evaluated and discussed, and in the meantime numerous studies had identified other death pathways, related to ionic deregulation, independent from the activation of glutamate receptors (Hossmann, 2009; Leng et al., 2014). Many reasons induced researchers to move the research about stroke pathophysiology from the study of mechanisms activated by glutamate axis to the identification of novel molecular pathways. The reasons of the failure of

excitotoxicity theory have been summarized in the so-called “Hossmann postulates” (Hossmann et al., 1996; Annunziato, 2009, Springer):

- Ischemic injury and glutamate exposure evoke different metabolic and biological responses;
- Increase of glutamate levels is not necessary in ischemia models for the induction of the pathological process;
- The ischemic penumbra is structurally preserved, so it’s not affected by its trophic environment;
- There is a time discrepancy between glutamate toxicity in vitro, that is a delayed phenomenon, and ischemic penumbra survival, that occurs from the beginning of injury;
- The action of inhibitory neurotransmitters, whose release is enhanced by ischemia, should reduce the harmful excitatory effect elicited by glutamate.

For these reasons, over the years, several other mechanisms of ionic imbalance have been investigated in order to identify new neuroprotective agents against stroke and brain injury. Thus, recently, the attention of scientific community has been focused on some plasma-membrane proteins controlling Ca^{2+} ,Na^{+}, K^{+} and H^{+} ion influx or efflux, indeed, expression or activity changes of these proteins could represent the molecular basis for the variation of ionic homeostasis independent by glutamate excitotoxicity (Figure 1.2).

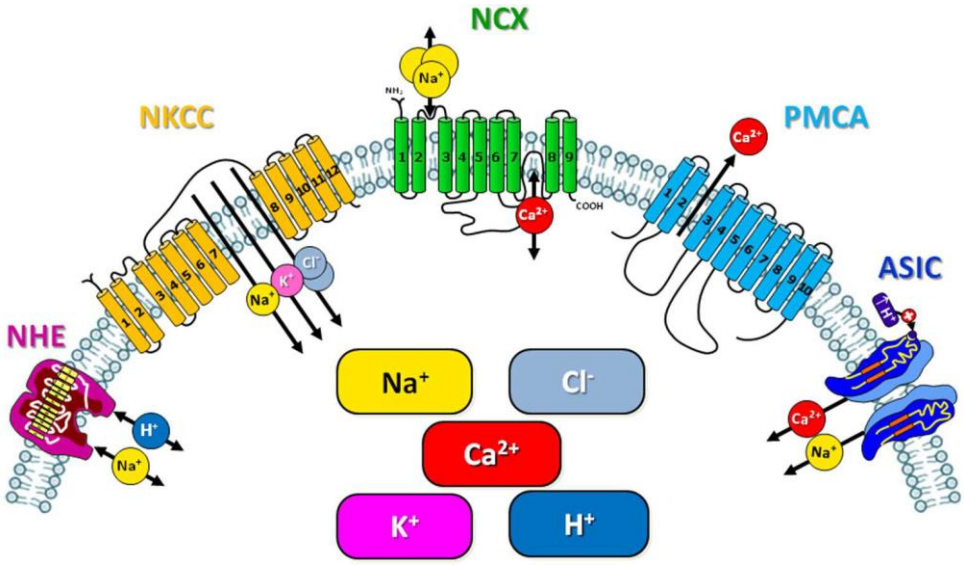

Figure 1.2: *Schematic representation of the ionic exchangers/transporters involved in ionic homeostasis maintenance during brain ischemia (by Cuomo et al., 2015).*

In particular, cytosolic Ca^{2+} plays a key role in intracellular signaling in all types of cells. Indeed, in order to activate mechanisms necessary for survival and cellular functioning, variations in cell calcium levels are indispensable. These oscillations must be short-lived, because it is necessary that calcium is quickly removed by cytosol. Moreover, in some cells, such as neurons, calcium variations must also be spatially confined in order to avoid simultaneous activation of the various calcium-controlled processes and of unnecessary pathways (Blaustein and Lederer, 1999). Calcium increase required by the cells is mediated by transport systems through the cell membrane, or from intracellular calcium stores, such as the endoplasmic reticulum. Under normal conditions, these systems unlikely cause a massive increase in cytosolic calcium levels, because specific buffering systems work to detect the smallest calcium variations, causing a rapid reduction of Ca^{2+} levels, thus allowing local responses to the exact points of calcium variation.

Neuronal stimulation induces changes of intracellular calcium concentration that in turn triggers several mechanisms mediating numerous nerve cell functions. In order to avoid that Ca^{2+} cell levels are high for a long time thus becoming toxic and in order to allow cells to be able to respond to a new stimulus, several pathways work together to restore calcium levels. Among them there are Ca^{2+} binding proteins that avoid abnormal intracellular Ca^{2+} through sequestration into the endoplasmic reticulum and mitochondria, and through the extrusion across the plasma membrane (Zaidi, 2010). The latter process is operated mostly by the low affinity-high capacity Na^{+}/Ca^{2+} exchanger (NCX) and by the high affinity-low capacity plasma membrane Ca^{2+}-ATPase (PMCA) (Brini et al., 2011; Brini et al., 2013). In some conditions, Ca^{2+} flux across membrane is the predominant mechanism of Ca^{2+} removal from cytosol compared to refilling of stores, for example after activation of localized signals in the dendritic spines of neurons. In particular, in these cases, while PMCA transport proteins control the resting levels of this ion, NCX proteins play a role in calcium homeostasis following the increase of levels during a signal event. For these reasons, a great deal of studies are being indicated to clarify the role of NCX in calcium homeostasis in specific conditions of dysregulation such as it occurs in the ischemic stroke.

1.2 NCX

NCX gene family belongs to the superfamily of Ca^{2+}/cation antiporter genes (CaCA), which comprises five major members groups: YRBG, in bacteria; CAX (Ca^{2+}/anion exchanger), mostly in plants and yeasts; NCX (Na^{+}/Ca^{2+} exchanger) and NCKX (Na^{+}/Ca^{2+}-K^{+} exchanger), both comprising almost exclusively vertebrate members; and, finally, CCX (Ca^{2+}/cation exchanger), which is still partially characterized (Lytton, 2007; He and O'Halloran, 2014). All members of this superfamily of transporters are characterized by 10 transmembrane domains organized in two clusters of five hydrophobic putative helices, connected by a cytoplasmic loop, and specific amino acid sequences called α-repeat regions, that represent key factors to form the ion-binding pocket, important for the function of the transporters.

Regarding the NCX family (Solute like carrier 8A, SLC8A), in mammals three isoforms encoded by different genes located in different chromosomes were identified: NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996). However, alternative splicing mechanisms result in the production of multiple isoforms from each primary transcript causing NCX expression in a tissue-specific manner (Lee et al., 1994; Schulze et al., 1996). The attention for the present study was focused on NCX1 isoform, which is the most broadly expressed member of the SLC8 family, and on NCKX2, both involved in stroke pathophysiology.

1.2.1 NCX1 Physiology

NCX is expressed in various tissues with the highest expression found in cardiac and skeletal muscle, brain tissue and vascular smooth muscle and urinary bladder smooth muscle (Murata et al., 2010). Specifically, NCX1 localizes at both the presynaptic and postsynaptic endings and in the endoplasmic reticulum membrane of neurons (Canitano et al., 2002), but high expression was also reported in axons, dendrites, growth cones (Luther et al., 1992), and in the inner membrane of the nuclear envelope. Recently, NCX1 brain localization has been well characterized. In the cerebral motor cortex, particularly in the pyramidal neurons of layers III and V, NCX1 isoform is intensively expressed (Canitano et al., 2002). In the hippocampus, NCX1 protein expression is particularly intense in the granule cell layer and in the hilum of the dentate gyrus (Annunziato et al., 2004; Papa et al., 2003). Moreover,

NCX isoforms are also expressed in crucial areas for the extrapyramidal control of motor coordination, for example NCX1 mRNA is detectable in the substantia nigra pars compacta, whereas protein product is present in the striatum. Finally, the analysis of cerebellum showed the highest density of cells positively stained for the mRNAs encoding for all three NCX isoforms, specifically distributed in the various neuronal populations.

In addition, NCX1 plays an important role also in non-neuronal cells, maintaining ion homeostasis over long term and under conditions of prolonged or acute ionic dysregulation in astrocytes, oligodendroglia and microglia (Parpura et al., 2016). In this scenario, this antiporter takes part to the mechanism of induced Ca^{2+}-dependent exocytotic release of glutamate from astrocytes (Reyes et al., 2012), a phenomenon important for the modulation of some neuronal functions, such as synaptic transmission and plasticity.

The human NCX1 gene includes 12 exons, but the major part of the mature protein is encoded by exons 2, 11, and 12 (Kraev et al., 1996), and at least different 15 splice variants. NCX1 protein is composed of about 900 aminoacids (938 in the canine heart), with a theoretical molecular mass of 120 kDa (Annunziato et al., 2004; Annunziato et al., 2009, Springer). In fact, with electrophoretic gels and under non-reducing conditions, two NCX1 bands are visible at 120 and 70 kDa. The first band corresponds to the native protein, whereas the other band represents a proteolytic fragment (Saba et al., 1999; Van Eylen et al., 2001). This membrane protein is composed of 10 transmembrane segments (TMS) (Ren and Philipson, 2013), with a large intracellular loop of 550 amino acids between TM5 and TM6, named f loop (Nicoll et al., 1999) (Fig. 1.3). The first five TMS are grouped into an N-terminal hydrophobic domain, with the amino terminus located in the extracellular space, whereas the C-terminal hydrophobic domain is composed of the last five TMS and a carboxyl terminus located intracellularly (Sharma and O'Halloran, 2014). The f loop is not directly implicated in Na^+ and Ca^{2+} translocation, though it is responsible for the regulation of NCX activity elicited by several factors, such as the same Na^+ and Ca^{2+} ions (Khananshvili, 2013). Indeed, this loop contains two stimulatory Ca^{2+}-binding domains named CBD1 and CBD2, which are regulatory domains required for intracellular ion sensing and binding (Giladi et al., 2016). CBD1 displays a high affinity for Ca^{2+} and so it's able to detect small increases in cytosolic Ca^{2+}, thus

inducing a conformational change that is thought to be responsible for the known regulatory effect of Ca^{2+} binding on NCX1 activity (Boyman et al., 2011). On the other hand, CBD2 binds Ca^{2+} with lower affinity and without a corresponding large conformational change (Hilge et al., 2006). The intracellular loop also contains the exchange inhibitory peptide (XIP) domain, a 20 amino acid sequence, located on the N-terminus side, which confers sodium-dependent inactivation properties; the mutation of this sequence can increase or remove NCX Na^{+}-dependent inactivation (Matsuoka et al., 1997). The action mode of XIP on NCX inactivation is mediated by a C-terminal portion of the f loop, that seems to interact with XIP domain when Na^{+} concentration increases, causing NCX inhibition (Maack et al., 2005). Other important regulatory domains are α1-repeat and α2-repeat located between TM2 and TM3 on the extracellular space, and between TM7 and TM8 on the intracellular space, respectively. These α-repeat domains are membrane loops that contain residues essential for cation binding and transport (Winkfein et al., 2003). In particular, experiments of mutational analysis suggest that these domains may form a portion of the ion translocation pathway (Ottolia et al., 2005).

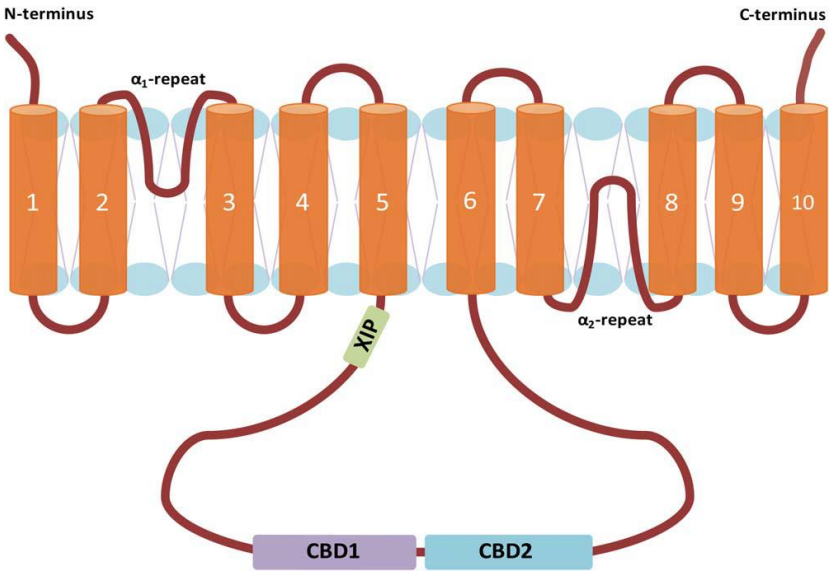

Figure 1.3: *Schematic representation of NCX1 structure (by Sharma and O'Halloran, 2014)*

NCX is a ‘‘low affinity-high capacity’’ system that can rapidly allow to respond to transient changes in Ca^{2+} concentration, exploiting the Na^{+} gradient across the plasma membrane as a source of energy, working with a stoichiometry of three or four Na^{+} ions for one Ca^{2+} ion depending on their cell concentration (Reeves and Hale, 1984; Hilgemann, 2004). In addition, this exchanger mediates both Ca^{2+} and Na^{+} flow in a bidirectional way through the plasma membrane (Blaustein and

Lederer, 1999; Philipson and Nicoll, 2000). Indeed, according to the intracellular and extracellular [Na^+] and [Ca^{2+}] and the membrane potential, it can operate either in forward mode (inducing Ca^{2+} extrusion) or reverse mode (causing Ca^{2+} entry). In resting excitable cells, when intracellular [Ca^{2+}] rises and it's necessary the return to resting levels, NCX works in forward mode, coupling the extrusion of Ca^{2+} to the influx of Na^+ ions into the cells down their electrochemical gradient, and keeping low the [Ca^{2+}] across the cell membrane. However, in other physiological circumstances, or in some pathological conditions, in which depolarization occurs and [Na^+] rises, transmembrane Na^+ electrochemical gradient is reduced and NCX begins to work in reverse mode, with subsequent increase of intracellular [Ca^{2+}]. This event is not always associated to a detrimental consequence, but is also the triggering factor of some physiological processes. In rat cortical astrocytes, NCX reverse mode of action induces a calcium increase that is the main mediator of glutamatergic glial transmission (Reyes et al., 2012). The same result was described in Purkinje neurons, in which calcium influx through NCX enhances the neurotransmitter release with subsequent postsynaptic effect (Roome et al., 2013). The choice of operating in forward or reverse mode depends on the difference between the reversal potential of NCX (ENCX) and the membrane potential (VM). In particular, if VM is more negative than ENCX, the exchanger works in forward mode. In contrast, when the VM value is more positive than reversal potential ENCX, NCX operates in Ca^{2+} entry mode.

Cytosolic Ca^{2+} and Na^+ ions can regulate the activity of NCX proteins acting in sites that are not directly involved in ion translocation (Matsuoka et al., 1995; Dipolo and Beaugé, 2006; Khananshvili, 2013). Ca^{2+} allosteric activation of NCX1 is really important in excitable tissues in order to allow rapid changes in cytosolic [Ca^{2+}]. This ion interacts directly with CBD domains of the brain and the cardiac isoform of NCX1, inducing its activation, and just submicromolar concentrations (0.1 – 0.3 μM) of intracellular Ca^{2+} can be picked up to activate the antiporter (Hilgemann et al., 1992). Moreover, when intracellular Ca^{2+} ions are removed NCX1 activity is blocked (Philipson and Nicoll, 2000). Conversely, intracellular Na^+ is also able to regulate NCX1, but induces an inactivation process of the exchanger (Hilgemann et al., 1992), named Na^+-dependent inactivation. When intracellular Na^+ increases, it binds to the transport site of the exchanger, with subsequent inactivation of ionic transport. This process is mediated by the XIP region in the f loop, characterized by a negative charge. When this site is occupied, a conformational change occurs in the f loop,

resulting in the inhibition of NCX1 (Li et al., 1991; Pignataro et al., 2004; Molinaro et al., 2015).

Lastly, NCX1 activity is strongly affected by pH, indeed a mild increase of intracellular H^+ inhibits NCX1 activity under steady-state conditions (Doering and Lederer, 1993), as well as under ischemia conditions (Dipolo and Beaugé, 2006).

Different studies suggest that metabolic state of the cell can influence NCX1 activity through ATP levels (Annunziato et al., 2004; Khananshvili, 2013; Dipolo and Beaugé, 2006). In particular, ATP functions as a phosphoryl donor molecule and can activate numerous pathways that can stimulate NCX activity. One of this mechanisms is mediated by PKA and PKC (Dipolo and Beaugé, 1998), activated by G-protein-coupled receptors. Moreover, the same ATP molecules are important for the operation mode of protein kinases. In addition, another pathway involved in NCX1 modulation by ATP is represented by phosphatidylinositol 4,5-bisphosphate (PIP_2) (Hilgemann and Ball, 1996), that seems to regulate only the cardiac isoform of NCX1. This mode of action of PIP_2 on NCX1 occurs in the XIP region, so preventing the Na^+- dependent inactivation and stimulating NCX function.

The mammalian NCX1 gene transcript is composed of six exons, named A through F, and it is subjected to alternative splicing to produce different and tissue specific mRNA. Indeed, all transcripts with exon A are present in excitable cells, such as muscle cells and neurons (Schulze et al., 2002; Sharma and O'Halloran, 2014). The cardiac isoform of NCX1 seems to be particularly regulated by the serum response factor (SRF) and GATA-4 (Xu et al., 2006), which work as *cis* elements. The first one is a transcription factor that regulates genes involved in the early stage of embryonic development; the other one takes part to embryogenesis and myocardial differentiation. Moreover, NCX1 promoter contains consensus sequences for the zinc finger transcription factor Sp-1 and binding sites of AP-2 (Annunziato et al., 2009, Springer). Experiments performed on PC12 neuronal cell lines demonstrated that extracellular-signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPKs) regulate the expression and the activity of NCX1 (Sirabella et al., 2012). In particular, upon Neural growth factor (NGF) stimulation, NCX1 is upregulated by ERK1/2 and p38. This MAPK-dependent transcriptional regulation of ncx1 gene seems to be mediated by Sp1 and CREB1

transcription factors, and after transfection with siRNA against CREB1 and Sp1, NGF-induced expression changes are prevented.

Finally, NCX1 expression is also affected by hormones and metabolites (Herchuelz et al., 2007): in pancreatic β cells, in which the extrusion of intracellular Ca^{2+} is mediated by PMCA in normal conditions, after glucose exposure the Ca^{2} efflux from cells is mediated by NCX.

The Na^{+}/Ca^{2+} exchanger protein may play a relevant function in different neurophysiological conditions. In neurons, expression of NCX is particularly high in those sites where a large movement of Ca^{2+} ions occurs across the plasma membrane, as it happens at the level of synapses. In fact, in this cell compartment, Ca^{2+} entry is essential to promote neurotransmitter exocytosis, but then it's rapidly extruded by the plasma membrane Ca^{2+} ATPase and by NCX. Notably, NCX represents the dominant Ca^{2+} extrusion mechanism when intracellular $[Ca^{2+}]$ is higher than 500nM, as it happens after a train of action potentials in the nerve terminals. Few studies have evaluated the role of NCX isoforms in learning and memory. Mice deficient for NCX2 function in hippocampal neurons showed prolonged time for clearance of the increased $[Ca^{2+}]$ induced by neuronal activation, and exhibited alteration of Long Term Potentiation (LTP) and synaptic plasticity; moreover, the animal's capacity for learning and memory enhanced (Jeon et al., 2003). These studies demonstrated that NCX2-mediated Ca^{2+} homeostasis is critical for the control of synaptic plasticity and cognition. On the other side, a most recent study investigated the potential involvement of NCX3 for LTP modulation, evidencing that NCX3 knock-Out mice displayed detrimental consequences on basal synaptic transmission, LTP regulation, spatial learning, and memory performance (Molinaro et al., 2011).

NCX is the major Ca^{2+} efflux mechanism of ventricular cardiomyocytes, hence the exchanger plays a critical role for the cardiac contractility and in the excitation-contraction (EC) coupling, maintaining a dynamic balance between Ca^{2+} entry and Ca^{2+} exit during the action potential (Ottolia et al., 2013). Consequently, reductions in Ca^{2+} efflux by the exchanger raise sarcoplasmic reticulum stores, causing increased cardiac contractility. By contrast, enhanced NCX-mediated Ca^{2+} efflux activity, for example during heart failure, affects the excitation-contraction coupling.

Furthermore, studies with the NCX knockout models provided information on NCX contributions to smooth muscle vasoconstriction (Zhang et al., 2010) and to blood pressure regulation (Zhang, 2013).

1.2.2 NCX1 in Pathophysiological Conditions

The pivotal role played by NCX in controlling Ca^{2+} and Na^{+} homeostasis confers to this transporter particular relevance in various pathophysiological conditions.

White matter injury after anoxia or trauma is known to be induced by Ca^{2+} influx mediated by NCX, forced to operate in reverse mode. Thus, it was demonstrated that inhibition of NCX activity was able to reduce white matter degeneration in different experimental models of axonal injury, such as optic nerve anoxia (Stys et al., 1990), spinal cord injury (Tomes et al., 2002), and stretch injured axons (Wolf et al., 2001). In experimental allergic encephalomyelitis and multiple sclerosis, evidence showed that massive sodium influx into axons through voltage-gated Na(v)1.6 sodium channels triggers reverse operation of the exchanger and subsequent enhancement of intra-axonal calcium that is associated with the axonal degeneration of the spinal cord (Craner et al., 2004).

Impairment of Ca^{2+} homeostasis is closely related to the process of brain aging (Annunziato et al., 2002). Indeed, studies performed on cortical nerve endings of aged rats have showed that NCX activity is significantly decreased (Canzoniero et al., 1992), and this seems to be the consequence of a reduced affinity of the antiporter for Ca^{2+} ions (Michaelis et al., 1984). Moreover, in aged brain showing accumulation of aggregated β-amyloid peptides, recent studies demonstrated that aggregated peptides could interact with the hydrophobic intracellular loop of the exchanger inhibiting its calcium extruding function (Wu et al., 1997). In fact, in Alzheimer's disease, Amyloid β peptide 1-42 in mouse neurons induces reversal of NCX function and calcium influx (Pannaccione et al., 2012). At the same time, a large number of studies have shown that the neurotoxicity exerted by the amyloid protein is intimately related to intracellular Ca^{2+} concentrations, and the attenuation of intracellular $[Ca^{2+}]$ enhancement caused a reduction of neural damage induced by the amyloid protein (Weiss et al., 1994). Recently NCX expression evaluated in Alzheimer patients resulted in a variable expression of the three transporters in the

parietal cortex cells: NCX1 and NCX2 expression was increased whereas NCX3 expression was reduced (Sokolow et al., 2011).

Outside the nervous system, maintenance of calcium homeostasis is extremely important during pregnancy and labor where it plays a significant role in uterine smooth muscle contraction and fetal implantation (Daston and Naciff, 2005; Salamonsen et al., 2001). Developmental processes like fetal bone mineralization are dependent on the placental transfer of calcium and phosphorous ions from maternal blood to fetal blood and calcium exchange is high during late gestation to meet the developing needs of the mineralizing fetal skeleton. NCX1 expression was observed in the placenta; in particular, NCX1 mRNA expression is altered during the second and third trimester and a rapid increase is observed at the 40 week stage (Yang et al., 2011).

Altered expression and function of the cardiac isoform has been reported in various forms of heart disease such as ischemia and heart failure (Bers, 2002; Sipido et al., 2002, 2006). Indeed, overexpression of NCX1 protein and/or its increased activity can contribute to contractile dysfunction and arrhythmogenesis. Similarly, upregulation of vascular NCX1 has been implicated in human primary pulmonary hypertension and in several salt-dependent hypertensive animal models (Iwamoto et al., 2004; Zhang et al., 2010).

Under pathological conditions, e.g. in muscular dystrophies, an excessive increase in cytosolic-free Ca^{2+} concentration leads to muscle degeneration (Sokolow et al., 2004). Thus, in human Duchenne Muscular dystrophy, characterized by genetic defect in dystrophin production, the result is an intracellular Ca^{2+} overload and muscle degeneration (Kuyumcu-Martinez and Cooper, 2006).

The activity of NCX in controlling Ca^{2+} levels is also important for pro-inflammatory functions in human macrophages and monocytes (Staiano et al., 2009). Finally, pancreatic β-cells express specific isoforms of NCX1 (Herchuelz et al., 2002, 2007), which display a high capacity and contribute to controlling insulin release. In particular, downregulation of NCX1 leads to an increase in β-cell function, proliferation, mass, and resistance to abnormal metabolic changes occurring in diabetes; therefore, development of selective inhibitors of NCX1 pancreatic isoforms may have clinical interest also in this pathology.

In the last fifteen years, several studies have evidenced the neuroprotective role of the isoform 1 of NCX in brain ischemia. Indeed, the idea that calcium and sodium dysregulation has a key role in stroke physiopathology induced many researchers to focus their studies on the possible neuroprotective role of NCX1 in brain ischemia (Annunziato et al., 2009, Springer; Khananshvili, 2013; Pignataro et al., 2004; Shenoda, 2015). After middle cerebral artery occlusion (MCAO), an animal model mimicking human brain ischemia with a large cerebral infarction involving basal ganglia and cortex, NCX1 protein expression is strongly reduced in the damaged area, and the percentage of infarct volume enhances when NCX is further reduced by genetic ablation with antisense oligodeoxynucleotides (Pignataro et al., 2004; Annunziato et al., 2007; Pignataro et al., 2014). NCX1 transcript analysis mirrors what happened with protein levels, being downregulated in the ischemic core, including prefrontal cortex and part of the striatum (Boscia et al., 2006). Similar results were obtained by NCX1 silencing experiments (Formisano et al., 2013) and NCX1 knockout mice (Molinaro et al., 2016). Moreover, pharmacological inhibition of NCX (Annunziato et al., 2004; Iwamoto et al., 2004) was demonstrated to increase infarct volume, as suggested by *in vivo* experiments after administration of the NCX blockers GLU-XIP, CB-DMB and Bepridil, that caused a worsening of the brain infarct lesion (Pignataro et al., 2004). On the other hand, NCX1 pharmacological activation (Pignataro et al., 2004a; Molinaro et al., 2013) ameliorates stroke consequences, as well as stimulation of NCX activity by redox agents (Amoroso et al., 2000) and NCX1 overexpression experiments (Molinaro et al., 2016).

In summary, an explanation of the neuroprotective action of NCX in ischemia and of the effect exerted by its inhibitors concerns the different mode of action of this antiporter in the ischemic core and in the penumbra regions (Figure 1.4). Indeed, it can be hypothesized that NCX1 still works in forward mode in the penumbra region, in which pump ATPase activity is still preserved. However, after its inhibition, the extrusion of Ca^{2+} ions in this area is reduced, thus enhancing Ca^{2+}-mediated cell injury. On the other side, in the ischemic core, in which energy failure leads to reduction of ATP levels and so to Na^+/K^+-ATPase inactivation, intracellular Na^+ ions massively accumulate promoting NCX1 to operate in the reverse mode. This mode of operation, that certainly does not improve the general situation in the ischemic core, is important to help cells to survive to the high intracellular levels of Na^+ and to water overload.

During brain ischemia, different factors work to modulate SLC8A1 gene expression. Under anoxic conditions in neurons, NCX1 is up-regulated by the transcription factor "nuclear factor kabba B" (NF-kB) (Sirabella et al., 2009), inducing a Ca^{2+} increase within the endoplasmatic reticulum. Furthermore, NCX1 is a target gene of the Hypoxia Inducible Factor-1 (HIF-1), that is required for the transcriptional activation of many genes implicated in stroke (Valsecchi et al., 2011). Notably, HIF-1α inhibition reverts SLC8A1 upregulation occurring during brain ischemic preconditioning (see paragraph 1.3). Finally, SLC8A1 gene is reported to be a REST target gene (Formisano et al., 2013), that negatively regulates NCX1 expression under conditions of cerebral ischemia.

Taken together, all these results have demonstrated that NCX1 activation could represent an important mechanism of neuroprotection, and in the last decade many works have been conducted to discover molecular pathways and mechanisms that regulate NCX1 function and activation, in order to identify new molecular targets able to induce neuroprotection.

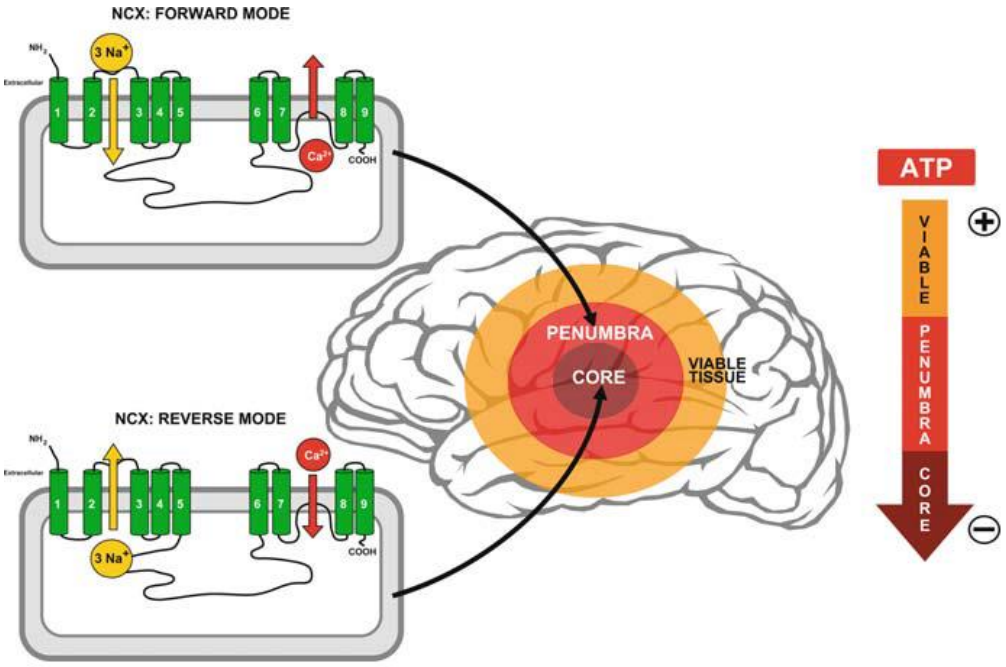

Figure 1.4: *Hypothetical mode of operation of NCX in the penumbra and core regions of ischemic Brain (by Annunziato et al., Springer, 2009)*

1.2.3 NCKX2 Physiology

The K^+-dependent Na^+/Ca^{2+} exchanger (NCKX) is the second group of proteins belonging to the superfamily of cation/Ca^{2+} plasma membrane exchangers. It promotes the exchange of Na^+ with Ca^{2+} and K^+, in a bidirectional way and with a stoichiometry of 4 Na^+ ions for 1 Ca^{2+} and 1 K^+ ion (Cervetto et al., 1989; Dong et al., 2001). It was originally described in the outer segments of vertebrate retinal rod photoreceptors (Schnetkamp et al., 1989) as the major Ca^{2+} extrusion pathway.

The NCKX gene family (named SLC24 in human) includes at least five distinct members, whose the fifth was identified but not yet characterized as a functional exchanger. In particular the transcript of isoform 1, NCKX1, has the most restricted tissue distribution and is only found in retinal rod photoreceptors and in platelets; NCKX2 is found in brain, in retinal ganglion cells and in cone photoreceptors; finally NCKX3 and NCKX4 transcripts are more widely expressed (Schnetkamp, 2004; Lytton et al., 2002).

The second member of the K^+-dependent family of Na^+/Ca^{2+} exchangers, NCKX2, was first cloned from rat brain (Tsoi et al., 1998) and subsequently from chick and human cone photoreceptors (Prinsen et al., 2000). This gene is broadly expressed in brain neurons (Schnetkamp, 2004) and co-expressed with NCX1 in many brain regions (Tsoi et al., 1998). Regarding cell localization, in situ hybridization experiments revealed that its expression is confined to neuronal cell bodies, particularly in all six layers of the parietal cortex, the molecular layer of the cerebellum, and throughout the neurons of the hippocampus, with highest levels contained in CA3 neurons (Lytton et al., 2002). In some regions, such as neocortex and hippocampus, expression of NCKX2 is overlapped with that of NCX1, but its activity is predominant in hippocampal CA1 neurons, whereas in forebrain the activity is higher than that of NCX1.

The various NCKX isoforms share the same predicted membrane topology, with eleven transmembrane segments (TMs 0-10), grouped in two sets (TMs 1-5 and TMs 6-10) that are connected via a large cytoplasmic loop (Kinjo et al., 2003) (Figure 1.5). The first segment (TM 0) is thought to be cleaved by a signal peptidase after protein translation (Kang et al., 2003; Shibukawa et al., 2007), and so in the mature protein, both the N-terminus and C-terminus are extracellular. The resulting N-terminal extracellular domain is glycosylated (Lytton et al., 2007).

The TMs also contain the two alpha repeats, two sequence elements that are thought to have arisen from an ancient gene duplication event and that are shared between members of the NCKX gene family and members of the NCX gene family. Scanning mutagenesis of the α-repeats of human NCKX2 showed that mutation of about 25% of these residues led to impaired NCKX2 proteins (Winkfein et al., 2003), and mutations in specific key residues induced a decrease in affinity for both cations (Kang et al., 2005). The exchanger protein likely contains a single cation binding pocket that can accommodate either 4 Na^+ ions or 1 Ca^{2+} and 1 K^+ ion, with a conformational change from inward facing to outward facing or *vice versa* (Visser and Lytton, 2007). In this way, NCKX2 performs its exchange activity through a sequential transport mechanism.

NCKX2 was recently shown to become inactivated in a time- and Na^+-dependent manner, suggesting that NCKX2, like NCX1, is also subjected to ionic regulation (Altimimi et al, 2007). Moreover, NCKX gets several consensus sites for protein kinase C (PKC) phosphorylation in its putative cytoplasmic loops (Lee et al., 2006) (Figure 1.6).

In acute preparations of axon terminals from rat neurohypophysis, it has been shown that more than 60% of the Ca^{2+} clearance is mediated via Na^+/Ca^{2+} exchangers, whose 90% is dependent on K^+ (Lee et al., 2002), therefore NCKX appears to be able to extrude Ca^{2+} more efficiently compared to other members of NCX family. Furthermore, other evidences demonstrated that NCKX contribution for Ca^{2+} homeostasis was specifically localized to the axon terminals (Kim et al., 2003) (Figure 1.6).

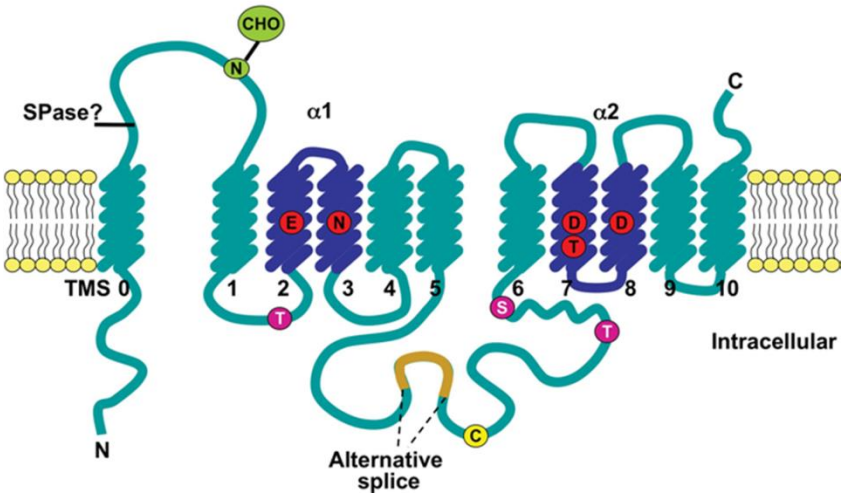

Figure 1.5: *Schematic representation of NCKX2 structure (by Lytton, 2007).*

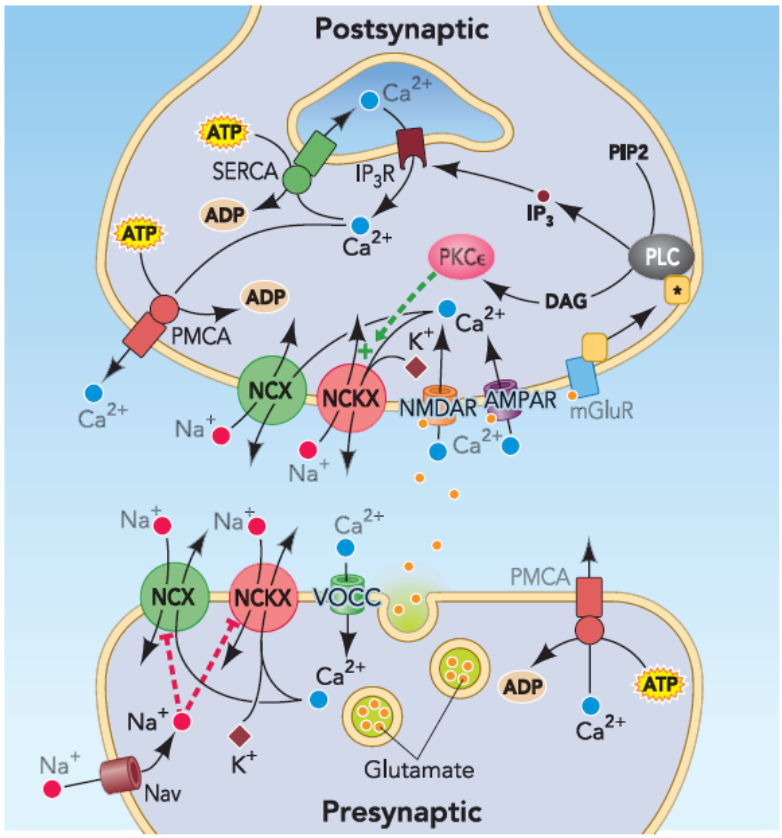

Figure 1.6: *Physiological contexts for NCKX function in Ca2+ signaling (By Visser and Lytton, 2007).*

1.2.4 NCKX2 in Pathophysiological Conditions

At present, only few studies have been conducted in order to assess the involvement of NCKX2 in human diseases. Notably, a clear physiological role was established in retinal rod and cone photoreceptors, where NCKXs represent the only Ca^{2+} extrusion protein present in the plasma membrane. Changes in calcium and sodium concentrations within the photoreceptor outer segments are crucial for phototransduction. Indeed, in the dark, calcium ions enter the rod and cone outer segments through open cyclic guanosine monophosphate (cGMP)-gated cation channels and are extruded by the rod and cone NCKXs. In response to light, cGMP-gated cation channels close while NCKX continues to function, resulting in a reduction in the calcium concentration within the photoreceptor cell, with subsequent hyperpolarization of the plasma membrane. Thus, it was reasonable to have proposed that mutations in the rod and cone exchanger genes might be the cause of some hereditary retinal diseases. In particular, DNA from patients with retinal disease has been screened for rod NCKX1 and cone NCKX2 mutations, identifying mutated alleles of both the NCKX isoforms that appear to be possibly pathogenic (Sharon et al., 2002).

Concerning the role of NCKX2 in brain ischemia, in the single study conducted so far it has been shown that NCKX2 transcript and protein expression are reduced in the ischemic core and surrounding area of rats subjected to permanent ischemia (Cuomo et al., 2008). Moreover, after knockdown of NCKX2 protein expression, a remarkable enlargement of the infarct volume occurred. These results indicate that NCKX2 is involved in brain ischemia, and it may represent a new potential target to be investigated in the study of the molecular mechanisms involved in cerebral ischemia.

1.3 Ischemic Conditioning as Neuroprotective Strategy

The failure of numerous stroke clinical trials has prompted researchers to identify mechanisms of protection endogenously activated after cerebral ischemia, in order to exploit and apply them as new neuroprotective strategies for stroke.

It's well known that various kinds of living organisms, exposing to alteration of environment, can acquire transient tolerance to environmental changes, otherwise lethal. The explanation lies in the activation of cellular response mechanisms, which are observed in a wide variety of species, from bacteria to mammalian cells. For cells and organs living under aerobic conditions, a long period of anoxia or ischemia can be fatal. However also brain is able to respond to tissue and cell damage triggering mechanisms of endogenous protection. Indeed, in normal conditions, brain cells try to reduce cell death and injury by recruiting its defenses (Huber et al., 1999; Dirnagl et al., 2003). This idea is the pillar that holds the concept of conditioning, a phenomenon that consists in the induction of beneficial effects by exposure to low doses of otherwise harmful agents.

Neurons share the same genetic information with other cells, so it's understandable that also the brain can acquire transient tolerance by a preceding stress. The terms 'tolerance' and 'preconditioning' to explain this phenomenon were used for the first time by Janoff about fifty years ago (Janoff, 1964), and then they have been extended by Murry and Kitagawa to indicate ischemic conditioning respectively in the heart (Murry et al., 1986) and in the brain (Kitagawa et al. 1990). Practically, an injurious stimulus, applied below the threshold of damage, is able to induce protection against ischemia. For this reason, in analogy to Janoff's terminology, this phenomenon has been termed 'ischemic conditioning' (IC) or 'ischemic tolerance' (IT). A large number of studies performed in the following years confirmed the existence of this process in the penumbral region in focal ischemia models. Moreover, it was demonstrated through measurement of cerebral blood flow that after IT induction no improvement of regional tissue perfusion was observed, thus supporting the thesis that the state of ischemic tolerance is based on the alteration of neurons themselves at the cellular level (Matsushima and Hakim, 1995).

Actually, these terms span three interventions, temporally different, covering the three therapeutic windows in ischemic and reperfusion injury (IRI): preconditioning (applied before the IRI), perconditioning (during IRI) and postconditioning (after IRI

onset) (Wang et al., 2015; Fairbanks et al., 2010; Hausenloy and Yellon, 2016). Specifically:

- Ischemic Preconditioning (IP) consists in the application of a brief non-injurious episode of ischemia, inducing protection from a subsequent lethal insult (Meller and Simon, 2013).
- Ischemic Perconditioning (IPE) consists in the application of a brief period of ischemia during a prolonged ischemic insult (Wang et al., 2015). It's expressed mostly as Remote Conditioning, in which short periods of ischemia are applied in a distant organ (see paragraph 1.3.5).
- Ischemic Postconditioning (IPO) is a neuroprotective strategy mediated by a modified reperfusion, applied after a prolonged ischemic episode (Pignataro et al., 2008; Zhao, 2009).

When these strategies are performed, the resulting cellular or tissue response underlying the protection to a harmful stress is called ischemic tolerance (Dirnagl et al., 2003; Meller and Simon, 2013). According to the time required for the development of the protective response, two temporally distinct types of IT can occur: an immediate tolerance, in which the trigger induces protection within minutes (Perez-Pinzon et al., 1997), is mediated by posttranslational modifications and the effective duration is brief; a delayed tolerance that develops over hours and days (Kitagawa et al., 1990) and usually involves *de novo* protein synthesis.

Lastly, it was demonstrated that IT also preserves brain functions, such as memory acquisition (Ohno and Watanabe, 1996). Indeed, after the induction of IT in rats subjected to IP and ischemia, which in normal conditions was lethal to CA1 neurons, memory acquisition was not impaired.

1.3.1 Methods to Induce Ischemic Conditioning

Occlusion of the middle cerebral artery (MCAO) by intraluminal insertion of a nylon monofilament into the internal carotid artery is the most common model to induce focal cerebral ischemia in rats and mice (Takano et al. 1997; Durukan et al. 2008). This method was introduced first time in a rat ischemic tolerance experiment, applying 10 minutes of transient middle cerebral artery occlusion (tMCAO) as IP stimulus, and permanent MCAO (pMCAO) as the final ischemia (Barone et al. 1998).

Different time intervals of reperfusion were allowed after IP and before permanent MCAO to establish the optimal duration of reperfusion to achieve the best protection. Among all experimental procedures, hemispheric infarct and neurological deficits were significantly reduced only when IP was performed 1, 2 or 7 days before pMCAO. Repeated brief transient ischemia regimen was also proved as a preconditioning paradigm inducing early IT in mice subjected to permanent focal ischemia (Stagliano et al. 1999).

Later, a modified model of transient focal IP/transient focal MCAO, consisting in the MCAO of adult spontaneously hypertensive rats for 10 min as preconditioning and 1h as ischemia was described (Naylor et al. 2005). In another mouse model, delayed tolerance is achieved by inducing two periods of 5 min tMCAO as IP method against 90 min tMCAO applied in 3 days (Zhang et al. 2008).

According to another recent protocol to induce tolerance (the same used in our laboratory), rats are subjected to 30 min of tMCAO followed, 3 days later, by 100 min of injurious tMCAO. The protection is already evident if the time interval between the preconditioning stimulus and the injurious stimulus is of 1 day, but it reaches the maximum extent of benefit when the harmful stimulus is delivered 3 days after preconditioning (Lusardi et al. 2011; Pignataro et al. 2008, 2009). A similar protocol can be applied to mice, but in that case, the duration of the IP stimulus is 15 min, whereas the duration of the injurious middle cerebral artery occlusion is 60 min. Once again, in order to reach the best protection, the time interval between preconditioning and harmful ischemia must be 3 days (Lusardi et al. 2011).

IT induction can also be mediated by non-ischemic stimuli, a phenomenon termed “cross-tolerance”. In general, it implies that a noxious stimulus can confer cellular tolerance to a subsequent stress of a different nature from the first one.

Elevation of body temperature in rats led to acquisition of tolerance to subsequent forebrain ischemia (Chopp et al., 1989), and in this same model repeated hyperthermia is even more efficient in protecting neurons (Kitagawa et al., 1991).

Beside the myriad of new models proposed, it is interesting to underline the IP in neonatal animals. To settle up this model in 7 days rat pups, unilateral common carotid artery ligation followed 1h later by 8% oxygen hypoxia for 2h was performed. Practically, pups were placed in airtight 500-ml containers partially submerged in a 37°C water bath through which humidified 8% oxygen was maintained at a flow rate

of 3L/min for 2h. After completion of hypoxia, the rat pups were returned to their cage. It was demonstrated that, when the carotid artery ligation was performed 24 h before hypoxia, a remarkable neuroprotection was achieved (Lee et al., 2004). Moreover, anoxia can be used to induce ischemic tolerance in neonatal rats (Gidday et al., 1994). When the postnatal rats are exposed to normothermic hypoxia before being subjected to hypoxia–ischemia treatment, almost no brain injury is detectable.

Spreading depression (SD) is a phenomenon that is characterized by a gradual expansion of the area of electrical suppression in the cerebral cortex. The area of SD is characterized by massive depolarization of neurons and elevation of extracellular K^+. SD is another stress that is considered to confer IT (Matsushima et al., 1996). Another model proposed that electroconvulsive shock (ECS) can be used as a preconditioning stimulus. In particular, both single and repetitive ECS application confers neuroprotection from subsequent global ischemia. Single ECS treatment was applied 2 days before 8 min global ischemia, whereas the repetitive one was applied once a day for 9 consecutive days until 2 days before global ischemia induction (Mishima et al. 2005).

Interestingly, in human brain there is a natural phenomenon that can be considered analog to experimental IP strategies, which is represented by transient ischemic attacks (TIAs), at very brief duration. Analyzing the time course of patients presenting with transient ischemic attack (TIA) it was demonstrated that those who have endured more TIAs showed better recovery than those with a single TIA (Weih et al., 1999), thus suggesting that preceding ischemia naturally protects the human brain by a mechanism similar to ischemic tolerance.

1.3.2 Mechanisms Involved in Ischemic Preconditioning-Mediated Neuroprotection

The knowledge of molecular and cellular mechanisms underlying the induction and the maintenance of ischemic tolerance is still fragmentary; however, various studies have demonstrated that different events play key roles in the development of ischemic stroke (Pignataro et al., 2009; Kirino, 2002).

Recent evidence highlights that the maintenance of ionic homeostasis plays a key role in propagating this neuroprotective phenomenon (Cuomo et al., 2015). Several

experiments, performed both *in vivo* and *in vitro*, attested that an improved capacity to preserve cellular ionic and pH homeostasis represents a determinant factor for IT. For example, in cortical neurons exposed to brief non-injurious oxygen and glucose deprivation (OGD), impairment in voltage-gated potassium channels has been observed. Moreover, *in vivo* experiments showed that IP prevented the inhibition of Na^+/K^+-ATPase activity after brain ischemia in hippocampal and cortical neurons of rats subjected to ischemia (de Souza Wyse et al., 2000).

Regarding calcium homeostasis maintenance, IP seems to increase Ca^{2+}-ATPase activity (Ohta et al., 1996) and protein expression (Kato et al., 2005), and in general it's observed a reduction of intracellular [Ca^{2+}] (Shimazaki et al., 1998). In regular ischemic conditions, Ca^{2+} ions play an important deregulation role in mitochondria, enhancing the uncoupling of oxidative phosphorylation and causing the reduction of mitochondrial membrane potential with consequent mitochondrial permeability transitional pore (MPTP) opening (Dirnagl et al., 1999). The latter process represents a crucial event for cell death, but its inhibition was considered as an important step for cytoprotection observed in IP. In this scenario, nitric oxide (NO) and protein kinase have been proposed as possible MPTP regulators (Shiva et al., 2007; Zhao et al., 2006).

NO production may also be linked to ischemic tolerance. In a newborn rat model, the resistance to hypoxic ischemia by hypoxic preconditioning was dependent on NO production by endothelial nitric oxide synthase (eNOS) (Gidday et al., 1999).

Neuronal NO synthase (nNOS) also seems to play a relevant role in neuroprotection induced by ischemic preconditioning in mature neurons (Gonzalez-Zulueta et al., 2000), involving N-methyl-d-aspartate (NMDA) receptor activation, Ca^{2+} influx and new protein synthesis. In particular, increase of NO levels is related to the modulation of MPTP opening (Shiva et al., 2007) and the enhancement of activity and protein expression of mitochondrial Mn-SOD (Scorziello et al., 2007), thus affecting reactive oxygen species (ROS) production. These functions are mediated by two main pathways: the Raf/Mek/Erk cascade (Nandagopal et al., 2001) and the PI3K/Akt pathway (Brunet et al., 2001).

In literature there are different opinions about the role of kinases in IP. Ischemia activates a process of protein phosphorylation (Shamloo and Wieloch, 1999), that persists for a few days, involving calcium/calmodulin-dependent protein kinase II

(CaMKII) and mitogen-activated protein kinases (MAPK). This enhanced and excessive phosphorylation is blocked after IP. On the other hand, the activation of Akt/protein kinase B occurring after a sublethal ischemia may contribute to the induction of IT (Yano et al., 2001). Some studies suggest that the early phase of ischemic preconditioning is characterized by rapid post-translational modification of pre-existing proteins through signaling pathways that involve protein kinase C (PKC) (Speechly-Dick et al., 1994) and MAPK (Shamloo et al., 1999).

The late preconditioning is mediated by the synthesis of new protective proteins, regulated by the activation of transcriptional factors through PKC and tyrosine kinase signaling pathways. The regulation of gene expression leading to apoptosis process seems to be related to IT. Indeed, during IP, Bcl-2 protein expression is enhanced, thus preventing the delayed neuronal death that normally occurs in the penumbra region (Shimazaki et al., 1994), whereas p53 gene expression is markedly reduced (Tomasevic et al., 1999). Generally, protein synthesis is impaired by cerebral ischemia (Kleihues and Hossmann, 1971), but in the gerbil model it was established that preconditioning restores the general protein levels (Nakagomi et al., 1993; Furuta et al., 1993): autoradiography analysis by using isotope-labeled amino acids demonstrated that the pattern of amino acid incorporation in the CA1 neurons returned to a normal pattern within 24 hours.

Other defense mechanisms activated in the phenomenon of IP is represented by the inflammatory cytokines, particularly tumor necrosis factor alpha (TNF-α) or interleukin-1 beta (IL-1β) have been implicated in the mechanisms of ischemic tolerance (Ohtsuki et al., 1996; Wang et al., 2000). Furthermore, NF-kB, that is activated by various signals, such as oxidative stress and intracellular Ca^{2+} elevation, is involved in the induction of neuroprotective gene products, such as MnSOD and Bcl-2, and pretreatment with its inhibitor abolished the neuroprotective effect of preconditioning (Blondeau et al., 2001).

Finally, the stress response of the brain to a noxious environment through heat-shock proteins (hsp) is recognized to be an important process underlying IT induction. In fact, hsp gene expression is greatly enhanced, and their role it's known to be essential for cell survival because the stress response avoids the accumulation of denatured proteins that arise from various stresses. In post-ischemic hippocampal neurons the processing of denatured proteins is disturbed (Ide et al., 1999), leading

to their accumulation. However, when IP is induced by preceding ischemia, hsp70 increases in the hippocampal CA1 pyramidal cells of ischemic animals (Kirino et al., 1991), and its experimental manipulation abolishes IT (Nakata et al., 1993).

1.3.3 Mechanisms Involved in Ischemic Postconditioning-Mediated Neuroprotection

Rapid revascularization of the occluded vessels and early reperfusion is one of the most effective approaches currently used for acute ischemic stroke, which is made possible by administering the thrombolytic agent, tissue plasminogen activator (t-PA), or by inserting mechanical devices to remove the occluding clot. However, it has been repeatedly demonstrated that during the early reperfusion phase reactive oxygen species are generated and intracellular free Ca^{2+} overload may occur, potentially leading to additional injury, triggering apoptosis mechanisms and an inflammatory response (Kuroda and Siesjo, 1997; Chan, 1996). In this scenario, reperfusion is a double edged sword, and in an attempt to attenuate the injurious early hyperemic response after reperfusion, a pivotal role is played by the neuroprotective procedure of IPO (Pignataro et al., 2009; Zhao, 2009; Fairbanks et al., 2010). Ischemic postconditioning is defined as a novel strategy that induces brief interruptions in blood flow in the early phases of reperfusion, thereby protecting organs from ischemia/reperfusion injury (IRI) (Liu et al., 2007). If postconditioning is performed immediately or within 30 min after reperfusion it is considered as rapid postconditioning, whereas when performed hours or days after reperfusion it is defined as delayed postconditioning (Ren et al, 2008).

Brain neuroprotection induced by postconditioning is usually achieved by subjecting the brain to different cycles of short, non-dangerous ischemia applied after harmful ischemia. In focal ischemia, two models have been used to date to induce postconditioning: in the first model, permanent distal occlusion of the middle cerebral artery is followed by a series of occlusions of both common carotid arteries (CCAs) (Zhao et al., 2006; Gao et al., 2008); in the second model, harmful tMCAO is followed by a series of brief non-injurious middle cerebral artery occlusions and repeated reperfusions (Pignataro et al., 2008).

In addition, protective action elicited by IPO was also evaluated in *in vitro* models of ischemia. Postconditioning with a 10 min OGD initiated at 10 min of reperfusion

reduced neuronal death in cortical cultures (Pignataro et al., 2008). The same positive effect was achieved in rat organotypic hippocampal slices cultures with brief OGD (Scartabelli et al, 2008).

Even in postconditioning the cross-tolerance phenomenon has been demonstrated to have effect, therefore other pharmacological strategies previously used as preconditioning stimuli can be used for postconditioning induction. In this context, isoflurane application after OGD in slice organ cultures resulted to be a good method for IPO induction (Lee et al., 2008).

Recently it has been shown that rapid postconditioning improves cerebral blood flow (CBF) (Ren et al., 2008; Gao et al., 2008), and consequently it affects the associates events, such as free radical production, endothelial function and inflammation. Rapid postconditioning also has been shown to increase superoxide dismutase (SOD) activity, suggesting attenuation of lipid peroxidation via decreased levels of superoxide anions in the brain (Zhao et al., 2006). Furthermore, rapid IPO was recently shown to reduce cytochrome c release from the mitochondria to the cytosol, an event that is central to induction of apoptosis (Wang et al., 2008).

Inhibition of inflammation after ischaemic stroke is another protective pathway seen in IPO condition. During the inflammatory response, leukocytes move from vessels into the brain tissue, releasing ROS, thus damaging lipid membranes, DNA, and proteins (Chan, 1996), and inflammation process is mediated by cytokines, such as IL-1β and TNF-α, and adhesion molecules, such as ICAM-1. Rapid postconditioning likely inhibits leukocyte accumulation, because it results in attenuation of the expression of IL-1β and TNF-α mRNA, and the ICAM-1 protein expression in the ischemic cortex (Xing et al., 2008).

Several recent studies examine the involvement of protein kinases in IPO, especially a family of kinases called “reperfusion injury salvage kinases” (RISK). During harmful ischemia, Akt is transiently phosphorylated and so activated, only for a short interval of time after reperfusion. This activation is extended after postconditioning stimulation (Gao et al., 2008). ETK and P38 MAPK seem to show a similar profile of activation after IPO. Conversely, the results on ERK1/2 and pJNK in IPO have to be elucidated. Indeed, the expression of phosphorylated ERK and phosphorylated JNK is increased after postconditioning, but their inhibition can revert the subsequent IT only under

some experimental conditions (Gao et al., 2008; Zhao, 2009), suggesting that this increase could be related to a secondary event.

1.3.4 Remote Ischemic Conditioning

In the last years, several groups of investigators have directed considerable attention toward evidence that a short occlusion of a distant artery, such as femoral artery, is able to protect an organ undergoing an ischemic insult, a phenomenon called remote ischemic conditioning (RIC) (Zhao et al., 2012; Hausenloy and Yellon, 2016; Heusch et al., 2015; Hess et al., 2015; Lim and Hausenloy, 2012).

In 1993, Przyklenk et al. made the crucial discovery that applying the IPC stimulus in one coronary vascular territory conferred tolerance to acute IRI in a different territory, suggesting that cardioprotection elicited by IPC could be transferred from one region of the heart to another (Przyklenk et al., 1993). Afterwards, investigators reported that myocardial infarction size could be reduced by inducing brief ischemia and reperfusion to either the kidney (McClanahan et al., 1993) or small intestine (Gho et al., 1996) immediately before the sustained coronary artery occlusion.

In a clinical trial published in 2010, this procedure has led to excellent results in salvage the myocardium of patients with myocardial infarction, when applied in the ambulance before percutaneous coronary intervention (Bøtker et al., 2010). Clinical trials of RIC have now become more numerous in the fields of cardiology, cardiovascular surgery, and renal protection too (Pei et al., 2014; Davies et al., 2013).

Also for RIC, the conditioning stimulus can provide protection when administered either before ischemia (remote ischaemic preconditioning; RIP), or during (remote ischemic perconditioning; RIPE), or after reperfusion (remote ischemic postconditioning; RIPO).

The mechanisms underlying the phenomenon of RIC are classified in three steps:

1. The first events occurring in the remote organ or tissue, generated by the RIC stimulus (Lim and Hausenloy, 2012). In fact, occlusion with a tourniquet or blood pressure cuff on the arm can stimulate the release of autacoids, such as adenosine, bradykinin and calcitonin gene-related peptide (Hess et al., 2015), which can protect target organ or tissue from injury.

2. The protective signal which is transmitted from the remote organ to the target organ. To explain the functioning of this phenomenon, neural and humoral mechanisms have been proposed as ways required to transmit the peripheral signal from an organ as limb to a distant organ, as brain or heart (Figure 1.7).
3. The event occurring in the target organ which mediates the protective pathway.

It was suggested that autonomic nervous system is involved in some models by inhibition of conditioning using an antagonist of nicotinic acetylcholine receptors and an autonomic ganglia blocker (Malhotra et al., 2011). Moreover, transection of the femoral nerve (Streensrud et al., 2010) or spinal cord (Donato et al., 2013) abolished the effect of RIC in the heart. The current theory is that the release of endogenous autocoids, including neuropeptides such as CGRP, adenosine, and bradykinin (Hess at al., 2013), triggers the activation of local afferent nerves, which in turn communicate with efferent nerves terminating at the ischemic organ to mediate protection.

On the other side, transferring dialysate prepared from the blood of conditioned animals and humans to isolated hearts induced cardioprotection (Shimizu et al., 2009), suggested a role for humoral factors. Subsequent studies have revealed that the release of humoral factors is induced by peripheral nerves (Jensen et al., 2012). In this scenario, different circulating mediators have been proposed in cardiac injury models after RIC induction. Plasma levels of SDF 1, IL 10 and microRNA 144 increased after RIC in animal models, and microRNA 144 levels also increased in humans after RIC. Furthermore, the protective effects of RIC are abrogated if the action of these molecules was previously blocked (Davidson et al., 2013; Cai et al., 2012; Li et al., 2014).

Nitrite that serves as a storage pool of NO also appears to play a pivotal role in the neuroprotection mediated by RIC (Pignataro et al., 2013). Blood levels of nitrite are increased after RIC (Hess et al., 2016). Nitrite works as vasodilator and increases CBF, so its upregulation could explain why cerebral blood flow was enhanced after RIC.

RIC is able to trigger different pathways that converge on the mitochondria. Blood-borne factors released after RIC induction (such as adenosine, bradykinin-2, opioids, angiotensin-1) can stimulate G‑protein coupled receptors on the cell surface,

inducing activation of intracellular kinase signaling that leads to opening of ATP-dependent potassium channels, thus preventing formation of the MPTP (Ong et al., 2015). Moreover, opening of ATP-dependent potassium channels is shown to have an important role in RIC, as demonstrated by its blockage that deletes the neuroprotection (Sun et al., 2012). In addition, nitrite affects mitochondria, because mitochondrial proteins are subjected to a process of nitrosylation, that reduces the generation of ROS, and inhibits MPTP and cytochrome C release (Shiva, 2013). Among pathways that seem to be involved in the neuroprotection mediated by RIC, PKC activation and PI3K/Akt pathway were shown to confer cardioprotection (Serejo et al., 2007; Breivik et al., 2011). Moreover, a work conducted in our model of focal ischemia demonstrated that p-ERK and NO contribute to the neuroprotection elicited by RIPO, and that NOS isoform responsible for this NO production in the temporoparietal cortex is that neuronal (Pignataro et al., 2013).

RIC has been shown to evoke a systemic protective response involving modulation of immune cells (Saxena et al., 2010). In particular, microarray analysis experiments evidenced that RIP stimulus by transient forearm ischemia induced suppression of gene expression related to leukocyte activation, innate immunity responses, cytokine synthesis and apoptosis. On the contrary, anti-inflammatory genes such as HSP70 were upregulated (Konstantinov et al., 2004). Subsequent studies in mice also showed upregulation of genes associated with cytoprotection, growth and DNA repair by limb RIP (Konstantinov et al., 2005). Finally, a further confirmation of the role of gene transcription in mediating the protective effect of RIC was achieved in mice with deficiency in transcription factor NF-κB p105 subunit, in which delayed RIP-induced cardioprotection was abrogated (Li et al., 2004).

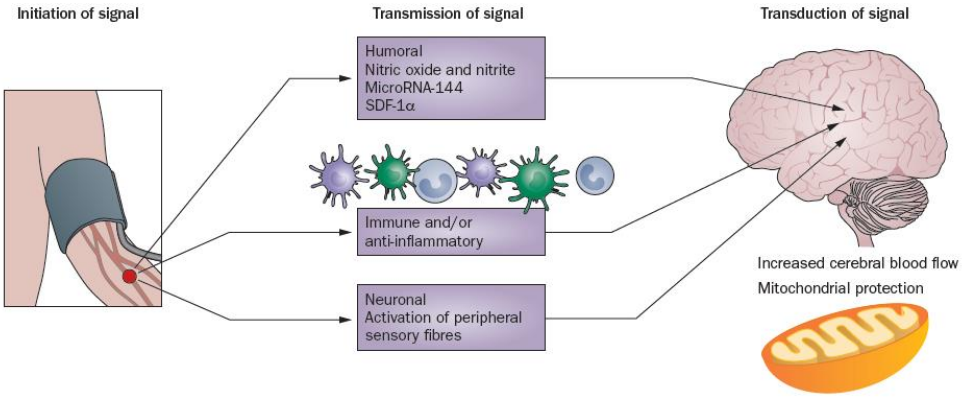

Figure 1.7: *Possible mechanisms by which the signal initiated by remote preconditioning is transmitted to the brain (by Hess et al., 2015)*

1.3.5 Role of NCX in the Neuroprotection Elicited by Ischemic conditioning

Because of the central role in regulating calcium homeostasis, it was reasonable to investigate the likely involvement of NCX in the neuroprotection mediated by the strategies of IP and IPO.

During cerebral ischemia NCX gene expression was reduced in rats in a different manner depending on the exchanger isoforms and on the region involved in the insult (Pignataro et al., 2004; Boscia et al., 2006). On the contrary, NCX1 and NCX3 protein expression increased after ischemic insult following IP induction (Pignataro et al., 2012), and their silencing partially prevented IP-mediated neuroprotection. Moreover, it has been shown that p-Akt, by acting on NCX1 and NCX3 (Formisano et al., 2008), represents a fundamental transducer of the neuroprotection exerted by preconditioning (Pignataro et al., 2012). These data are in accordance with previous researches concerning the relationship between NCX expression and brain damage. Indeed, when NCX3$^{-/-}$ mice were subjected to MCAO, the resulting brain damage was increased (Molinaro et al., 2008). In addition, the silencing of NCX1 and NCX3 expression by RNA interference increases cerebellar granule neuron vulnerability to Ca^{2+} overload and excitotoxicity (Bano et al., 2005; Secondo et al., 2007); similar results were achieved in baby hamster kidney (BHK) cells overexpressing NCX1 or NCX3, in which the vulnerability to chemical hypoxia considerably increased after either NCX1 or NCX3 silencing (Bano et al., 2005; Secondo et al., 2007). Taken together, all these data support the theory that preconditioning neuroprotection is also mediated by the action of p-Akt on the effectors NCX1 and NCX3 and that their increased expression could render the brain tissue ready to withstand subsequent and more severe brain conditions.

However, other cellular factors in normal conditions can regulate NCX, and are likely triggered from IP, such as Hypoxia-inducible factor-1 (HIF-1). This is a nuclear factor required for transcriptional activation in response to hypoxia, and regulates several genes involved in stroke, thus leading to vasculogenesis, increased glucose transport and increased glycolysis. This transcriptional factor is upregulated in rat brain during cerebral ischemia (Bergeron et al., 1999), or ischemic preconditioning (Taie et al., 2009). Recently it was demonstrated that NCX1 is a target gene for HIF-1α, and that after IP induction HIF-1α expression is strongly augmented, and exerts its prosurvival role through the upregulation of NCX1 transcript and protein (Valsecchi et al., 2011).

The involvement of NCX isoforms was even elucidated in IPO (Pignataro et al., 2011). In particular, regarding this aspect, different conclusions were obtained:

1. IPO induces an NCX3 overexpression in the penumbral region of cortex;
2. p-Akt expression after postconditioning increases;
3. NCX3 silencing reverts the neuroprotection induced by IPO;
4. the selective p-Akt inhibition prevents NCX3 upregulation.

Moreover, NCX1 downregulation induced by siRNA was not able to revert the postconditioning-induced neuroprotection, like it occurred in IP, underlining that NCX1 does not play a relevant role in this phenomenon (Pignataro et al., 2011). This capability of NCX3 isoform to maintain intracellular [Ca^{2+}] and [Na^{+}] homeostasis in anoxic conditions might be correlated to its ability to operate when ATP levels are reduced (Secondo et al., 2007), unlike the other two NCX isoforms. In addition, NCX1 and NCX3 promoters show structural differences, and isoform 3 gene is the only target for the prosurvival kinase cAMP response element-binding protein (CREB), an Akt downstream player (Gabellini et al., 2003).

1.4 Non-Coding RNA: Focusing on MicroRNA Function

The “central dogma” dominating molecular biology for many years asserted that the protein production was mediated by the DNA-RNA-protein axis, which only involves the mechanisms of transcription and translation to allow the human genome to be decoded for the production of specific proteins. However, over recent years, the discovery of the world of non-coding RNA has radically revolutionized this theory, defining new mechanisms involved in regulating protein synthesis. The human genome project revealed that protein-coding sequences only constitute the 1.5% of the entire genome, whereas the remaining 98.5% of the genome is composed of introns, regulatory DNA sequences, interspersed elements and non-coding RNA (ncRNA) molecules (International Human Genome Sequencing Consortium, 2001). Indeed, the majority of mammalian genomes are transcribed into ncRNAs, many of which are alternatively spliced or processed into smaller products. To date, two types of non-coding RNAs have been identified and academically classified: short non-coding RNA and long non-coding RNA molecules. The short non-coding RNA molecules can further be subdivided into microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), piwi-interacting RNA (pi-RNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and other uncharacterized small molecules. Among all the regulatory molecules, miRNAs are the most studied and better characterized, particularly as regulators in human diseases, and one of the main proofs is that miRNA expression profiles very often show significant modifications in response to a disease state, suggesting that miRNAs represent key regulators of disease-associated pathways. The first miRNA was discovered over 30 years ago in the nematode Caenorhabditis elegans with the identification of the developmental regulator lin-4 (Lee et al., 1993). Currently, miRNAs are defined as RNA molecules of 18-24 nucleotides in length, transcribed from specific genes or from intronic regions of other genes, and able to regulate gene expression either by promoting mRNA degradation or by inhibiting protein translation (Watson, Zanichelli, 2015). Based on computational prediction, it has been estimated that more than 60% of mammalian mRNAs are targeted by at least one miRNA (Friedman et al., 2009).

Recent developments in the field of miRNA, in relation to human diseases, have revealed that miRNAs represent valuable tools as biomarkers and as potential disease-modifying agents (Basak et al., 2016).

1.4.1 MicroRNA Biogenesis and Function

MiRNAs are evolutionarily conserved regulatory molecules that are synthesized after processing by both nuclear and cytosolic proteins (Hammond, 2015). In the nucleus, non-coding RNA molecules, called primary miRNAs (pri-miRNA), are transcribed from miRNA-encoding genomic sequences by RNA polymerase II. These primary miRNA transcripts are often several hundred nucleotides long and are modified similarly to protein-coding transcripts by the addition of a 5' cap and a 3' poly-A tail. The defining feature of all miRNA genes is the stem-loop precursor RNA structure, with one (but sometimes both) of strands of the stem that represents the source of the mature miRNA (Figure 1.8).

Following this initial transcription event a protein complex comprised of the RNase III enzyme, Drosha and DGCR8 (in addition to several cofactors), recognizes the pri-miRNA and cleaves the 50 and 30 arms of the pri-miRNA hairpin to form the premature miRNA (pre-miRNA) of 70–110 nucleotides. Then, Exportin-5 recognizes and transports the pre-miRNA to the cytosol for further processing via a RAN-GTP-dependent mechanism. In the cytosol, a protein complex consisting of Dicer (RNase) and double-stranded RNA binding domain proteins TRBP, PACT and Ago2 further processes the pre-miRNAs into 22 nucleotide mature miRNA duplexes. Typically, one strand of this mature miRNA duplex, termed the guide strand, associates with the RNA-induced silencing complex (RISC), in particular with Ago2. While it is generally believed that upon incorporation into the RISC complex, the other strand (passenger strand) is separated from the guide strand and degraded, there is evidence that in some cases, both strands of the miRNA duplex can be functional. Ago2 directly binds the mature miRNA and seeks target mRNAs that have complementarity to the miRNA (Watson, Zanichelli, 2015).

Alternatively, miRNAs can be generated without the RISC involvement, but from short hairpin introns, snoRNAs, tRNAs and endogenous short hairpin RNAs as a result of splicing, debranching and complex processing mechanisms (Miyoshi et al ., 2010).

An increasing number of studies have shed considerable light on biological pathways that involve protein-protein and protein-RNA interactions to regulate the expression and the function of miRNA (Krol et al., 2010). Moreover, several transcription factors, as well as activators and repressors, were demonstrated to take part in the regulation of miRNA biogenesis (Finnegan and Pasquinelli, 2013).

MicroRNAs are considered to be downregulators of gene expression via two principal mechanisms: mRNA cleavage and translational repression. MiRNA-RISC complexes interact with mRNA targets through partial sequence complementation, typically within the 3' untranslated region of target mRNAs. In particular, nucleotides 2-7 of the miRNA (starting from 5' end), termed the "seed" region, are important for target association. It is currently thought that the extent of base pairing between the miRNA and its mRNA target determines whether the mRNA is degraded or translationally repressed (Fabian et al., 2010) (Figure 1.9). On the one hand, miRNAs have been shown to bind complementary regions of protein-coding mRNA sequences resulting in RISC-mediated cleavage. In this case, the cut precisely occurs between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, and in the absence of appropriate complementarity, miRNAs also have the ability to bind to 3'UTRs and block translation.

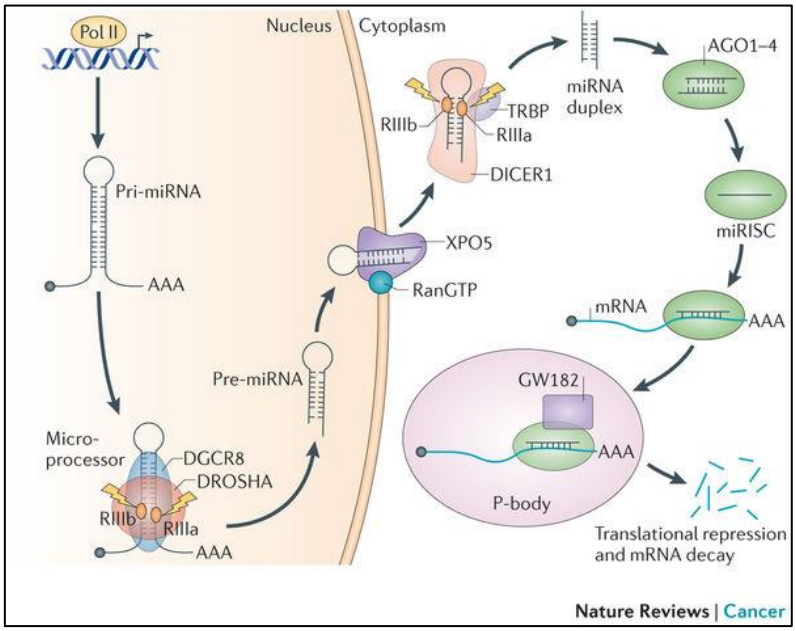

Figure 1.8: *Schematic representation of miRNA biogenesis process (by Lin and Gregory, 2015).*

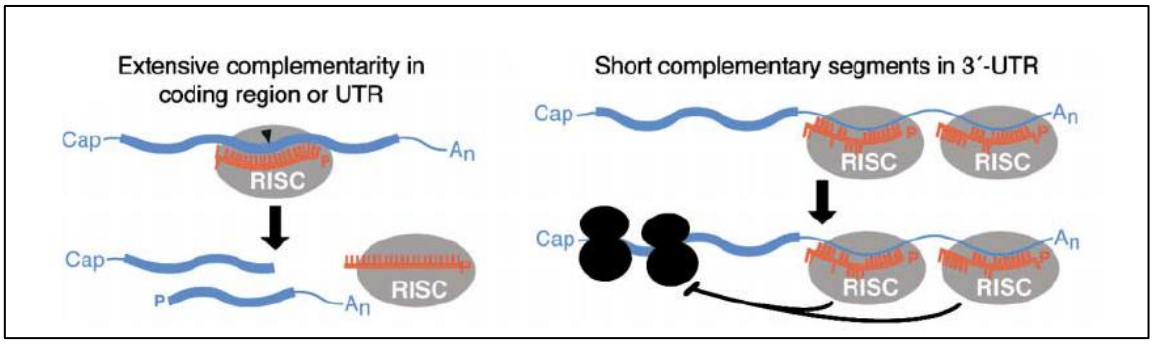

Figure 1.9: *Schematic representation of the action modes of miRNA (by Bartel, 2004). On the left: mRNA cleavage; on the right: translational repression.*

1.4.2 Nomenclature of MicroRNA

The discovery of an increasing number of microRNA has led researchers to adopt a uniform system of annotation to ensure uniformity and ease the cataloguing of miRNAs (Bhaskaran and Mohan, 2014; Ambros et al., 2003; Griffiths-Jones, 2004). First of all, miRNAs are numbered sequentially in the time order that they are discovered. In particular, when a new miRNA molecule is confirmed, it is assigned a number that is attached to the prefix “miR” followed by a dash (eg, miR-1). To give an indication of the organism in which the novel miRNA has been identified, three letters are added before the word “miR” (eg, “hsa” for Homo sapiens, “mmu” for mouse and “rno” for rat). Moreover, the mature miRNA is denoted with a capitalized “R” (eg miR-1); on the contrary, the uncapitalized “r” refers to both the miRNA gene and the miRNA precursor (eg mir-1). It can often occur that identical mature miRNA sequences originate from different precursor sequences and genomic loci: in this case a numeric suffix will be used to distinguish them, such as miR-103-1 and miR-103-2. This situation is different from that in which mature sequences differ by few nucleotides (1 or 2), and are named with a lettered suffix. This would mean that, for example, hsa-miR-130a and has-miR-130b are derived from specific and discrete precursors. In addition, deep sequencing studies have revealed that individual pre-miRNAs can give rise to multiple mature miRNA species, “termed isomirs”, with little differences in length or sequence because subjected to a variety of modifications. Finally, in some cases, miRNA precursors could give rise to two mature miRNAs: if the predominantly expressed miRNA specie can be definitively established, it is named as mentioned above (eg miR-136), while the one originating from the opposite strand of the precursor is designated the same name but with an asterisk next to the number (miR-136*). However, determination of the predominantly

expressed species often is not possible and so identifiers such as miR-223-5p (from the 5′ strand) and miR-223-3p (from the 3′ strand) are assigned.

1.4.3 MicroRNA Local Expression

As with mRNAs, some miRNAs are differentially expressed among tissues or developmental stages. Indeed, although all tissues harbor miRNA, their levels differ between different tissues types. Examples include miR-122, which is preferentially expressed in the liver (Lagos-Quintana et al., 2002), miR-133a and miR-133b, which are highly enriched in muscle (Sempere et al., 2004), and the miR-302 family members which are specific to stem cells (Barroso-del Jesus et al., 2009).

There are many examples of miRNAs that are specifically expressed in brain tissue and play a critical role in regulating neuronal activity (Kuss et al., 2008). The tissue specificity of miRNAs is especially evident in neurons, as they regulate and influence key neuronal features such as neurogenesis, synaptic plasticity, neuronal differentiation and neuronal proliferation. In this scenario, significant number of miRNAs were identified to be enriched in a cell compartment-specific manner. For example, miR-132, miR-134 and miR-138 are abundant in nerve endings and have important regulatory roles in the synapses, such as the development of dendritic spines (Bicker et al., 2014; Schratt et al., 2006). Furthermore, miR-9, miR-124 and miR-128 represent brain-specific miRNAs, and their expression patterns change in disease states. Interestingly, miR-124a regulates neuronal differentiation and maintenance affecting the levels of many hundred non-neuronal gene transcripts (Conaco et al., 2006).

1.4.4 MicroRNA Target Prediction

The development of precise and fast assays for miRNA target identification has played a significant role in the study of miRNA functions and the biological processes in which they are involved. Several effective algorithms have been developed for the prediction of miRNAs targets in animals (Huang et al., 2010). In particular, there are different prediction criteria that are exploited from these software. First of all, the complementarity of miRNA sequence to the 3′-UTR sequence of potential target mRNAs. Indeed, the strong binding of the 5′ end of the mature miRNA to the 3′-UTR

sequence of mRNA is very important for targeting. In particular, there are three structural types of target sites (Figure 1.10):

- The condition in which canonical sites have good or perfect complementarity at both the 5′ and 3′ ends of the miRNA, with a characteristic bulge just in the middle (Figure 1.10A);
- A situation in which there is perfect seed complementarity in the 5′ end of the miRNA, but poor 3′ complementarity (Figure 1.10B);
- The possibility that compensatory sites have a mismatch or wobble in the 5′ seed region of miRNA, that are compensated through excellent complementarity at the 3′ end (Figure 1.10C).

Another very important aspect to be considered concerns the thermodynamic properties of miRNA-mRNA duplexes, that are assessed by calculating free-energy (ΔG) of the putative binding. The energetically more favorable state is when the complementary miRNA and mRNA are hybridized. Lower values of free energy of two paired RNA strands correspond to more energy that is needed to disrupt this duplex formation. Thus, when the free energy is low, the binding of the miRNA to the mRNA is stronger (Lewis et al., 2005). Moreover, the conservation of miRNA target sites among different species is another important element to be considered for an optimum prediction, because using predicted binding sites that are conserved in multiple species allows to reduce the number of false positives.

The software "**miRanda**" screened 3' UTRs considering the three principles discussed above. It was initially designed to predict miRNA target genes in D. melanogaster (Enright et al., 2003), and subsequently improved for the identification of miRNA targets (John et al., 2004). In particular, it's required almost perfect complementarity in the seed region, and only a single wobble pairing is allowed; in addition, only for the highest scoring alignments, the thermodynamic stability of the complex is calculated and reported.

TargetScan is a computational method to predict the targets of conserved vertebrate miRNAs, integrating the model of interaction miRNA-mRNA on the basis of thermodynamics and sequence alignment analysis between miRNA binding sites among different species (Lewis et al., 2003). In this way many false positives are filtered from the beginning of the prediction process, so resulting to be estimated in a range between 22% and 31%. This method was then developed into **TargetScanS**

(Lewis et al., 2005), that requires a conservation of target sites in five genomes (human, mouse, rat, dog and chicken), and a six-nucleotide seed (position 2–7 in the 5′ end of the miRNA) followed by an additional 3′ match of adenosines surrounding the miRNA seed, thus decreasing the false-positive rate to 22% in mammals.

The algorithm of **DIANA-microT** integrates computational and experimental approaches (Kiriakidou et al., 2004). The search is performed in the UTRs for stringent seed to the miRNA, but also putative target sites with 6 consecutive base pairs or with seed matches containing one G:U wobble are accepted if they are compensated by a pairing to the 3′ end of the miRNA. Currently, the software considers also the conservation of the sites and the binding type. The total score of a target is the sum of the individual scores of each target site on the 3′-UTR.

RNAhybrid is a tool characterized by the application on a single genome, basing on analyzing the secondary structure of the miRNA/mRNA duplex (Rehmsmeier et al., 2004; Krüger and Rehmsmeier, 2006). This algorithm calculates the most favourable hybridization miRNA-mRNA in terms of energy, but user must specify the portion of the miRNA that should form a perfect helix corresponding to the seed site.

RNA22 is a method for identifying miRNA binding sites (Miranda et al., 2006), but it doesn't exploit a cross-species sequence conservation filter. So the user, to restrict the number of the results, that in this way is increased, has to choice putative target sites considering the maximal free energy, the minimum number of base-pairs between miRNA and target, and the maximum number of unpaired bases of the miRNA seed region.

PicTar uses the criteria of co-expression in space and time of miRNAs and their targets, through combinations of different microRNAs (Krek et al., 2005). This algorithm requires that the binding stability of the putative miRNA:target interaction, measured by thermodynamic binding energy, is higher than a specified threshold.

Finally, **MicroRNA.org** is a common source of microRNA target predictions and expression profiles (Betel et al., 2008). Target predictions are based on a development of the miRanda algorithm which in integrated with current biological knowledge on target rules and on the use of an up-to-date compendium of mammalian microRNAs. Indeed, MicroRNA expression profiles are derived from a comprehensive sequencing project of a large set of mammalian tissues and cell lines of normal and disease origin. Therefore, the application of this method allow to

explore the numerous genes that are potentially regulated by a particular microRNA, to compare multiple microRNAs acting on the same particular mRNA, and to merge this information with microRNA expression profiles in various tissue.

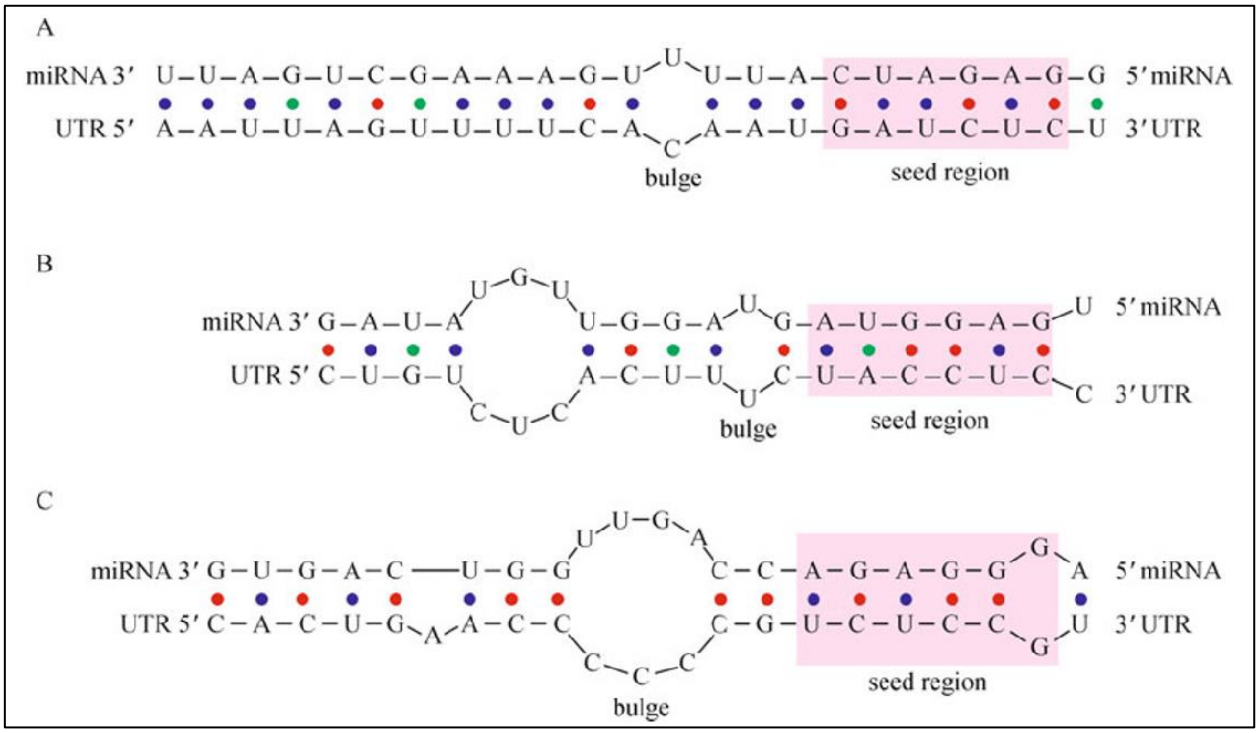

Figure 1.10: *Secondary structures of the three main types of target site duplex (by Huang et al., 2010).*

1.4.5 MicroRNA as Therapeutic Target

There are several expanding efforts to develop therapies that directly can target microRNA, and according to the type of miRNA, its expression and its function. Different approaches are used to overexpress or inhibit miRNA, therefore these drug candidates can be either miRNA mimics or antisense inhibitors (Hammond, 2015).

If the therapeutic goal is to replace the miRNA concentration, that was previously reduced, for example in pathologic conditions, a miRNA mimic is applied. This approach was developed in 2007 as gain-of-function tool for specific miRNAs and consists of synthetic double-stranded RNAs that are directly recognized by RISC (Wang, 2009). These miRNA-like RNA fragments have a 5’ end that gets a motif with partial complementarity to the 3’UTR of the target genes, thus miming microRNA operate mode. This technology has been further developed by pharmaceutic

companies, and a library of miRNA mimics is available for human miRNAs discovered until now. Moreover, these molecules undergo modifications in backbone and ribose to promote stability *in vivo* and to reduce problems related to pharmacodynamics. Nevertheless, important advances are being made using adeno-associated virus (AAV) vectors and tissue-specific promoters to enhance tissue specificity (van Rooij et al., 2012).

On the other hand, blocking a miRNA also can be useful in terms of disease intervention. AntimiRNAs are antisense oligonucleotides that would bind to the mature guide strand of the miRNA, causing its inhibition (Basak et al., 2016). In order to create stable and deliverable miRNA inhibitors, several methods are used to add modifications, such as cholesterol conjugation and the use of Locked Nucleic Acid (LNA) (Elmen et al., 2008), generating a class of antagonists known as antagomirs. LNA modification increases the stability and nuclease resistance of antisense oligonucleotides, but also increases the efficiency of hybridization to single stranded RNA. The most promising candidate as therapeutic drug to date is the Regulus drug RG-101, an antisense inhibitor of miR-122 that is being developed for the treatment of hepatitis C virus (HCV) infection (Hammond, 2015). In phase 1B a single subcutaneous dose of this drug induced a significant reduction in viral load in patients with HCV within 4 weeks of treatment (van der Ree et al., 2017). Phase 2 studies are underway to establish the efficacy of a combination of RG-101 with direct-acting antivirals to potentially shorten treatment duration.

An evolution of antimiRNA is represented by miRNA sponges, that are able to inhibit multiple miRNAs simultaneously (Ebert et al., 2007). These molecules can contain a seed sequence for an entire miRNA family, but could be also used to target multiple mRNA.

Finally, an emerging mechanism for controlling miRNA activity is through the use of miR-Masks (Wang et al., 2011). They are single stranded 2'-*O*-methyl-modified antisense oligonucleotide that recognize the miRNA target site on the 3'UTR of mRNA, thereby masking this binding site.

1.4.6 Role of MicroRNA in Ischemic Stroke

In the past decade, the role of miRNAs in several human diseases has been gradually emerging (Hammond, 2015; Bhaskaran and Mohan, 2014). Gene expression profile studies have shown alterations in miRNA expression in several diseases. Indeed, many cancer types are related to alterations in specific miRNA expression, and in-depth studies have evidenced the role of some of these non-coding RNA, that can function as tumor suppressors or can promote tumor development (oncomiRs) depending on the functions of the target proteins they regulate. Luckily, each day a new miRNA is presented as a novel candidate for cancer diagnosis and prognosis, and an increasing number of studies are achieving good results for miRNA applications (by overexpression o by inhibition) in cancer treatment (Farazi et al., 2011). In cardiovascular diseases, the role of miRNA was investigated evidencing different expression profiles in hypertrophy and heart failure (van Rooij et al., 2006). MiRNA dysregulation was also observed in autoimmune diseases (Qu et al., 2014) and in other diseases involving immune system (Contreras et al., 2012), such as in infectious diseases. In this context, miRNAs can influence the manifestation and pathogenesis of infectious diseases modulating the pathogenicity of individual pathogens and regulating the expression of genes that have a pivotal role in innate and adaptive immune responses. Neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis were also found to have characteristic miRNA expression profiles (Karnati et al., 2015).

Over the last 10 years, the role of miRNAs in stroke has been widely discussed and evaluated, focusing attention on stroke risk factors (Koutsis et al., 2013) and mechanisms activated and elicited by ischemic insult (Khoshnam et al., 2017).

1.4.6.1 MicroRNAs Regulating Stroke Risk Factors

As concern the role of miRNA in stroke and in stroke-related diseases, several studies have highlighted the potential role in the progression of these diseases.

Little is still known about the correlation of miRNAs and patients with hypertension, nevertheless some evidences suggest that miR-155 and miR-637 have an important role in regulation of expression of genes related to hypertension risk, such as

ATP6V0A1 (Wei et al., 2011) and the AT1R (Ceolotto et al., 2011), acting on their specific polymorphisms.

The concept of atherosclerosis, that is a relevant stroke risk factor, is tightly related to the structural condition of endothelial cells that compose the inner arterial wall. In this scenario, many studies have been conducted on the various stages of the atherosclerotic process. For example, miR-126 has been shown to regulate VCAM-1, an adhesion molecule that plays an important role in white cell recruitment (Harris et al., 2008), while miR-21 was found to be induced by shear stress (Weber et al., 2010). The activity of vascular smooth muscle cells, which has been associated with atherosclerosis, was demonstrated to be mediated by the expression of miR-143 and -145 (Quintavalle et al., 2011). Moreover, miR-27 appears to be implicated in the progression of atherosclerosis (Chen et al., 2012).

Different microRNAs were found to be important for the induction and the maintenance of atrial fibrillation. An opposite course was evidenced between miR-1 and the channel Kir2.1, responsible for the conduction of inward rectifier K^+ current: while miRNA expression is reduced, the channel is upregulated (Girmatsion et al., 2009). This type of K^+ current was also demonstrated to increase after downregulation of miR-26 (Wang et al., 2011). Finally, miR-328 expression was particularly reduced in atrial fibrillation patients, and its forced expression shortened atrial action potential duration (Lu et al., 2010).

Numerous studies evidenced a differential expression of miRNA both in animal models of hyperglycemia and in patients with type 2 diabetes (Zampetaki et al., 2010), revealing lower plasma levels of several microRNAs, in particular miR-126, that is an endothelial-enriched microRNA. Dyslipidemia is another risk factor for stroke induction, and the most important miRNAs involved in lipid homeostasis are miR-33, targeting ABCA1 and ABCG1, which have a key role in cholesterol efflux (Rayner et al., 2010), and liver-specific miR-122, whose inhibition resulted in decreased plasma cholesterol levels and a significant improvement in liver steatosis (Esau et al., 2006) (Figure 1.11).

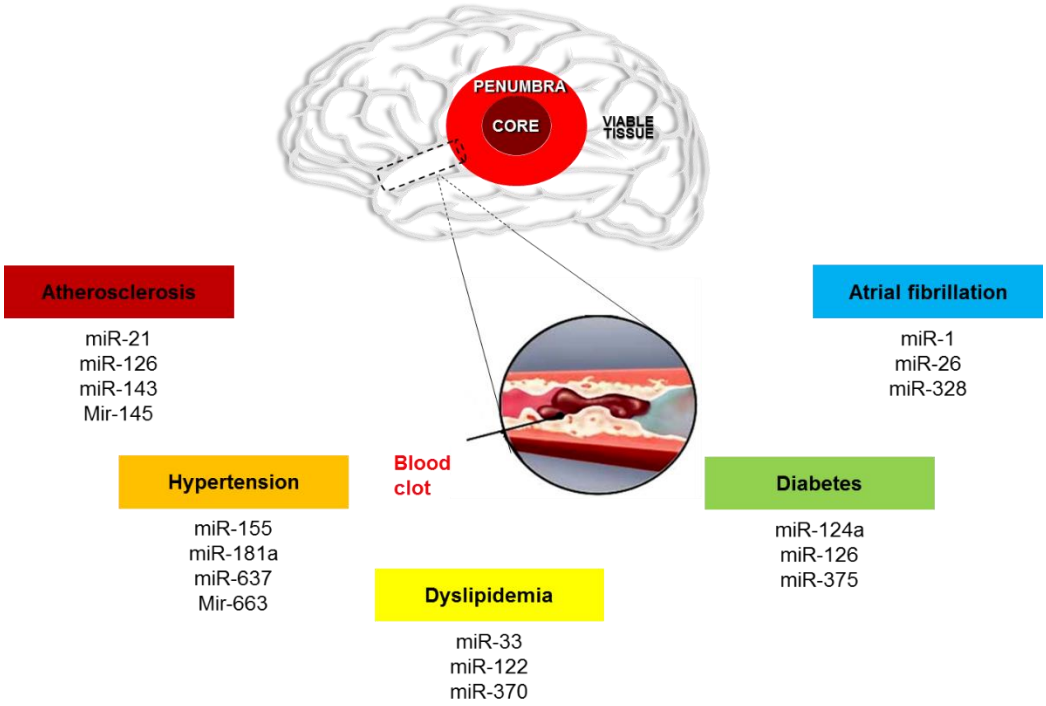

Figure 1.11: *List of microRNAs implicated in risk factors for stroke (modified, by Koutsis et al., 2013).*

1.4.6.2 MicroRNAs Regulating Stroke Pathogenic Processes

One of the mechanisms by which ischemic stroke mediates the damage of brain tissue is glutamatergic excitotoxicity (Krnjević, 2008), a process of cell death induced by excessive synaptic release of glutamate and overactivation of its receptors. In particular, under basal synaptic transmission, activation of the synaptic NMDA receptors (predominantly containing subunit NR2A) stimulates the signaling components of the neuronal survival signaling complex (NSC) that promotes neuronal survival. Conversely, after stroke induction, the increase of extracellular glutamate concentration causes excitotoxic activation of NMDA receptors (with subunit NR2B), that promotes active death-associated protein kinase (aDAPK). This latter, in turn, stimulates the neuronal death-signaling complex (NDC) and suppresses synaptic NSC activity, thus mediating neuronal death. On the other hand, glutamate binding to mGluR receptors induces calcium release from intracellular store, leading to changes in the osmolarity and to the activation of endogenous enzymes for protein, lipid and DNA cell content. Different miRNA are shown to take part to the pathway of glutamate excitotoxicity (Figure 1.12). MiR-107 overexpression occurring after ischemic stroke leads to inhibition of GLT-1 expression, with subsequent elevation of glutamate accumulation (Yang et al., 2014). On the other

hand, miR-29a has a protective role targeting PUMA, a proapoptotic factor that is known to cause also the impairment of astrocyte GLT-1, thus reducing neuronal vulnerability (Ouyang et al., 2014). Moreover, using in vitro and in vivo models of ischemic reperfusion brain injury and excitotoxic neuronal death, some researchers evidenced that miR-223 targets the subunit GluR2 of AMPA receptor and the subunit NR2B of NMDA receptor (Harraz et al., 2012). Instead, miR-125b negatively regulates NR2A, and its overexpression could cause a worsening of ischemic damage (Edbauer et al., 2010).

During ischemia several mechanisms induce oxidative stress, either by increasing ROS production or by decreasing anti-oxidative stress defense systems. In the context to discover microRNA regulating antioxidant genes, great attention has been focused on Nrf2, a nuclear factor that is crucial for transcriptional activation of ARE elements located on these genes. MiR-424 was demonstrated to induce the upregulation in MnSOD, extracellular SOD and Nrf2 expression (Liu et al., 2015), whereas miR-93 appears to target directly the 3′-UTR sites of Nrf2 (Wang et al., 2016). Furthermore, miR-210 is involved in the beneficial action mediated by vagus nerve stimulation after ischemia, by increasing SOD levels (Jiang et al., 2015). The same result was achieved by studies on miR-23a-3p, which increases the expression on MnSOD and reduces that of NO (Zhao et al., 2014). Conversely, miR-145 overexpression cause suppression of SOD2 protein levels. Finally, cyclooxygenase 2 (COX2), that triggers ROS production, was shown to be targeted by miR-101 (Strillacci et al., 2009) and by miR-146a (Iyer et al., 2012).

It has been clarified that a number of miRNAs target several genes that are involved in inflammation processes activated in ischemia. Studies demonstrated that microglia activation is prevented by miR-424 (Zhao et al., 2013), miR-let-7c-5p (Ni et al., 2015) and miR124 (Ponomarev et al., 2011). Ischemic inflammatory process may be the result of activation of Toll-like receptors (TLRs), that works via NF-kB. MiR-181c negatively regulates TLR4 expression (Zhang et al., 2015), and it can directly modulate TNF-α production in microglia (Zhang et al., 2012). In addition, it is determined that in ischemic cerebral tissue, miR-155 induces the upregulation of TLR4 and downregulates the expression of inflammatory mediators (Wen et al., 2015). MiR-146a has been found to suppress expression of IL-1β and IL-6, and during ischemia its levels are reduced (Iyer et al., 2012).

Preventing the apoptotic cascade occurring in the penumbra region is one of the main therapeutic strategies to arrest the ischemic injury. Therefore, many studies were performed to find microRNAs able to modulate cerebral ischemia/reperfusion damage through downregulation of genes involved in the apoptosis pathways. For example, several miRNAs were found to interfere with the expression of Fas/FasL pathway, such as miR-25 (Zhang et al., 2016) and miR-29 (Schickel et al., 2010). Moreover, when overexpressed in vitro, miR-21 reduces neurons' sensitivity to apoptosis by targeting Fas ligand gene (Buller et al., 2010). Caspase-3 expression, that gets a key role in the extrinsic pathway-mediated apoptosis activation, is also reported to be modulated by miRNAs: miR-99a, miR-155 and miR-let-7c-5p were found to suppress caspase-3 (Khoshnam et al., 2017), whereas experiments with miR-34a led to inhibition of caspase-3 expression (Hu et al., 2012). Therefore, modulating levels of these miRNAs could contribute to increase neuronal survival. Furthermore, anti-apoptotic function of Bcl2 proteins can be repressed by Bcl2L11, which is shown to be targeted by miR-9: in the ischemic human brain, miR-9 expression is downregulated, thus promoting apoptosis (Wei et al., 2016). Members of Bcl-2 family are also modulated negatively by miR-106b-5p, miR-497, miR-181a and miR-134 (Khoshnam et al., 2017). On the contrary, other miRNAs are known to target members of intrinsic pathway of apoptosis, for example miR-125b and miR-124 could target proteins of p53 family.

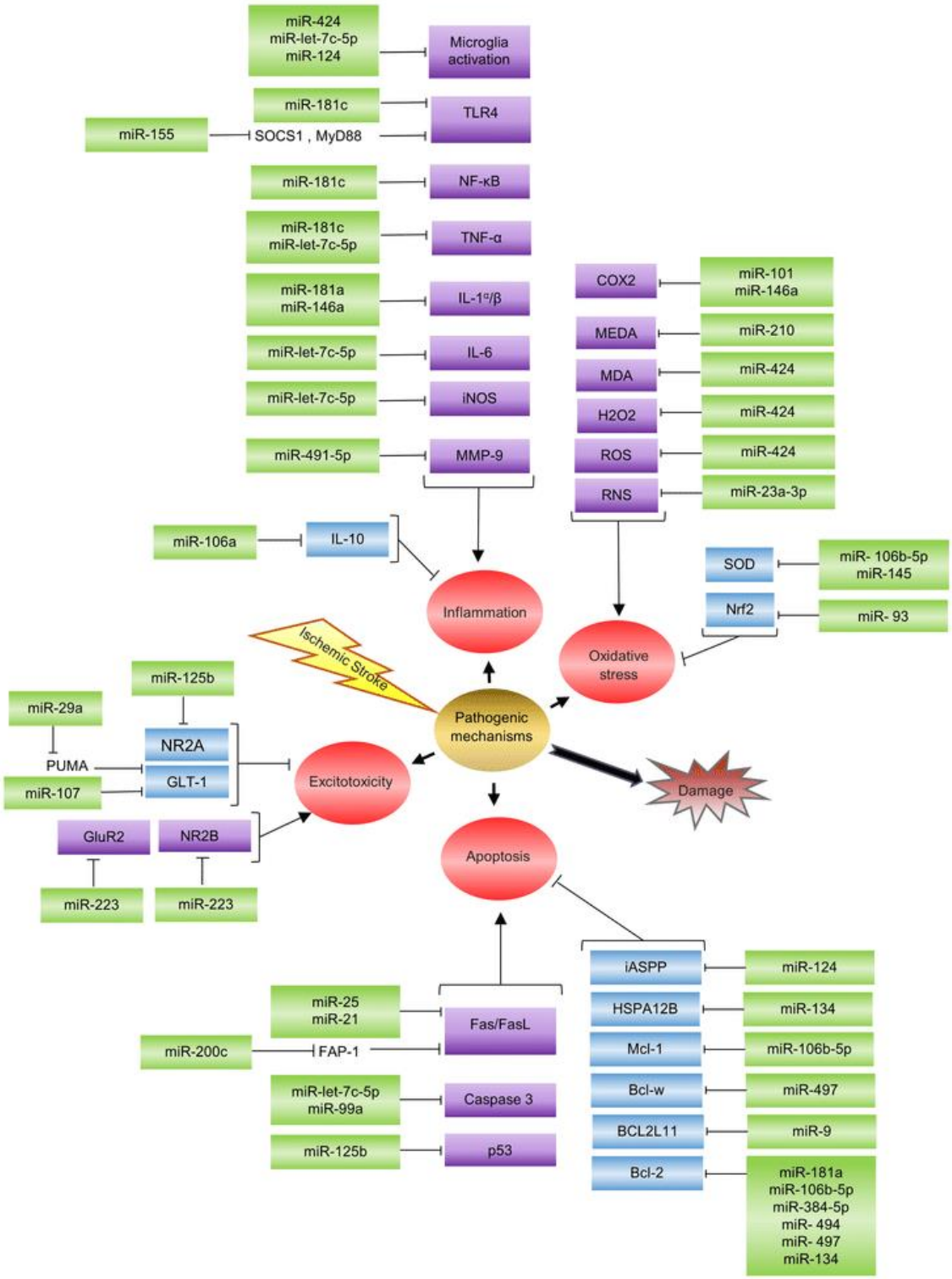

Figure 1.12: *Scheme of microRNAs involved in detrimental and protective pathways that are activated by ischemic stroke (by Khoshnam et al., 2017).*

1.4.6.3 MicroRNAs as Therapeutic Agents in Stroke

Basing on their ability to regulate gene expression, microRNAs can contribute to mediate some phenomena, such as neuroprotection, neurogenesis and angiogenesis, which lead to enhancing recovery and to repair mechanisms in ischemic stroke patients (Khoshnam et al., 2017; Wang et al., 2013) (figure 1.13).

Neuroprotective strategies have the aim to limit the ischemic damage, and therefore involve the activation of mechanisms able to save penumbra region from secondary tissue loss. Many studies on neuroprotective strategies involve applications on glutamate antagonists, and as described above miR-125b and miR-223 could be interesting regulators of NMDA receptors (Harraz et al., 2012; Edbauer et al., 2010). Nevertheless, their potential therapeutic application against stroke was not yet demonstrated.

Restoration of ionic homeostasis in the penumbra region after cerebral ischemia is another important neuroprotective strategy, in order to prevent the worsening of the damage due to the disruption of intracellular concentration of ions, such as Na^+ and Ca^{2+}. In this scenario, particular attention was focused on NCX1, whose activation improves the consequences of ischemic brain damage. MiR-103-1 was showed to target NCX1 mRNA and its inhibition induced amelioration of ischemic damage (Vinciguerra et al., 2014).

Other studies to induce neuroprotection were performed by targeting inflammatory mediators. For istance, treatment with miR-181a antagomir caused a reduction of NF-κB activation and an improvement of neurological deficits in mice (Xu et al., 2015). Moreover, suppression of TLR4, mediated by miR-181c, appeared to have therapeutic effect for ischemic stroke, interfering on microglia activation (Zhang et al., 2015). Other miRNAs were found to play a regulatory role working on microglial activation, such as miR-424, whose overexpression is beneficial on cerebral ischemia injury (Zhao et al., 2013). On the other side, miR-106a and miR-124 induced the increase of levels of IL-10 and TGF-β, respectively, that are important factors involved in the suppression of adhesion molecules in endothelial cells and in the production of pro-inflammatory cytokines (Khoshnam et al., 2017).

Another possible aspect to achieve neuroprotection consists in the ability of counteracting apoptosis process. Negative regulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-w, mediated by miR-497, promotes ischemic neuronal cell death.

Knockdown of cerebral levels of this miRNA attenuated brain infarction in *in vivo* experiments (Yin et al., 2010). Similarly, miR-145 antagomir was shown to be closely related to increased protein levels of SOD2 in the post-ischemic cortex (Dharap et al., 2009). Other studies have demonstrated that the neuroprotective effects mediated by miR-106b-5p antagomir on cerebral IRI are associated with inhibition of apoptosis and oxidative stress (Li et al., 2016). Conversely, the overexpression of miR-99a and miR-let-7c-5p reduced neuronal damage following cerebral IRI, regulating cell cycle progression and preventing apoptosis (Tao et al., 2015).

In the adult central nervous system, neurotrophic factors have important roles in the survival and maintenance of neuronal cells by activating cell survival genes and inhibiting those genes regulating apoptosis. Since the deprivation of these molecules in the ischemic penumbra zone can promote the onset of neuronal apoptosis leading to cell death, various preclinical studies evidenced the neuroprotective role of different neurotrophic factors in reducing the infarct size in animal models (Cheng et al., 2004). 3'UTRs of multiple IGF signalling pathways, comprising the same IGF gene, get binding sites for miR-1 and miR-let-7f, and their antagomirs conferred protection against ischemic stroke (Selvamani et al., 2012). BDNF and Bcl-2 expression appeared to be regulated by miR-134, whose inhibition was shown to be a therapeutic strategy by both *in vitro* and *in vivo* experiments (Huang et al., 2015). In addition, miR-107 and miR-30-5p were reported to be regulators of BDNF (Mellios et al., 2008). Signaling pathways involving Notch, Wnt and TGF-β were found to be responsible for proliferation, migration and differentiation of neuronal stem and precursor cells (NSC and NPC) to promote neuronal repair of the injured areas after ischemic stroke. MiR-21 and miR-34a could act as NPC negative regulator (Liu et al., 2013). Alternative strategies to induce neurogenesis could be associated to Sox9 gene suppression; this gene was demonstrated to prevent neuronal differentiation, that on the contrast is promoted by miR-124 overexpression targeting SOX9 mRNA (Cheng et al., 2009). MiR-9 has been revealed to limit migration and promote proliferation in human NPCs, while its downregulation allowed neuronal migration.

Angiogenesis is a biological process involving the growth of new blood vessels from pre-existing vessels, and re-establishment of the functional cerebral microvasculature network is a beneficial event that improves regional blood supply, thus promoting stroke recovery (Yin et al., 2015). Although angiogenesis is completely suppressed

under normal physiological conditions in adult brains, studies from human and experimental stroke indicate that neovascularization is present in the adult brains after cerebral ischemia, and promoting post-ischemic angiogenesis may become a useful therapeutic strategy for treatment of acute ischemic stroke. Furthermore, an increasing number of individual miRNAs have recently been identified as regulators of angiogenesis signaling pathways, involved from vascular endothelial migration to proliferation. In this scenario, angiogenesis-related miRNAs can be classified into two groups: pro-angiogenic miRNAs and anti-angiogenic miRNAs. MiR-210 overexpression promotes focal angiogenesis in the adult mouse brain, and this action is appeared to be related to VEGF upregulation (Zeng et al., 2011, 2014). Conversely, miR-92a works as anti-angiogenic miRNA targeting several pro-angiogenic proteins (Bonauer et al., 2009). Similar results were achieved on miR-15a studies, whose overexpression suppressed post-stroke angiogenesis via direct inhibition of endogenous endothelial FGF2 and VEGF activities (Yin et al., 2012). Moreover, this miRNA is upregulated after stroke in the ischemic penumbra, thus representing an interesting target for potential therapeutic strategies against stroke. Other microRNA were found to target VEGF, such as miR-16, miR-20a and miR-20b (Hua et al., 2006). Instead, miR-126 plays a pivotal role for maintaining vascular integrity during angiogenesis, targeting SPRED1 and PIK3R2, that are negative regulators of VEGF signaling pathway (Caporali and Emanueli, 2012). Parallel studies have demonstrated that miR-130a downregulated growth arrest-specific homeobox (GAX) and homeobox A5 (HOXA5), which both are anti-angiogenic transcription factors and are involved in the inhibition of endothelial cell function (Chen et al., 2008). Finally, miR-221 and miR-222 have been found to inhibit angiogenesis reducing tube formation and migration by targeting both KIT and endothelial NOS (Suàrez et al., 2007).

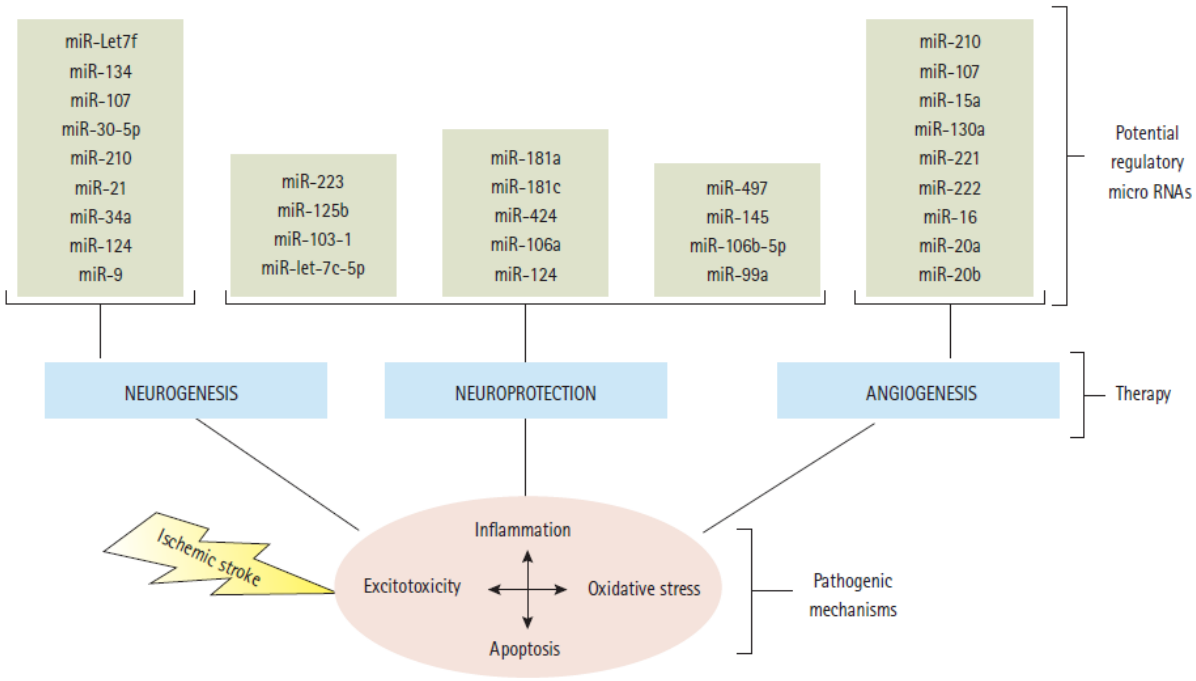

Figure 1.13: *Overview of processes involved in ischemic stroke and high potential therapeutic microRNAs (by Khoshnam et al., 2017).*

1.4.6.4 MicroRNA Expression Profiles in Post-Ischemic Brain

Several laboratories have showed that cerebral ischemia can alter miRNA profile in the brain of rodents (Koutsis et al., 2013; Vemuganti, 2013). The first miRNA expression profiling study in cerebral ischemia was performed in 2008, by using microarray analysis and real-time quantitative PCR (qPCR) techniques, on brains of rats subjected to MCAO with reperfusion for 24 or 48 hours (Jeyaseelan et al., 2008). 106 and 82 transcripts were detected at 24 and 48 hours respectively, and the result achieved was that various miRNAs were differentially expressed between the normal and ischemic brain and their expression pattern changed with reperfusion time. Interestingly, some miRNAs resulted specifically expressed in a reperfusion-time dependent manner: 8 miRNAs were found to be present exclusively in the 48-hours samples (rno-miR-99a, -181 (a, b, and c), -195, -328, -379, and -539); similarly, 32 miRNAs were found to be exclusively present in the 24-hours samples (rno-miR-16, -17, -20a, -21, -24, -25, -30a-3p, -34a, -92, -124a, -130a, -132, -134, -151*, -210, -215, -324-3p, -322, -329, -342, -361, -374, -382, -383, -422b, -433, -451, -497, -505, -664, let-7d, and let-7f). On the other hand, miRNAs that were highly upregulated during both ischemia/reperfusion periods comprised rno-miR-206, -214, -223, -290, -292-5p, -298, -327, and -494. The authors also draw attention to members of the let-

7 family implicated in neural cell specification: as described above, let-7d and let-7f were expressed in the 24-hour brain samples only; instead, let-7a, b, c and e miRNAs were downregulated in the 24-hour-reperfused MCAO rat brains but were subsequently upregulated after 48 hours from reperfusion.

A further miRNA profile study was performed in brain samples from spontaneously hypertensive rats following transient MCAO at 3, 6, 12, 24 and 72 hours post reperfusion (Dharap et al., 2009). In this case, 238 miRNAs were screened and, among these, 24 miRNAs showed an increased expression while 22 miRNA expression was significantly decreased at one or more reperfusion time points compared with sham. The most interesting evidence is that the 5 miRNAs with the highest upregulation at 72 hours (miR-206, -214, -223, -290 and -292-5p) were also found to be highly upregulated in the study of *Jeyaseelan et al. (2008)*.

1.4.6.5 MicroRNA Expression Changes in Ischemic Tolerance

Phenomenon of ischemic preconditioning (IP) is known to be associated with increased protein synthesis and altered expression of many protein-coding genes (Meller and Simon, 2013). This idea led researchers to investigate whether cerebral ischemic tolerance could be related to changes in the expression of miRNAs in brain (Saugstad, 2015; Koutsis et al., 2013). In particular, the effect of IP on miRNA expression profiles of rat and mouse brain was mainly evaluated by three studies. In the first study, from a total of 360 miRNAs that were detected in rats at 3 and 24 hours following the only IP, 8 miRNAs were upregulated selectively in the IP 3-hour group, compared with the normal group and the ischemia 3-hour group (Lee et al., 2010). These miRNAs were categorized into 2 miRNA families: miR-200 family (comprising miR-200a, -200b, -200c, -429 and -141) and miR-182 family (comprising miR-182, -183 and -96). Interestingly, these microarray results were confirmed by using real-time polymerase chain reaction, which showed an upregulation approximately from 10- to 1000-fold compared with the normal brain, whereas their levels in ischemic group were not different from those of the normal group. Moreover, miR-200b, -200c and -429 were predicted to target the 3'-UTR of prolyl hydroxylase 2 gene (PHD2), an enzyme involved in HIF1α metabolism, and this could explain the increasing levels of HIF1α after IP.

In another study, again by microarray analysis, was examined the effect of IP only, ischemia and post IP tolerance to ischemia on brain miRNA expression profiles in male mice (Lusardi et al., 2010). In particular, a large subset of miRNAs were uniquely dysregulated in the IP group, including members of the miR-200 and -182 families, which resulted to be upregulated. In addition, target mRNA of miRNAs dysregulated following ischemic tolerance were predicted, and among these, attention was focused on methyl-CpG binding protein 2 (MeCP2), which is a global regulator of transcription. Indeed, immunohistochemical staining of MeCP2 protein showed increased expression during the development of IT, whereas miR-132, known to control MeCP2 expression, was significantly downregulated.

The third main study of evaluation of miRNA expression changes after ischemic conditioning was conducted in brain samples of spontaneously hypertensive rats at 6, 24 and 72 hours following 10 minutes of MCAO (Dharap and Vemuganti, 2010). By microarray analysis 265 miRNA were screened, and among these, 11 were significantly upregulated and 9 downregulated. Analysing the possible targets of downregulated miRNAs, the protein that resulted targeted by a greater number of miRNA was MeCP2, as suggested by the studies of *Lusardi et al.*. Instead, the major pathways targeted by upregulated miRNAs included the MAP-kinase and mTOR signaling pathways, which are reported to be implicated in ischemia and hypoxia, while genes targeted by downregulated miRNAs included members of Wnt and GnRH signaling pathways, involved in cell regulation, proliferation and apoptosis.

Little is known about the involvement and the expression patterns of microRNA in remote ischemic conditioning. The most important work was conducted on a mouse model of limb remote ischemic preconditioning (RIP) against myocardial infarction (Li et al., 2014). Li and colleagues found that brief episodes of limb ischemia evoked a significant increase in cardiac expression of miR-144, identified by microarray analysis in hearts harvested at 15 min from the end of RIP stimulus. Moreover, *in vivo* pretreatment with an antisense oligonucleotide against miR-144 abolished the protective effect of RIP in isolated buffer-perfused hearts subjected to global ischemia-reperfusion, while cardioprotection achieved with RIP in isolated hearts was mimicked by *in vivo* administration of a miR-144 mimic. Finally, plasma miR-144 levels in mice and humans increased after myocardium ischemia, but this plasma mature miRNA was not protected from RNase-mediated degradation, suggesting that

this miRNA did not flow in blood through exosomes or microparticles (see paragraph 1.4.7), but rather these increased levels were observed in the exosome-poor plasma supernatant and was stabilized and protected against degradation via binding to the argonaute protein AGO-2.

1.4.7 Circulating MicroRNA

A significant number of miRNAs have been observed outside the cells, including various body fluids (Weber et al., 2010). Indeed, up to now, miRNAs have been detected in plasma, serum, milk, tears, saliva, urine, amniotic fluid, cerebrospinal fluid and semen fluid. Given the instability of most RNA molecules in the extracellular environment, the presence and apparent stability of miRNAs here was surprising. In particular, they resulted to be very stable and resistant to RNases, freezing and pH variations. Many studies demonstrated that this stability is related to the different carriers that mediate microRNA transport (Makarova et al., 2015). MicroRNA can be released by cells through microvesicles, that origin by outward budding and fission of the plasma membrane (Colombo et al., 2012). Moreover, a specific type of vesicles with a characteristic process of biogenesis, called exosomes, were showed to be enriched of miRNA (Zhang et al., 2015). This membrane-bound vesicles are present in all biofluids and are involved in phenomena of cell-to-cell communication. The current idea is that exosomes can regulate the bioactivities of recipient cells by transporting lipids, proteins and nucleic acids, such as miRNAs, while circulating in the extracellular space, and several reports have shown that exosomes play important roles in immune response, tumor progression and neurodegenerative disorders. Alternative mechanisms of miRNA transport concern the activity of apoptotic bodies, formed during the programmed cell death, and high-density lipoproteins (HDL). Finally, miRNAs were also found to travel in the extracellular medium not in vesicles, but in complex with AGO proteins to form ribonucleoproteins.

The presence of microRNA in blood and the ability to measure their levels in a non-invasive way has opened new doors in the search for peripheral biomarkers for the diagnosis and prognosis of diseases such as brain ischemia. Since the recommended therapeutic window is very limited, biomarkers for stroke have the potential to expedite diagnosis and institution of treatment. Moreover, in the last decade it has been evidenced that expression levels of miRNAs in blood are

reproducible and indicative of several diseases. For all these reasons, in order to identify peripheral markers for stroke, several studies were conducted to evaluate the expression changes in circulating miRNAs following stroke, in both animal models and human patients (Martinez and Peplow, 2016).

The first demonstration of the possibility to exploit circulating miRNA as markers for cerebral ischemia was published in 2008 and performed on blood samples withdrawn from rats subjected to transient focal ischemia, within 24 and 48 hours of reperfusion (Jeyasseelan et al. 2008). Authors compared the expression profiles of microRNA in brain and blood, showing that miRNAs dysregulated equi-directionally in both the two tissues at 24 and 48 hours were miR-290 and miR-494, which resulted upregulated, and miR-let-7i, that conversely was downregulated. On the other hand, some miRNA showed an opposite trend in expression at 24 and 48 hours (miR-150, -195 and -320). It is important to note that these experiments were conducted on the whole blood sample, not in plasma or serum.

Later on, a different study was performed on blood samples of chronic stoke patients 18 to 49 years of age, within 6 to 18 months from the stroke onset (Tan et al., 2009). Distinct patterns of miRNAs indicative of the outcome of cerebral ischaemia have been observed. It is noteworthy that several miRNAs showed changes during the progression of a disease. Another interesting observation is that more microRNAs are downregulated in all good outcome stroke samples compared to normal controls, irrespective of subtype of stroke. However, microRNA expression profiles exhibited differential fold change values among the different subtypes; for example, patients with small artery (SA) stroke had a distinctly different pattern from that of the large artery (LA) stroke samples. In particular, among the highly upregulated miRNAs in small artery stroke samples, 7 miRNAs (miR-130b, -29b, -301a, -339-5p, -532-5p, -634 and 886-5p) showed more than 2-fold change.

Recently, three brain-enriched microRNAs (miR-107, miR-128b, miR-153) were detected in plasma samples of patients suffering ischemic stroke within 24 hours oh hospital admission and in healthy volunteers, demonstrating that their levels were upregulated after stroke onset and positively correlated with the severity of cerebral ischemic injury (Yang et al., 2016). Interestingly, no correlation was found between age and smoke with the levels of miRNAs, thus suggesting that this upregulation was mediated only by ischemic insult. In parallel, a further study examined the levels of

two atherosclerosis-related miRNAs, miR-185 and -146a, in plasma of ischemic stroke patients in the acute (1 to 5 days) or subacute phase (6 to 30 days) (Li et al., 2015). While miR-185 was downregulated in both the acute and subacute phases, miR-146a resulted to be downregulated in the acute phase but upregulated in the subacute phase. Finally, it has been demonstrated that eight miRNAs were differentially expressed in blood collected 28 hours from stroke onset between acute stroke patients and vascular risk factor controls (Jickling et al., 2014). Indeed, miR-122, miR-148a, let-7i, miR-19a, miR-320d, miR-4429 appeared to decrease, whereas miR-363 and miR-487b were showed to increase in blood cells of patients with acute ischemic stroke. These miRNA were predicted to regulate different genes and pathways associated with cerebral ischemia and involving immune activation, leukocyte extravasation and thrombus formation. For these reasons, they may be important regulators of leukocyte gene expression in acute ischemic stroke.

1.4.8 MicroRNA-15/107 Family

As described above, the 5′ end-portion of miRNAs is particularly important to define the function of microRNA. Comparing the sequence of this portion, from human genes in the miRBase miRNA Registry (Griffiths-Jones et al., 2004), it was evidenced that different miRNAs share the sequence that is crucial for the interaction with 3'UTRs of target genes. An example is represented by miR-15/107 family, whose members are a group of 12 paralogous evolutionarly-conserved miRNAs, with the sequence AGCAGC in common, starting from the first of the second nucleotide of the 5' end of the mature miRNA (Finnerty et al., 2010; Wang et al., 2014). In the figure 1.14 it's illustrated the list of miRNAs belonging to this family and human genomic context of their origin. MiR-103 and miR-107 paralog genes reside in the pantothenate kinase (PANK) genes introns; these genes get important metabolism-related cell functions, indeed they are central enzymes in the regulation of cellular Co-enzyme A levels. The location of miR-103 and -107 genes within introns of the PANK genes may be physiologically relevant, and some studies have showed that both miRNAs act symbiotically with the PANK proteins (Wilfred et al., 2007). Moreover, expression levels of this pair miRNAs and their host genes often are highly correlated, presumably because they are co-transcribed. On the other hand, miR-15 and miR-16 are located in genes related to cell proliferation, SMC4 and DLE2 (the

latter is a non-coding transcript). The function of the genes related to the other members of miR-15/107 family is still unknown. Nevertheless, studies collected in the scientific literature provide clues that these miRNAs play key roles in gene regulation involved in physiological mechanisms, such as cell division, metabolism, stress response and angiogenesis, and implicated in pathological processes including cancers, cardiovascular disease and neurodegenerative diseases. All these function are due to the capacity of miR-15/107 group to target various mRNAs, including GRN, DICER1, BCL2, CDK5R1, BDNF, CDK6 and BACE1 (Finnerty et al., 2010).

MiRNA profiling experiments have shown that miR-15/107 group miRNAs are expressed at moderate-to-high levels in many mammalian tissues. The most recent expression study has evidenced that similar anatomical tissues were generally clustered together; for example heart and skeletal muscle samples demonstrated similar expression patterns (Wang et al., 2014). However, generally, the highest-expressing miRNAs in humans tend to be miR-15a, miR-15b, miR-16, miR-103 and miR-107. Moreover, in RNA isolated from different human brain samples (cerebral cortex, frontal cortex and primary visual cortex), miR-16, miR-103, miR-107 and miR-497 are the most highly expressed miRNAs among the family members; in particular miR-103 and -107 appear to be brain-enriched miRNAs, whereas the other members show higher levels in non-brain tissues.

The expression of miR-15/107 family miRNAs was also investigated in different brain cell types. In primary rat neuron, astrocyte and microglia cultures, obtained from rat pups at embryonic day 18, all tested miRNAs showed different expression patterns between neuronal and non-neuronal cells, and astrocytes and microglia share relatively similar expression profiles. Specifically, 4 members (miR-103, miR-107, miR-195 and miR-497) were among the neuron-enriched miRNAs.

As anticipated above, members of miR-15/107 are involved in different physiological functions, for example regulation of pathways related to mitosis. Specifically, it was shown that miR-15a, miR-16 miR-103 target a highly disproportionate number of cell cycle genes, and transfection with these miRNAs led to G0/G1 arrest (Linsley et al., 2007). However, the main studies about the role of these miRNAs were performed on their involvement in the regulation of cellular metabolism. First of all, miR-107 expression was demonstrated to be altered following increased extracellular glucose (Tang et al., 2009) and after dietary intake of various lipids (Davidson et al., 2009).

Furthermore, miR-103 and -107 are shown to be upregulated in obese mice, and their silencing led to improved glucose homeostasis and insulin sensitivity (Trajkovski et al., 2011). Indeed, caveolin-1, a critical regulator of the insulin receptor, was identified as a direct target gene of miR-103/107, suggesting that this protein is upregulated upon inactivation of miR-103 and miR-107 in adipocytes, contributing to the stabilization of the insulin receptor and enhance of insulin signalling.

Multiple stress conditions have been associated with miR-15/107 function. MiR-16 expression increased in cultured cells exposed to ultraviolet light and then appeared to participate in regulating the DNA-damage response (Pothof et al., 2009). On the other hand, miR-16 and miR-15 expression decreased in mice subjected to exposure with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a common environmental toxicological contaminant (Zhang et al., 2009). In addition, after induction of rodent traumatic brain injury miR-107 expression is down-regulated, and this reduction could mediate the activation of adaptive responses (Redell et al., 2009).

Finally, MiR-103 and miR-107 are strongly induced by hypoxia in vascular endothelial cells and by targeting AGO1 they are shown to promote angiogenesis (Chen et al., 2013), and more specifically microRNA-107 contributes to post-stroke angiogenesis by targeting Dicer-1 (Li et al., 2015). On the other hand, miR-16 appears to regulate VEGF, thus affecting angiogenesis (Karaa et al., 2009).

The involvement of miR-15/107 cluster in various human cell pathways suggests that the dysregulation of its gene expression may contribute to, or even cause, human diseases. For example, miR-15a and miR-16 constitute key tumor suppressors whose deletion contributes to cancer: in particular they are located in regions that when subjected to deletion might contribute to susceptibility to chronic lymphocytic leukemia (Calin et al., 2005). Moreover, granulin (GRN), which is regulated by miR-15/107 gene group members, is an active mitogen and growth factor relevant to many cancers.

MiRNAs get a direct relevance to human neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and frontotemporal dementia (FTD). Members of the miR-15/107 group have been shown to be downregulated in AD. Specifically, miR-107 is associated to the early stage of AD pathogenesis (Nelson et al., 2010; Wang et al., 2008), and in addition it targets the beta-amyloid cleavage enzyme 1 (BACE1) gene, so its downregulation may mediate the

pathogenetic consequences. Moreover, miR-107, but also other group members, strongly regulate the expression of GRN, which is a gene that increases predisposition to FTD (Wang et al., 2010). Members of the miR-15/107 group are also predicted to target neurodegeneration-related genes such as the β-amyloid precursor protein (involved in AD) and α-synuclein (involved in PD). Finally, miR-195 may regulate the gene expression if the brain growth factor BDNF, with pathogenic consequences leading to schizophrenia (Mellios et al., 2008).

About the implication of miR-15/107 cluster in cardiovascular diseases, the most studied the main studies were conducted on miR-195. In particular, overexpression of miR-195, a stress inducible miRNA that was up-regulated during cardiac hypertrophy, in transgenic mice resulted in pathological cardiac remodelling and heart failure (van Rooij et al., 2006).

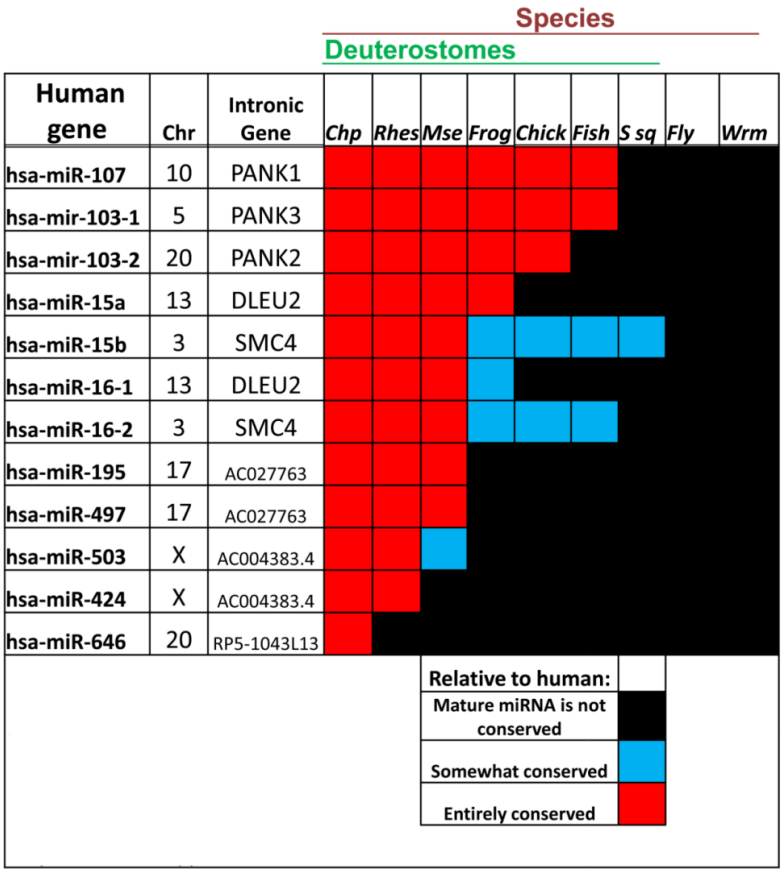

Figure 1.14: *Human genomic context including chromosome (Chr) and evolutionary conservation for the miR-15/107 gene group between human and Pan troglodytes (Chimpanzee; Chp), Macacca mulatta (Rhesus; Rhes), Mus musculus (Mouse; Mse), Xenopus tropicalis (Frog), Gallus gallus (Chicken; Chick), Danio rerio (Zebrafish; Fish), Ciona intestinalis (Sea squirt; Ssq), Drosophila melanogaster (Fruitfly; Fly), and Caenorhabditis elegans (Worm; Wrm) (by Finnerty et al., 2010).*

1.4.8.1 MicroRNA-103 – Mediated Regulation of NCX1 Expression

The experimental work described in this thesis is born following a study conducted a few years ago in our laboratory and published in 2014 (Vinciguerra et al., 2014). By bioinformatic analysis, miR-103-1 was supposed to target NCX1 transcript and investigated by in vitro assays. Indeed, using a luciferase gene reporter assay the interaction between miR-103-1 and 3′UTRs of NCX1 mRNA was demonstrated. Further confirmation was obtained by transfecting miR-103-1 mimic in BHK-NCX1 cells, PC12 cells and cortical neurons: in all three cell types the NCX1 protein expression was reduced following transfection.

In vivo experiments were performed to evaluate the expression of NCX1 protein in ischemic animals subjected to antimiRNA treatment. First of all, a time-course analysis of miR-103-1 levels and NCX1 expression after stroke revealed that miR-103-1 levels, which increased 24 hours after stroke induction, inversely correlated with NCX1 expression that conversely was reduced. As expected, anti-miR-103-1 upregulated NCX1 protein expression in the ipsilateral cortex and striatum of rats subjected to ischemic stroke, compared to rats treated with the negative control and to sham-operated animals. Finally, treatment with anti-miR-103-1 exerted a strong neuroprotective effect on ischemic damage: infusion with antimiR-103-1 in ischemic animals reduced the extent of brain ischemia and improved general and focal scores.

2. AIM

The purpose of the present experimental study is to evaluate the diagnostic and therapeutic action of microRNAs involved in gene regulation of proteins that play a key role in ionic homeostasis maintenance during brain ischemia.

In particular, the experiments described in the following paragraphs were grouped in four parts.

1) The first aim was to investigate the possible role of plasma miR-103-1 as biomarker of cerebral ischemia, assessing its expression changes in plasma samples of an animal model of stroke and on plasma samples withdrawn from human patients. In fact, as described above, treatment benefit against cerebral ischemia is time-dependent, because of the short therapeutic time window, related to stroke pathophysiology and penumbra restoring (Donnan, 2008), and for these reasons just a small portion of patients can receive thrombolytic therapy. Therefore time to diagnosis is critical and biomarkers for stroke have the potential to expedite diagnosis and institution of treatment. Recently, different predicted biomarkers were examined in positive and negative studies from experimental research and clinical trials. Actually, difficulties in biomarker discovery are primarily related to the slow or missed release of glial and neuronal proteins across the blood brain barrier after stroke. In addition, cerebral ischemia markers can lack diagnostic specificity and are increased in a variety of stroke mimic situations. Ideal stroke biomarkers should exhibit features that include diagnostic specificity and sensitivity to infarcts, differentiation between hemorrhagic and ischemic stroke, early and stable release shortly after infarction, predictable clearance, potential for risk assessment and guidance of therapies, and the ability to be quantitatively and rapidly measured by cost-effective methodologies. In this scenario, the presence of microRNA in blood and the ability to measure their levels in a non-invasive way has opened new doors in the search for peripheral biomarkers for the diagnosis and prognosis of ischemia. Moreover, in the last decade it has been evidenced that expression levels of miRNAs in blood are reproducible and indicative of several diseases.

The identification of miR-103-1 as miRNA modulated after cerebral ischemia onset and involved in the regulation of NCX1 gene expression (Vinciguerra et al., 2014),

evoked further interest and curiosity about its potential role as a peripheral marker of ischemia.

2) The second aim of this study was to investigate whether NCX1 is activated after remote ischemic conditioning (RIPO) induction and the possible involvement of miR-103 in this regulation. In fact, despite advances in preclinical studies that have established mechanisms of cell death in brain ischemia and identified potential strategies to prevent or treat brain injury following stroke, just few of these advances have successfully translated into clinical practice. Preconditioning model was largely exploited to study mechanisms elicited by endogenous neuroprotective responses, and previous studies in our laboratory evidenced that NCX1 activation observed during this neuroprotective strategy is related to the improvement of ischemic damage. Nevertheless, more recent efforts have focused on RIPO, that allows to apply a short occlusion of a distant artery after a stroke, which is more relevant to the clinical performance in human patients, but little it's still known about physiological processes that are activated from this phenomenon.

3) In order to identify other potential therapeutic targets for ischemia, next step of this study han been to search other miRNAs modulated in opposite direction by stroke and by RIPO. In this way two types of information can be achieved: firstly, to investigate miRNAs that could mediate the protection, and so to examine whether current gain-of-function or suppression strategies can induce protection from stroke; but also to predict target genes that could be regulated by miRNA modulated, with the intention to define new pharmacological therapies to activate or repress the proteins involved.

4) In the last part of this thesis the attention han been focused on NCKX2, that has an important role in the extrusion of intracellular Ca^{2+} and some studies suggest that it works more efficiently compared to other members of NCX family. In particular, it was investigated the possible role of miR-223 in the regulation of NCKX2 protein expression (predicted target of this miRNA). Moreover, to better elucidate the involvement of this miRNA in stroke progression, experiments of miR-223 manipulations were performed to test the effect of its inhibition on ischemic injury and to confirm the crucial impact of this miRNA in stroke progression.

3 MATERIALS AND METHODS

3.1 Animals

Male Sprague–Dawley rats (Charles River), weighting 200g to 250g, were housed under diurnal lighting conditions (12 h darkness/light) and in a conditioned room (23°C). Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of “Federico II”, University of Naples, Italy. Animals, during any surgical or invasive procedure, were anesthetized using 2% sevofluorane, 60% N2O, and 38% O2, and the rectal temperature was maintained at 37±0.5°C with a heat-controlled mat (Harvard Apparatus).

3.2 Ischemic Patients

Human plasma samples were taken from ischemic patients hospitalized at the stroke unit of the “Santa Maria della Misericordia” Hospital in Perugia, Italy, in collaboration with Dr. Fiorella Guadagni (“San Raffaele” University of Rome) and Dr. Valeria Caso (Hospital of Perugia). The study protocol of human plasma samples was approved by Ethic Committee of the same hospital. Samples of healthy controls were taken by volunteers from our Department, with sex and age characteristics similar to those of ischemic patients. For prognostic purposes, to verify that the amount of microRNA correlated with the severity of the damage, patient samples were pooled and analyzed separately, based on the National Institutes of Health Stroke Scale (NIHSS) values recorded just after hospital admission. Specifically, the samples were grouped for NIHSS index greater than 15 (serious deficit), ranging from 8 to 14 (moderate deficit) and less than 7 (mild deficit). Furthermore, in order to investigate a possible involvement of reperfusion in miRNA expression, patients were again grouped in those that underwent to thrombolysis treatment and those that did not undergo to thrombolysis. All blood samples were collected immediately after hospitalization (within 24 hours from ischemic damage).

3.3 Transient focal ischemia

Stroke model used for this experimental work has been transient middle cerebral artery occlusion (tMCAO), a surgical procedure that consists in the insertion of a suture filament into the internal carotid artery until to the MCA (Longa et al., 1989), modified and readapted in our laboratory (Pignataro et al., 2008). Briefly, under an operating stereomicroscope (Nikon SMZ800, Nikon Instruments, Florence, Italy), the first step is the identification and the exposition of right carotid bifurcation, by using surgical pincers (Dumont #7, FST). Then, the external carotid artery near the bifurcation is cut and electrocauterized to create a stump on the artery. A silicon-coated nylon filament (Doccol, Ca, USA) is inserted through the stump and it's gently advanced 19 mm into the right internal carotid artery until it blocks the origin of the MCA. At this point, the surgical wound can be closed and the filament is left in place for 100 minutes and kept here by using a braided silk suture (FST); when the time of occlusion is expired, the filament is gently removed in order to allow reperfusion. Induction of ischemia is confirmed by monitoring regional cerebral blood flow in the area of the right MCA through a disposable microtip fiber optic probe (diameter 0.5mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001; Perimed, Sweden) and analyzed using PSW Perisoft 2.5 (Kawano et al., 2006).

3.4 Limb Remote Ischemic Postconditioning

Remote ischemic postconditioning was induced by subjecting ischemic animals to a brief cycle of femoral artery occlusion (FAO), as previously described and demonstrated (Pignataro et al., 2013). Briefly, after 20 minutes of reperfusion from tMCAO, femoral artery was identified, isolated and occluded with two microserrafine clips (FST) to stop the blood flow for a duration of 20 minutes. The achievement of femoral artery blockade was verified by measuring blood flow. Throughout this text, the occlusion of the femoral artery, without the previous tMCAO, will be indicated as FAO; conversely, the condition of femoral artery occlusion following 100 minutes tMCAO will be indicated as RIPO.

3.5 Evaluation of the Infarct Volume

The ischemic volume was evaluated by 2,3,5-triphenyl tetrazolium chloride (TTC) staining. Specifically, the brains were cut into 1 mm coronal slices with a vibratome (Campden Instrument, 752 M). Coronal slices were incubated in 2% TTC for 20 min and in 4% Paraformaldehyde overnight. The infarct area of the sections (about six in total) was calculated with image analysis software (Image-J 1.50c) (Bederson et al., 1986). The total infarct volume, corrected for edema, was calculated as sum of the single infarcted areas and expressed as percentage of the volume of the hemisphere ipsilateral to the lesion. Edema was calculated by difference between the volume of hemisphere ipsilateral to the lesion and that of hemisphere contralateral to the lesion.

3.6 Evaluation of Neurologic Deficit Scores

Neurological scores were evaluated before the sacrifice according to 2 scales: a general neurologic scale and a focal neurologic scale (Clark et al., 1997). In the general score, the following 6 general deficits were measured: (1) hair conditions, (2) position of ears, (3) eye conditions, (4) posture, (5) spontaneous activity, and (6) epileptic behavior. For each of the 6 general deficits measured, animals received a score ranging between 0 and 12 depending on the severity of signs. The scores of investigated items were then summed to provide a total general score ranging from 0 to 28. In the focal score, the following 7 areas were assessed: (1) body symmetry, (2) gait, (3) climbing, (4) circling behavior, (5) front limb symmetry, (6) compulsory circling, and (7) whisker response. For each of these items, animals were rated between 0 and 4 depending on severity. The 7 items were then summed to give a total focal score ranging from 0 to 28.

3.7 Intracerebroventricular Administration of AntimiRNA

The continuous release of antimiRNA into brain lateral ventricle was achieved by using osmotic pumps (alzet® MICRO-OSMOTIC PUMP MODEL 1003D), which ensure a continuous and closed circulation between the cannula and the cerebrospinal fluid, at the speed of 1µl/1h. The osmotic pumps were prefilled with 80µl of antimiRNA or negative control antimiR, and were immersed in saline solution (0.9% NaCl) in Falcon conical tubes over night at 37°C. After an incubation of 24

hours, implantation of the osmotic pump frame was carried out in rats positioned on a stereotaxic apparatus 2 hours before the induction of transient ischemia. The osmotic pump was connected to a brain infusion kit (alzet® 3-5mm) made of a stainless steel cannula that was implanted into the right lateral ventricle using the stereotaxic coordinates from the bregma: 0.4mm caudal, 2mm lateral, and 2mm below the dura and secured to the skull with dental cement. The pump was placed in the skin fold on the neck of the rat. The 72-hour anti-miR intracerebroventricular (icv) infusion allowed us to overcome problems related to the short half-life of miRNA.

3.8 Drugs and Chemicals

For *in vivo* loss of function experiments, antimiRNA for miR-223-5p (miRCURY LNA™ microRNA inhibitor, 5 nmol, for rno-miR-223-5p, Exiqon) and antimiR control (miRCURY LNA™ microRNA inhibitor control, 5nmol) were used. Both the molecules, purchased in dried down pellets, were diluted to the final concentration in saline solution (0.9% NaCl) previously filtered (Microglass filters), and were icv administered at the concentration of 10μM (9μg/kg body weight) starting 2 hours before ischemia induction.

3.9 Plasma Samples Collection

Blood samples were withdrawn from tail vein of anesthetized rats before the ischemia induction and at different time intervals from reperfusion. Using a 1ml syringe with a 23G needle blood was withdrawn and collected in BD Vacutainer tubes (K3 EDTA 5.4mg). To separate plasma, blood samples were centrifuged in the same collecting tubes at 1500 x g (2900rpm) for 8 minutes at room temperature in ALC PK 120 centrifuge. The supernatant plasma was transferred to sterile eppendorf and centrifuged in Eppendorf centrifuge at 11,000rpm to clean the sample from any cellular residues. Prior to RNA extraction, the absorbance at 415nm of a 50μl aliquot for each sample was measured in a Bio-rad Microplate Reader to evaluate the presence of free hemoglobin because of a previous hemolytic process. The data reported in the literature suggest to work on plasma samples with absorbance values below 1.0OD. This restriction is necessary in order to obtain an evaluation of microRNAs that are present in exosome or free in plasma, and released after

ischemic lesion, excluding those present in the red and white blood cells and released following hemolysis.

3.10 Rat Cortical Neurons

Primary cortical neurons were prepared from 17-day-old Wistar rat embryos (Charles River) (Scorziello et al., 2007). Briefly, the rats were first anesthetized and then decapitated to minimize animals' pain and distress. Dissection and dissociation were performed in Ca^{2+}\Mg^{2+}-free phosphate-buffered saline (PBS) containing glucose (30mM). Tissues were incubated with papain for 10 minutes at 37°C and dissociated by trituration in Earl's Balanced Salt Solution (EBSS) containing DNAse (0,16U\ml), bovine serum albumin (10mg\ml), and ovomucoid (10mg\ml). Neurons were plated in plastic Petri dishes (Falcon™ Becton-Dickinson) pre-coated with poly-D-lysine (20µg\ml), were grown in MEM\F12 (Life Technologies) containing glucose, 5% of deactivated fetal bovine serum (FBS) and 5% of horse serum (HS, Life Technologies), glutamine (2mM\l), penicillin (50Units\ml), and streptomycin (50µg\ml) (Invitrogen). Within 48h of plating, cytosine arabinoside (arabinoside-C) (10µM) was added to prevent non-neuronal cell growth. Neurons were cultured at 37 °C in a humidified 5% CO2 atmosphere and used after 7-10 days of culture.

3.11 Transfection of Rat Cortical Neurons

Primary cortical neurons were transfected with 50 nM of rno-mir-223-5p Mimic, rno-miR-223-5p antimiRNA and respective Negative Controls. HiPerFect Trasfection Reagent was used as transfection agent, according to the manufacturer's protocol. After an incubation period of 24 hours, the medium was replaced and the cells were harvested and used for western blot analysis.

3.12 Analysis by Real-Time Polymerase Chain Reaction

Total RNA from brain tissues was extracted with Trizol following supplier's instruction (TRI Reagent® - Sigma). For miRNA analysis, 5ng of RNA were retrotranscribed in cDNA, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Taqman probes, following TaqMan Small RNA Assays Protocol (16˚C for 30 min,

42˚C for 30 min and 85˚C for 5 min); in this way, only RNA target was retrotrascribed in cDNA. Taqman technology (Thermo Fisher Scientific) consists in a pair of probes: one for retrotranscription and one for PCR. Probes used are the following: miRNA Assay hsa-miR-103 (ID: 000439); miRNA Assay mmu-miR-223-5p (ID: 007896); miRNA Control Assay 4.5S RNA (ID: 001716). Quantitative real-time polymerase chain reaction was performed with TaqMan Universal PCR Master Mix II (Applied Biosystems) in a 7500 Fast Real-Time PCR System (AB Applied Biosystems). cDNA samples were amplified simultaneously in triplicate in 1 assay run, following the protocol for Taqman assays: 50°C for 2 minutes, 95°C for 10, 40 cycles of amplification of 95°C for 15 seconds and 60°C for 1 minute. All reactions were run in triplicate. Results were analysed and exported with 7500 Fast System SDS Software.

For NCX1 mRNA, 1µg di RNA were retrotranscribed, using High Capacity cDNA Reverse Trascription Kit (Applied Biosystems), but following the manufacturer's protocol: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. Real-Time PCR was performed as described above, with GAPDH as endogenous control RNA. Probes used are the following: Gene Expression Assay Slc8a1 (ID: Rn04338914_m1); Gene Expression Assay Gapdh (ID: Rn01462661_g1).

MicroRNA isolation from plasma samples was performed with miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's protocol. For cDNA synthesis, not specific concentration, but precise volumes (5µl) of RNA were used. Retrotranscription and Real-Time PCR were performed as for brain tissues. As endogenous control for plasma samples was used the U6 snRNA (miRNA Control Assay U6 snRNA, ID: 001973).

3.13 Analysis by Western Blot

Rat cortical neurons and rat brain samples were homogenized in a lysis buffer (50mmol/L Tris-HCl, pH 7.5, 100mmol/L NaCl, 1% Triton X-100) containing protease and the phosphatase inhibitor. After centrifugation at 12,000g at 4°C for 15 minutes, the supernatants were collected. Protein concentration was estimated using Bradford method, by means of a spectrophotometer (Eppendorf). Then, 80-100µg of protein was mixed with a Laemmli sample buffer and boiled at 95 °C for 5 min. The samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and

transferred to nitrocellulose membranes. Blots were probed with antibodies to NCX1 (1:1000 Swant), NCKX2 (1:500, Vinci Biochem), β-actin (1:10000 Sigma) and α-tubulin (1:10000 Abcam) diluted in tris buffered saline (TBS-T) 1% bovine serum albumin overnight (4°C). Then, they were detected using horseradish peroxidase-conjugated secondary antibody (1:2000; mouse and rabbit Cell Signaling; 60min at room temperature in 5% non-fatmilk) and an enhanced luminescence kit (Amersham Pharmacia Biotech, NJ, USA).

3.14 MicroRNA Expression Profiling by Microarray

To obtain a general microRNA expression profile that is modulated after RIPO induction, a microarray for microRNA was performed. In particular, total RNA from the ischemic area was extracted with Trizol following supplier's instruction (TRI Reagent® - Sigma) and diluted to 2µg total. These samples were sent to Lc Sciences (Houston, Texas, USA), a global biotechnology company that is provided with a microRNA microarray service, using microarrays based on the developed µParaflo® technology platform. This Biochip technology consists in a high performance microfluidic custom microarray platform for all mature miRNAs described in the latest version of the miRBase database (Release 21, July 2014).

3.15 Validation of Microarray Results

Results obtained by microarray were validated by real-time PCR. RNA was extracted by brain tissues with Trizol following supplier's instruction (TRI Reagent® - Sigma). For retrotranscription we used High Capacity cDNA Reverse Trascription Kit (Applied Biosystems) but following the protocol for Creating Custom RT and Preamplification Pools using TaqMan® MicroRNA Assays. Briefly, 50ng of RNA were retrotranscribed in cDNA using RT Primer Pool containing a mix for all 13 miRNA targets and for 4.5S RNA, as endogenous control, and incubating the reaction mix at 16°C for 30 minutes, 42°C for 30 minutes and at 85°C for 5 minutes. Then, cDNA product was preamplificated using TaqMan PreAmp Master Mix and appropriate PreAmp Primer Pool mix, and letting react with the following protocol: 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, twelve cycles of two amplification steps, composed of 15 seconds at 95°C and 4 minutes at 60°C, and finally a phase of 10 minutes at

99.9°C. At the end of preamplification 25µl of DNA were obtained and diluted in 175µl di TE buffer 0,1X. Real-time PCR was performed in Taqman Custom Plates (Applied Biosystems) that already contained Taqman microRNA Assays on the bottoms of wells, using TaqMan® Universal Master Mix II (Applied Biosystems), as described above. Each Custom Plate (of 96 wells) contained all primers for 2 samples (in triplicate), so with one plate only two samples could be read: one sample of sham group and one sample of tMCAO or RIPO group.

3.16 Statistical Analysis

Values are expressed as means ± SEM. In particular, Real-Time PCR results are expressed as fold change ($2^{-\Delta\Delta Ct}$) compared to the control group setted to 1, following the instructions provided by the literature (Livak and Schmittgen, 2001). Briefly, difference between Ct values of gene of interest and internal control (ΔCt) is calculated for both control sample and target sample. Then, difference between ΔCt of target sample and control sample (ΔΔCt) is calculated. Fold change of gene expression of target samples compared to control sample is calculated as $2^{-\Delta\Delta Ct}$.

For Western Blot analysis, results are expressed as percentage of variation of target protein (already normalized for internal control) of test sample compared to control sample.

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA), using ANOVA followed by Newman–Keuls or Bonferroni post test for group more than 2. To compare two groups unpaired t-test was used. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

4. RESULTS

4.1 Evaluation of miR-103-1 Plasma Expression in Rats Subjected to tMCAO

The first step in this thesis work was to identify the presence of miR-103-1 in rat plasma and to evaluate any differences before and after the induction of ischemia. In particular, rats of 8-9 weeks were subjected to 100 minutes tMCAO and blood samples were withdrawn at different time intervals from reperfusion: 30 minutes from stroke onset (-70 minutes from reperfusion), and 30 minutes, 6 hours, 24 hours, 72 hours and 7 days from reperfusion. Compared to the expression of miRNA in healthy rats, miR-103-1 plasma levels did not change until 72 hours, whereas were strongly and significantly reduced at 7 days from reperfusion (FC = 0.11±0.03) (figure 4.1).

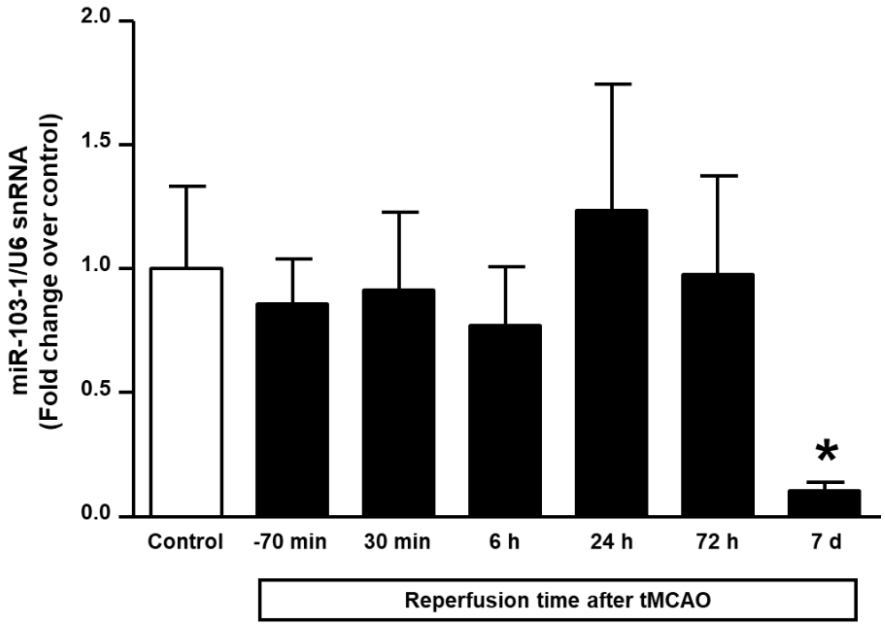

Figure 4.1: Time course of miR-103-1 expression in plasma samples of animals subjected to 100 minutes tMCAO. Blood was withdrawn from healthy rats before surgical procedure, (control, n=13) and after tMCAO at different time intervals from reperfusion: -70 minutes (corresponding to 30 minutes from ischemia induction, n=6); 30 minutes from reperfusion (n=6); 6 hours from reperfusion (n=11); 24 hours from reperfusion (n=9); 72 hours from reperfusion (n=10); 7 days from reperfusion (n=10). MicroRNA levels were normalized with respect to U6 snRNA and results were expressed as fold change over control animals. Each column represents the mean ± S.E.M. *p<0.05 versus control rats.

4.2 MiR-103-1 Expression Is Significantly Reduced in Human Plasma Samples of Ischemic Patients

In order to characterize the expression of miR-103-1 in stroke patients, experiments were carried out in human plasma samples withdrawn from ischemic patients

immediately after their hospitalization. Samples were stratified according to stroke gravity, based on NIHSS values recorded in hospital. Moreover, samples were organized so that each group comprised patients getting similar risk factors, i.e. smoke, alcohol, diabetes and obesity. Compared to healthy individuals, all patients showed very low expression of miR-103 in their plasma samples, regardless of stroke severity, reaching very low levels (figure 4.2 A, B and C). Furthermore, to investigate whether thrombolytic treatment could affect the release of this miRNA in the blood, a new set of plasma samples from patients subjected to thrombolysis was analyzed. Results showed that, miR-103 is still downregulated in plasma of ischemic patients also after thrombolysis treatment (figure 4.2 D, E and F).

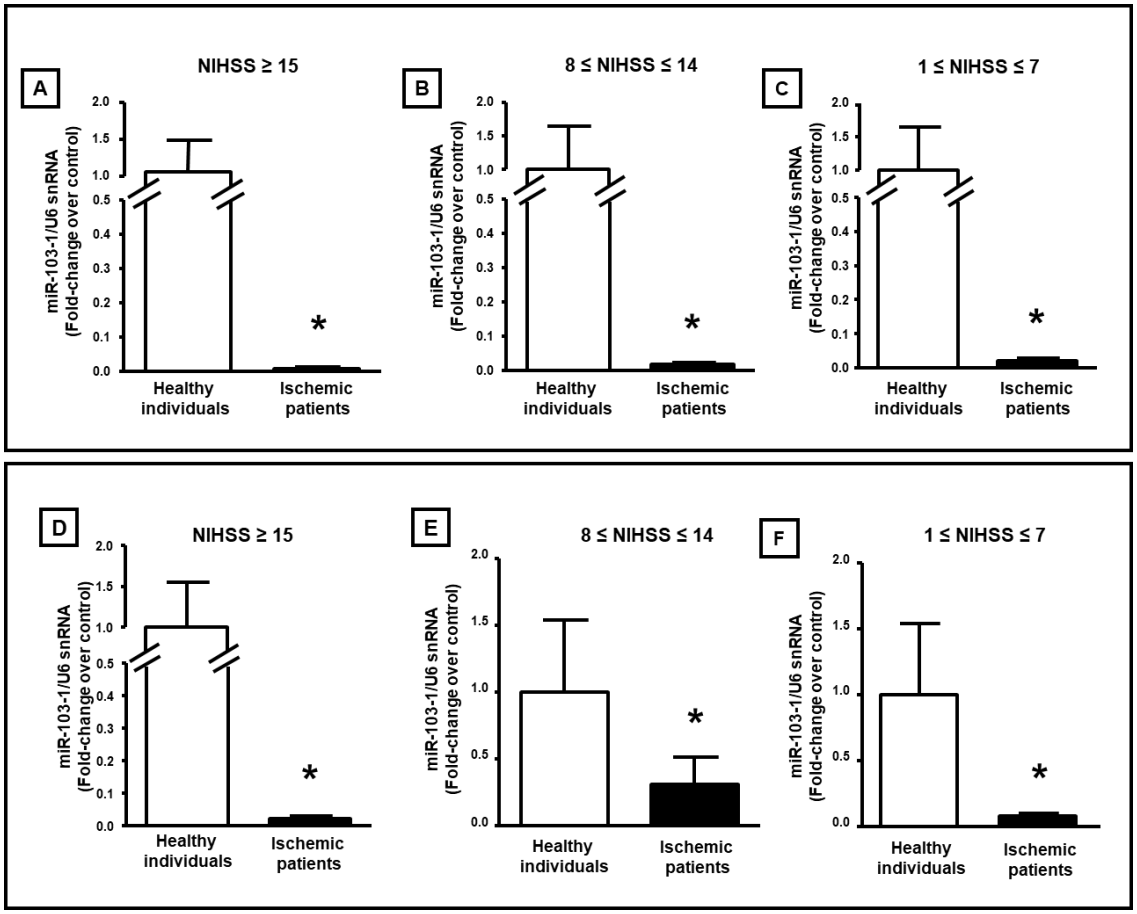

Figure 4.2: Relative expression analysis of miR-103-1 in plasma samples of ischemic human patients. MicroRNA levels are expressed as fold change over healthy controls. Each column represents the mean ± S.E.M. Results of microRNA expression were normalized with respect to U6 snRNA. *p<0.05 vs. sham-operated controls.

Panels A, B and C show miR-103 expression levels of patients which were not previously subjected to thrombolysis treatment:

(A) Expression levels in ischemic patients with serious deficits; n=4 samples per group.
(B) Expression levels in ischemic patients with moderate deficits; n=4 samples per group.
(C) Expression levels in ischemic patients with slight deficits; n=6 samples per group.

Panels D, E and F show miR-103 expression levels of patients which were previously subjected to thrombolysis treatment:

(D) Expression levels in thrombolysed ischemic patients with serious deficits; n=6 samples per group.
(E) Expression levels in thrombolysed ischemic patients with moderate deficits; n=6 samples per group.
(F) Expression levels in thrombolysed ischemic patients with slight deficits; n=6 samples per group.

4.3 Remote Ischemic Postconditioning Prevents the Increase of miR-103-1 Expression in the Ischemic Area Ipsilateral to the Lesion

In order to correlate the expression levels of miR-103-1 after ischemia and after the neuroprotection elicited by RIPO, real-time PCR analysis was performed on the whole ischemic region affected by damage. In particular, the brain area withdrawn for this analysis comprised a part of striatum and a part of prefrontal cortex (figure 4.3). Animals were sacrificed at 24 hours from surgical procedures. MicroRNA levels significantly increased in the tMCAO group (FC = 4.10±0.41) compared to the same region of sham-operated animals (FC = 1.06±0.20). Conversely, in animals subjected to the strategy of femoral artery occlusion following ischemia induction, miR-103 expression returned to baseline values (FC = 1.40±0.30).

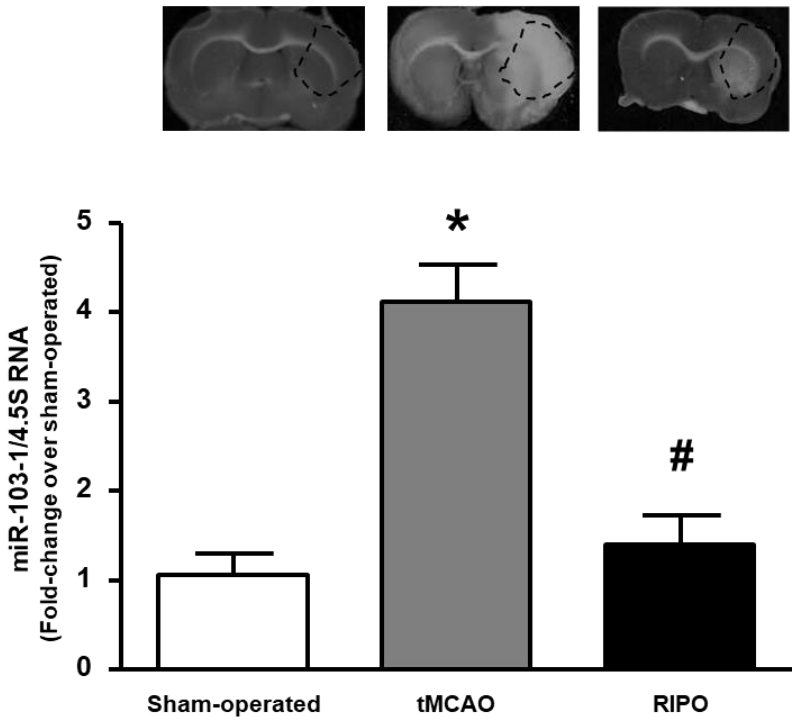

Figura 4.3: Relative expression analysis of miR-103-1 in the ischemic region of rats subjected to tMCAO and limb remote postconditioning (RIPO) at 24h from reperfusion. MicroRNA levels are expressed as fold change over sham-operated controls. Each column represents the mean ± S.E.M. Levels of microRNA expression were normalized with respect to 4.5S RNA. *p<0.05 vs. sham-operated controls. #p<0.05 vs. tMCAO; n=3 samples per each group.

On the top of the panel illustrative examples show the amplitude of the damage in the three different conditions and the area considered for the analysis (dashed).

4.4 Evaluation of miR-103-1 Expression in Ipsilateral Cortex and Striatum of Ischemic Rats Subjected to Limb Remote Postconditioning

Since neuroprotection elicited by Remote Postconditioning involves the preservation of the temporoparietal cortex, limiting the damage to the striatum only, we investigated whether miR-103 has a different expression pattern in these two cerebral regions.

A time course of miR-103 expression was performed on samples ischemic brain tissues taken from rats subjected to ischemic rats subjected to RIPO at different time intervals from reperfusion (3, 24 and 72 hours), comparing them with samples withdrawn from rats subjected to tMCAO only. As showed in figure 4.4, miR-103 was significantly reduced in ipsilateral cortex after a few hours from the ischemic event (FC = 0.51±0.15) and its levels remained low at 24 hours (FC = 0.47±0.10), until to rise again at 72 hours from reperfusion (FC = 1.12±0.25). On the other side, the expression of miRNA after limb remote postconditioning was still reduced after 3 hours from reperfusion (FC = 0.53±0.15), but it began to increase after 24 hours (FC = 0.79±0.15), reaching the same levels of the sham-operated, and significantly increased after 72 hours from reperfusion (FC = 3.08±1.13) (figure 4.4 A).

Time course experiments performed on cortex samples were reproduced on striatum samples of the same animals. Compared to the expression assessed in the sham-operated controls, in the tMCAO group, miR-103 was significantly reduced in ipsilateral striatum after 3 hours from reperfusion (FC = 0.55±0.08) (figure 4.4 B). Interestingly, its levels strongly increased with a maximum of expression at 24 hours from reperfusion (FC = 2.17±0.72). After 72 hours from ischemia induction the expression of miR-103 was still higher than sham-operated, even if it began to decrease (FC = 1.29±0.34). MiR-103 expression after RIPO was reduced after 3 hours from reperfusion (FC = 0.64±0.11), whereas it significantly increased at 24 hours (FC = 2.62±0.70) and 72 hours (FC = 1.73±0.39) from reperfusion, reflecting the same course of levels observed in tMCAO groups.

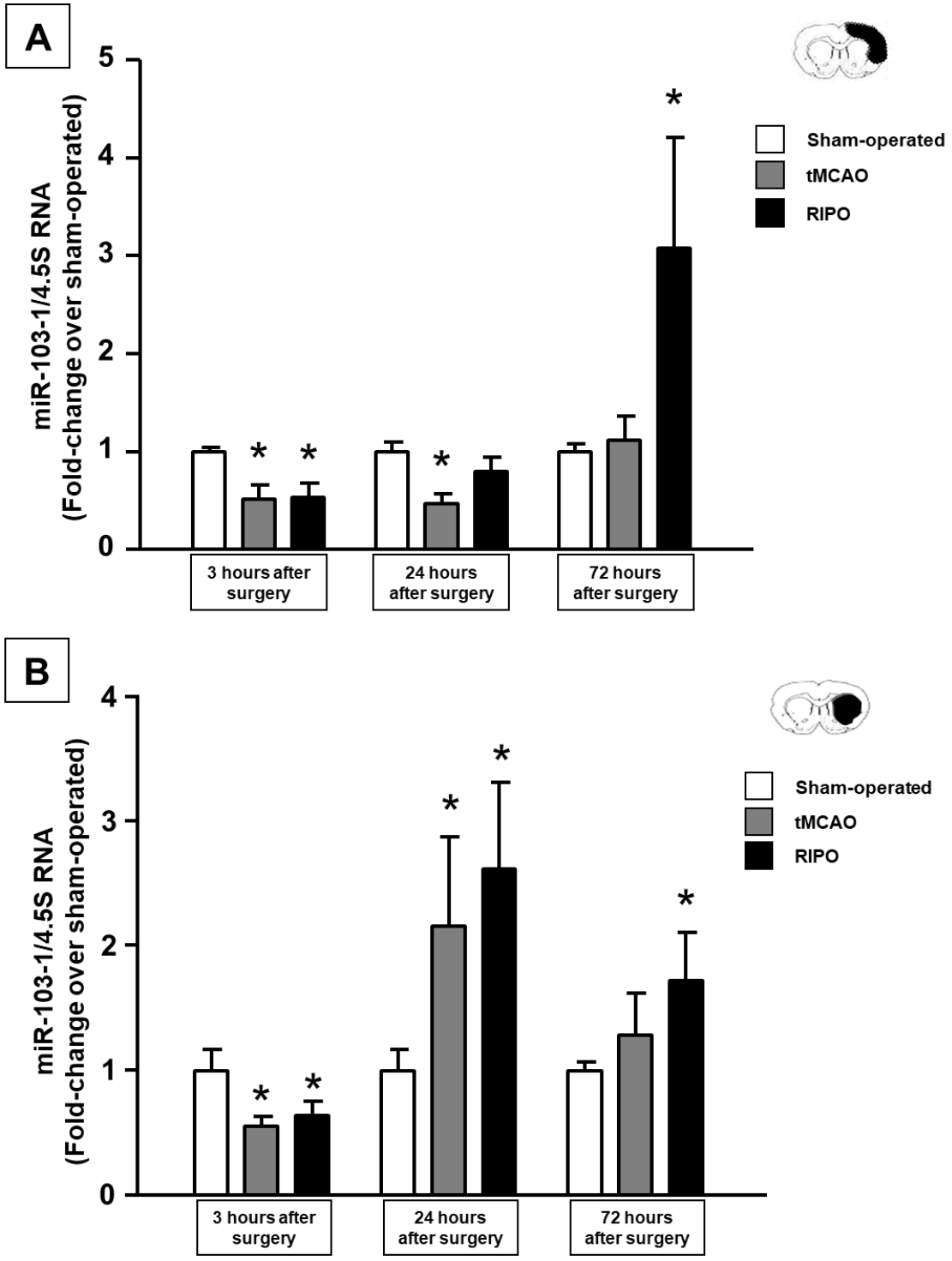

Figure 4.4: Time course of miR-103 expression in cortex and striatum of ischemic rats subjected to limb remote postconditioning. Three time windows were analyzed after reperfusion: 3h, 24h and 72h. MicroRNA levels of both tMCAO and RIPO groups are expressed as fold change over sham-operated controls. Each column represents the mean ± S.E.M. Results of microRNA expression are normalized with respect to 4.5S RNA. *p<0.05 vs. sham-operated controls.
(A) Expression levels of miR-103 in cortex: n=5 samples per each group at 3h; n=3 samples per each group at 24h; n=4 samples per each group at 72h.
(B) Expression levels of miR-103 in striatum: n=4 samples per each group at all times.

4.5 Limb Remote Postconditioning Restores NCX1 Levels Reduced after tMCAO in both Ipsilateral Cortex and Striatum

In order to clarify whether mir-103 levels correlated with NCX1 levels, its protein expression was assessed at 24 hours from reperfusion.

In particular, NCX1 was evaluated in cortex ipsilateral to damage or rats subjected to tMCAO, to FAO only and to RIPO. For each of these surgical procedures appropriate sham-operated animals were prepared to be compared with each treatment. Firstly, the expression of NCX1 does not change between the three kinds of sham-operated animals (data not shown). Moreover, as shown in figure 4.5 A, NCX1 protein levels were strongly and significantly reduced after tMCAO (45.16%±17.46%), as previously demonstrated (Pignataro et al., 2011). FAO did not induce changes in NCX1 expression, and the same result was observed also after RIPO induction, suggesting that RIPO strategy could prevent the downregulation of NCX1 induced by tMCAO (figure 4.5 A).

Assessment by Western Blot Analysis of NCX1 protein levels in the striatum of sham, tMCAO, FAO and RIPO groups has evidenced results that can be comparable with those achieved in the cortex. Indeed, NCX1 appeared to be reduced after 24h from tMCAO, while its levels did not change in the FAO group. Interestingly, also in the striatum the suppression of NCX1 expression after stroke induction was prevented by RIPO application (figure 4.5 B).

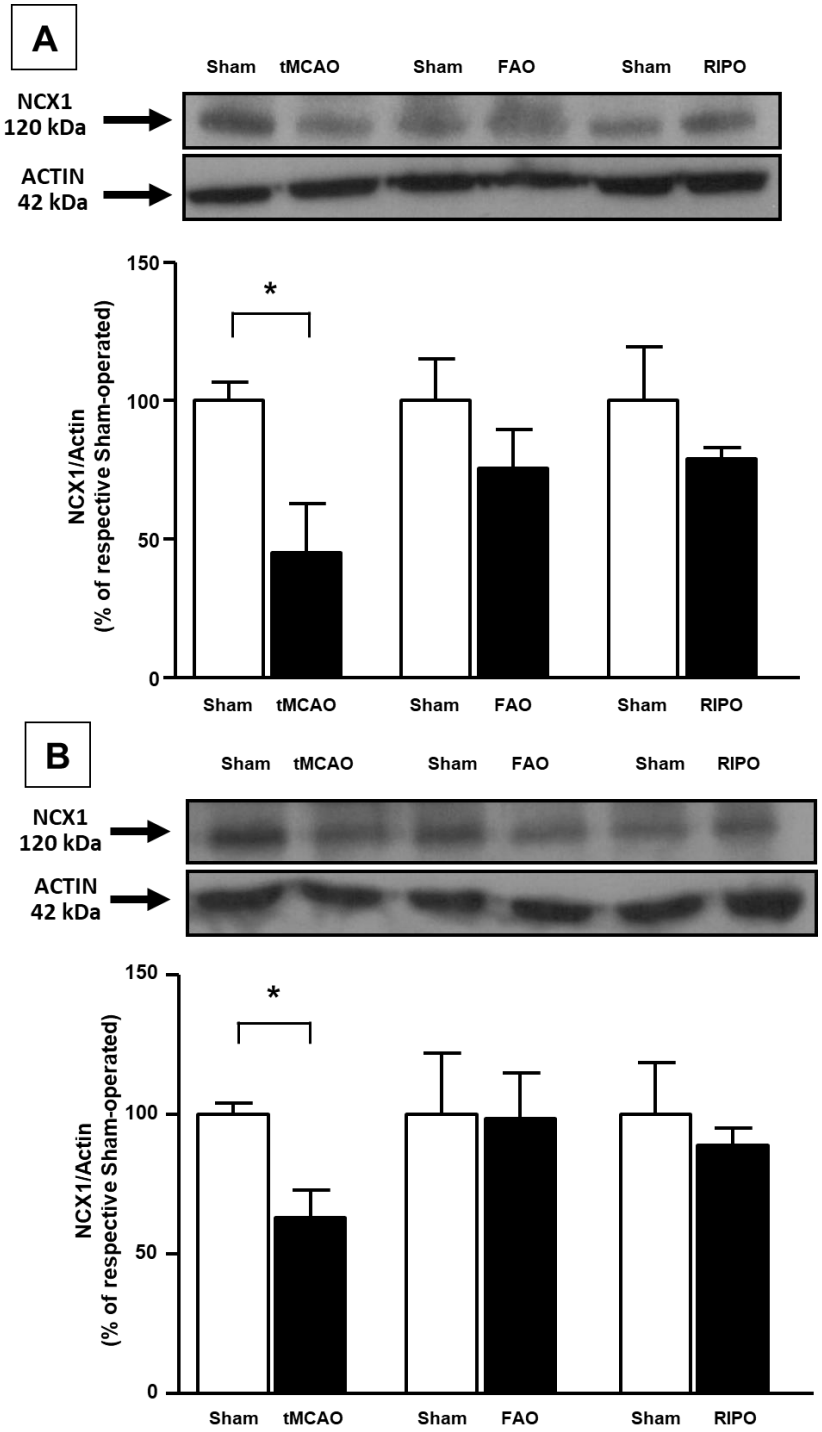

Figure 4.5: Evaluation of NCX1 protein expression in samples of ipsilateral cortex and striatum from ischemic rats subjected to limb remote postconditioning at 24h from reperfusion. NCX1 levels are expressed as percentage versus the respective sham-operated controls. For each surgical procedure specific sham-operated animals were subjected to the same conditions. Each column represents the mean±S.E.M. Results of NCX1 expression were normalized with respect to β-actin. *p<0.05 vs. sham-operated controls. On the top of each graph, representative blots of NCX1 and β-actin signals rats subjected to tMCAO, rats subjected to FAO only, and rats subjected to RIPO, with respective sham animals.
(A) NCX1 protein levels in cortex: n=3 samples per each group.
(B) NCX1 protein levels in striatum: n=3 samples per sham-tMCAO group, tMCAO group and sham-FAO group; n=5 samples per FAO group; n=4 samples per sham-RIPO group; n=6 samples per RIPO group.

4.6 NCX1 mRNA Expression Is Unchanged in Brain Ischemic Regions after Limb Remote Postconditioning Induction

In order to understand if the changes of NCX1 protein expression observed in cortex and striatum after tMCAO and RIPO are due either to the involvement of posttranscriptional mechanisms that suppress NCX1 protein synthesis or to suppression of the same ncx1 transcription, we have compared the levels of NCX1 mRNA in ischemic rats after tMCAO and after RIPO. As shown in figure 4.6, no difference was found in NCX1 mRNA expression in ipsilateral cortex after 24 hours from tMCAO and RIPO induction compared to sham-operated animals (figure 4.6 A). On the other side, in striatum NCX1 mRNA significantly increased after 24 hours from both tMCAO (FC = 4.38±0.93) and RIPO (FC = 4.15±0.90) (figure 4.6 B).

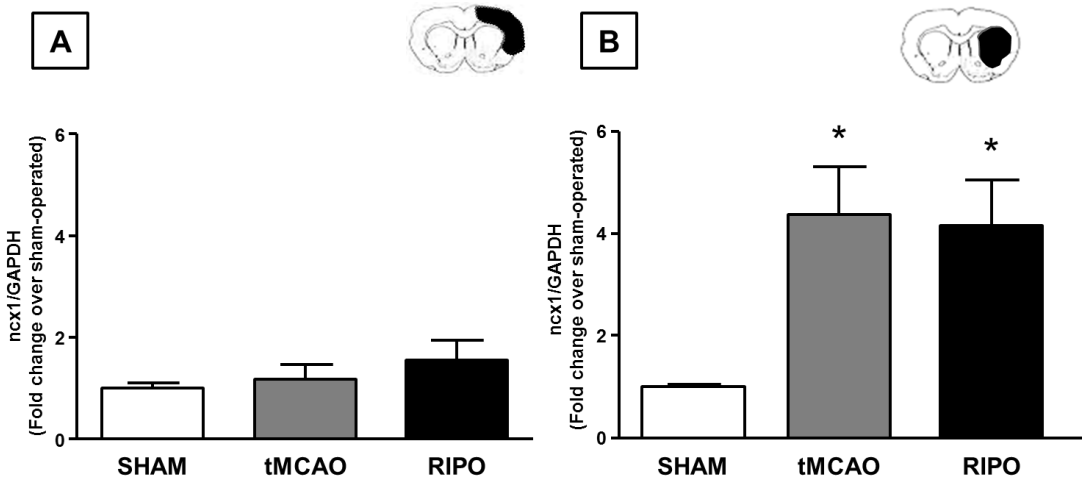

Figure 4.6: Relative expression analysis of *ncx1* mRNA in ipsilateral cortex and striatum of ischemic rats subjected to limb remote postconditioning at 24h from reperfusion. mRNA levels are expressed as fold change over sham-operated controls. Each column represents the mean ± S.E.M. Results of mRNA expression were normalized with respect to *gapdh* mRNA. *p<0.05 vs. sham-operated controls.

(A) Expression levels of *ncx1* mRNA in cortex; n=3 samples per each group at all times.
(B) Expression levels of *ncx1* mRNA in striatum; n=3 samples per each group at all times.

4.7 Remote Ischemic Postconditioning Enhances Plasma Levels of miR-103-1

To understand whether remote postconditioning strategy could induce changes in circulating levels of miR-103, a time course of plasma expression was assessed after FAO and after RIPO. As shown in figure 4.7, in plasma samples withdrawn from rats subjected to FAO only, with no previous induction of ischemia, miR-103 levels did not change at the three time points analysed (figure 4.7 A). By contrast, after RIPO application (figure 4.7 B), miR-103 significantly increased in plasma, already at 30 minutes from reperfusion, with a fold change at least greater than 3.

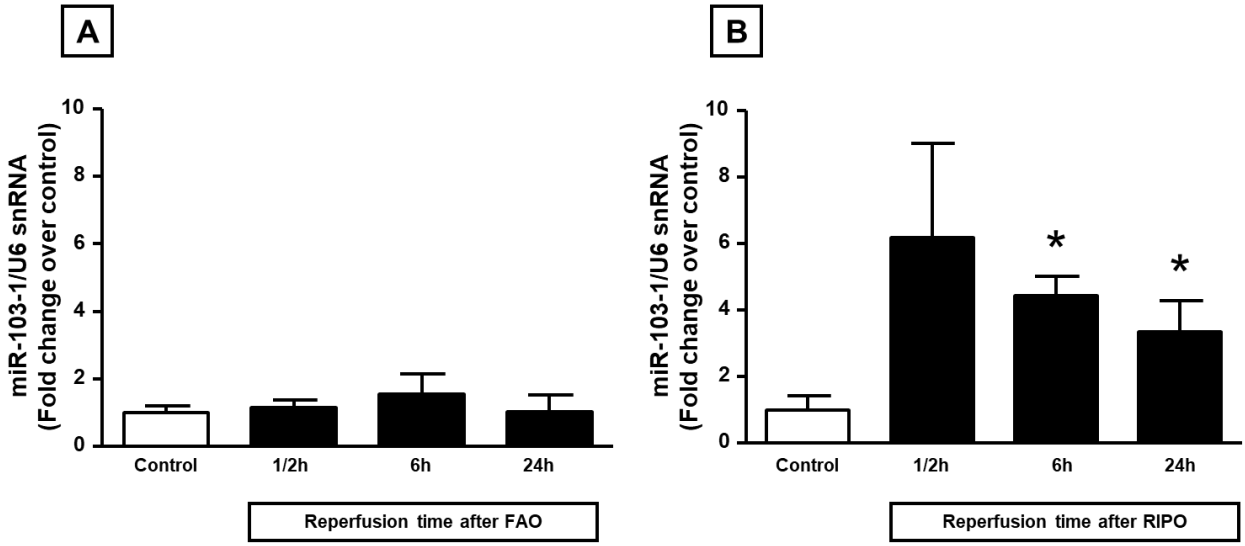

Figure 4.7: Time course of miR-103-1 expression in plasma samples of animals subjected to femoral artery occlusion only and to remote ischemic postconditioning. A) MiR-103 expression in plasma of rats subjected to FAO only; B) miR-103 expression in plasma or rats subjected to RIPO. Blood was withdrawn from healthy rats before surgical procedure, but under anesthesia (control, n=4 per FAO group and n=3 per RIPO group) and after FAO and RIPO at different time intervals from reperfusion: 30 minutes from reperfusion (n=4 per FAO group and n=3 per RIPO group); 6 hours from reperfusion (n=4 per FAO group and n=3 per RIPO group); 24 hours from reperfusion (n=4 per FAO group and n=3 per RIPO group). MicroRNA levels were normalized with respect to U6 snRNA and results of microRNA expression are expressed as fold change over control animals. Each column represents the mean ± S.E.M. *p<0.05 versus control rats.

4.8 MicroRNA Expression Profiling in the Ischemic Area of Rats Subjected to Limb Remote Postconditioning

In order to identify microRNA that could be exploited as a therapeutic target, a miRNA expression profile in brain tissue was performed to discriminate miRNAs modulated by the neuroprotective strategy of RIPO and that showed an opposite trend to that observed after ischemia. RNA samples were prepared from the ischemic region ipsilateral to damage, of a section of brain comprising striatum and taken from rats subjected to sham surgery, tMCAO and RIPO. From these samples a microarray analysis for microRNA was performed according to all mature miRNAs of all species available in the latest version of the miRBase database (2014). In figure 4.8 are shown the results of miRNAs with an expression pattern significantly different between tMCAO and RIPO groups, compared to sham-operated animals.

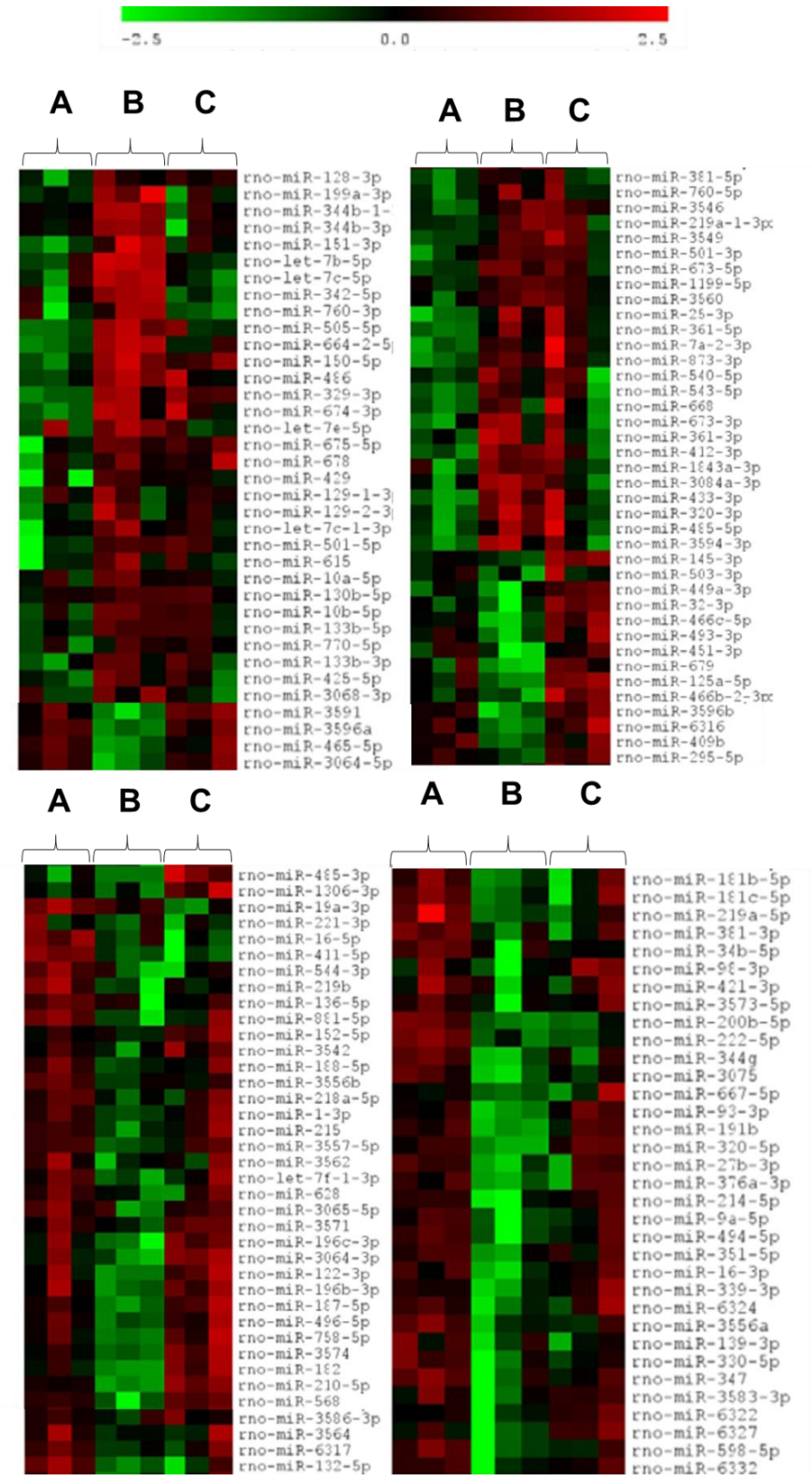

Figure 4.8: Heat map of microRNA expression profiles in the whole ischemic area of sham, tMCAO and RIPO groups. Only the average of signal intensities for each significantly expressed miRNA are reported ($p<0.05$). Red and green indicate up- and downregulation respectively. The vertical line A refers to the three samples of sham group for each miRNA; the line B indicates the tMCAO group; the line C refers to RIPO group. n=3 per each group.

4.9 Validation of MicroRNA Profiles by Real-Time PCR in the Ischemic Area of Rats Subjected to Limb Remote Postconditioning

Examining the results obtained from microarray analysis, we have selected 13 microRNAs whose expression resulted more significantly different between ischemic and neuroprotective rats, compared to sham group: let-7a-5p, miR-21-5p, miR-32-3p, miR-34c-3p, miR-103-3p, miR-125a-5p, miR-143-3p, miR-150-5p, miR-210-5p, miR-382-5p, miR-451-5p, miR-466b-5p and miR-485-3p. The expression of these miRNAs was validated by real-time PCR in the whole ischemic region of rats subjected to tMCAO and to RIPO (figure 4.9). Compared to the sham-operated animals, almost all thirteen microRNAs significantly increased in the tMCAO group, except for miR-125a-5p, whose expression appeared to increase but with a remarkable standard error. On the contrary, after RIPO induction three different situations were observed. Firstly, some miRNAs continued to be significantly unregulated as in tMCAO group, such as miR-21-5p, miR-32-3p, miR-210-5p, miR-382-5p and miR-466b-5p. For other miRNAs, real-time PCR showed a not significant increase of their levels, and it's the case of miR-34c-3p, miR-103-3p and miR-150-5p. Finally, RIPO treatment prevented the raise of expression occurring after ischemia induction in the remaining miRNAs: let-7a-5p, miR-143-3p, miR-451-5p and miR-485-3p in the RIPO group returned to the same levels of sham rats.

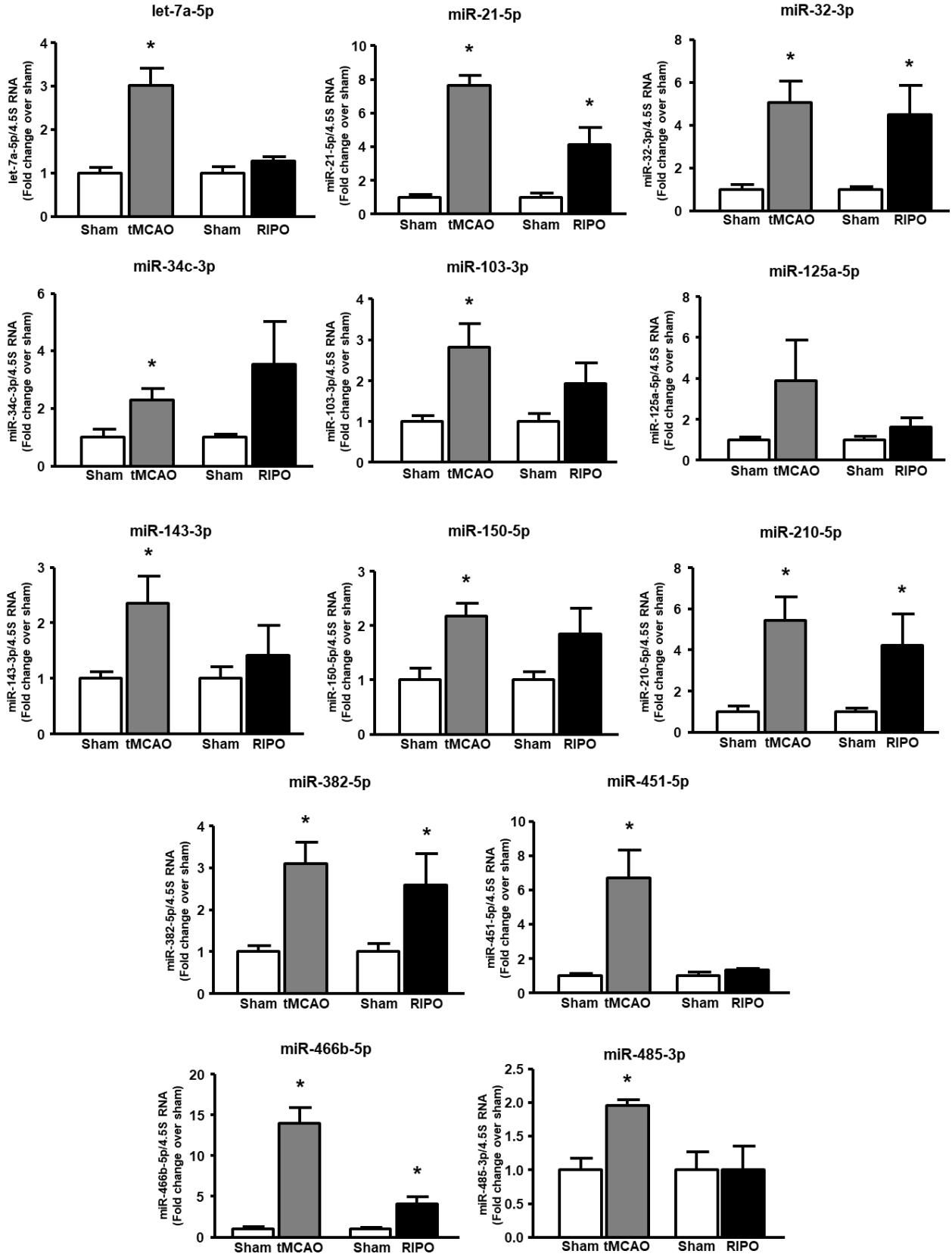

Figure 4.9: Validation of microarray results by real-time PCR. MicroRNA levels of rats subjected to tMCAO and RIPO are expressed as fold change over the respective sham-operated controls. Each column represents the mean ± S.E.M. Results of miRNA expression were normalized with respect to 4.5S RNA. *p<0.05 vs. sham-operated controls. n=3 or 4 per group.

4.10 Identification of Novel MicroRNA Involved in Ionic Homeostasis Regulation

The last part of this experimental thesis concerned the identification of novel miRNAs modulated in stroke and involved in calcium homeostasis. Comparing the results of different microRNA expression profiles analyzed in the experimental works that are cited in paragraph 1.4, it has emerged that miR-223 is one of the miRNAs that more increased by microarray analysis in many articles studied. We have analyzed the miRNA sequence and evaluated via bioinformatic analysis possible interactions with mRNA of genes that play a pivotal role in the regulation of ionic homeostasis in ischemia. This search led to identify NCKX2 as possible target of miR-223. In figure 4.10 are shown the predicted sites of interaction of NCKX2 3'UTR for miR-223, as indicated by latest version of TargetScan (v7.0; targetscan.org) (Agarwal et al., 2015).

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile
Position 330-336 of SLC24A2 3' UTR rno-miR-223-5p	5' ...AAAUAUUAUAAAUAUAUACACAU... 3' GUUGAGUCGAACAGUUUAUGUGC	7mer-A1	-0.07	61
Position 1745-1751 of SLC24A2 3' UTR rno-miR-223-5p	5' ...AAAUCGGCACACGUCAUACACAG... 3' GUUGAGUCGAACAGUUUAUGUGC	7mer-A1	-0.02	16
Position 2826-2832 of SLC24A2 3' UTR rno-miR-223-5p	5' ...UUAUUCUGUGCACAUAUACACAC... 3' GUUGAGUCGAACAGUUUAUGUGC	7mer-A1	-0.03	27
Position 3892-3898 of SLC24A2 3' UTR rno-miR-223-5p	5' ...ACCACACACACACACAUACACAC... 3' GUUGAGUCGAACAGUUUAUGUGC	7mer-A1	-0.02	16
Position 5819-5825 of SLC24A2 3' UTR rno-miR-223-5p	5' ...CUCAAAUCAAGUUACAUACACAC... 3' GUUGAGUCGAACAGUUUAUGUGC	7mer-A1	-0.02	24

Figure 4.10: Bioinformatic prediction of interaction between miR-223-5p and SLC24A2 3'UTR. A screen-shot of TargetScan during research for putative regulators of NCKX2, showing predicted target sites of SLC24A2 mRNA for rno-miR-223-5p.

4.11 Evaluation of miR-223-5p Expression in Ipsilateral Cortex and Striatum of Ischemic Rats

The first step of this study was to confirm that miR-223 expression increased in cortex and striatum in our model of stroke. Interestingly, in both cortex and striatum the expression of miR-223-5p resulted very low in sham-operated rats (with Ct values higher than 35); interestingly, after tMCAO induction its level significantly increased in a time-dependent manner. Indeed, in cortex, miR-223 levels raised at 24 hours (FC = 3.80±1.55) and increased at 48 hours (FC = 10.30±4.37) and 72 hours (FC =

4.80±2.51) from reperfusion (figure 4.11 A). In striatum, the expression began to enhance already at 6h from tMCAO (FC = 3.19±1.30), and remained high at 24, 48 and 72 hours from reperfusion (FC = 19.63±10.07, 8.94±6.55 and 36.02±6.21, respectively) (figure 4.11 B).

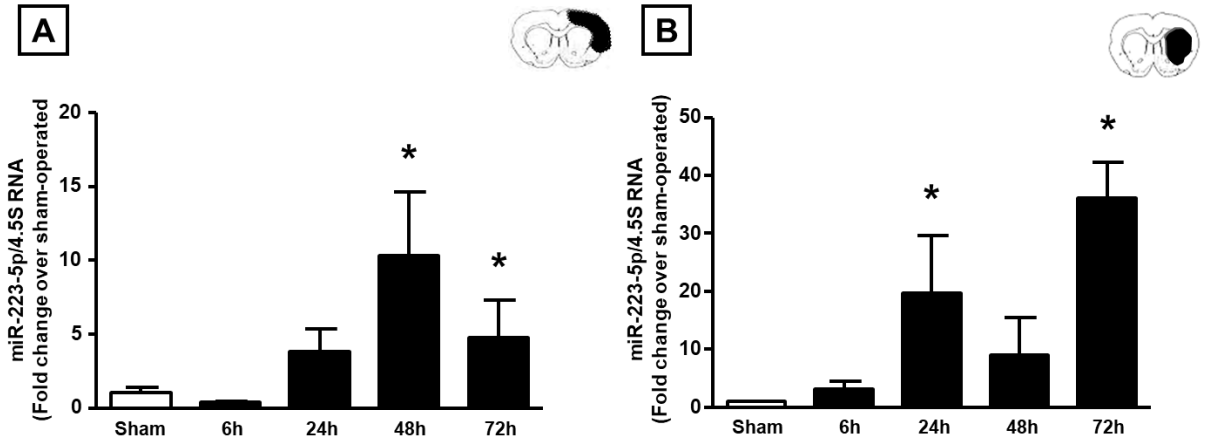

Figure 4.11: Time course of miR-223-5p expression in cortex and striatum of rats subjected to tMCAO. Four time windows were analyzed after reperfusion: 6h, 24h, 48h and 72h. MicroRNA levels of tMCAO groups are expressed as fold change over sham-operated controls. Each column represents the mean ± S.E.M. Results of microRNA expression are normalized with respect to 4.5S RNA. *p<0.05 vs. sham-operated controls. (A) Expression levels of miR-223 in cortex; n=3 samples per each group at all times. (B) Expression levels of miR-223 in striatum; n=3 samples per each group at all times.

4.12 Evaluation of NCKX2 Protein Expression in Ipsilateral Cortex and Striatum of Ischemic Rats

NCKX2 protein levels were assessed in the cerebral regions of cortex and striatum. Both tissues were harvested from animals subjected to tMCAO and sacrificed at 6, 24 and 72 hours from reperfusion. In cortex no change of expression was observed at any of the time intervals analysed (figure 4.12 A). Conversely, in striatum, 72 hours after reperfusion the expression of NCKX2 resulted significantly suppressed compared to sham control animals, reaching almost the 50% of downregulation (figure 4.12 B).

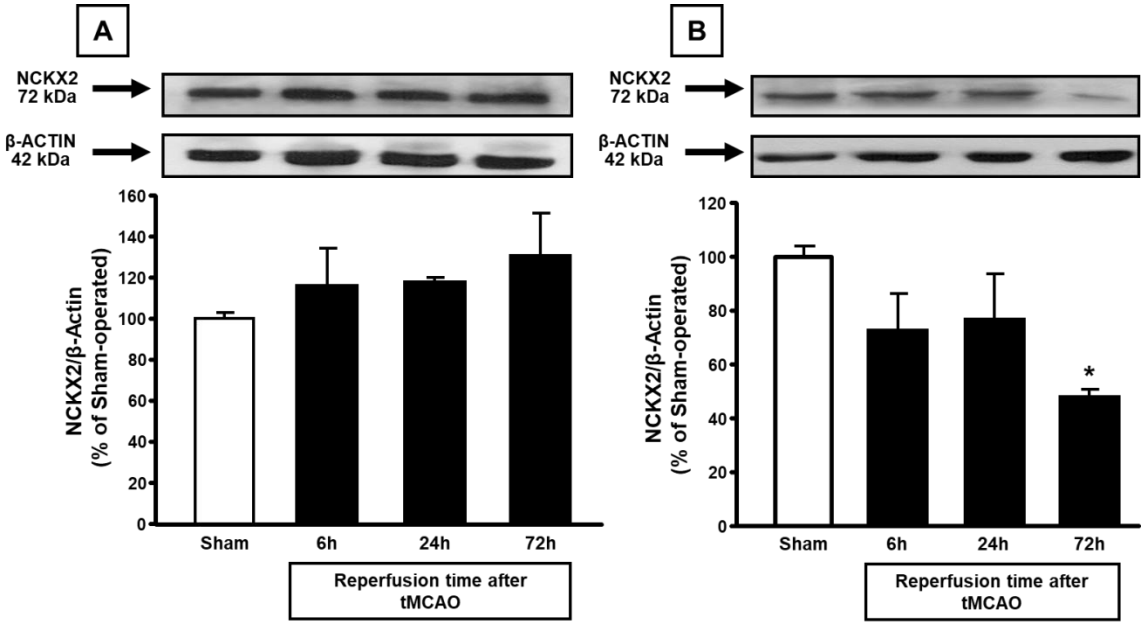

Figure 4.12: Evaluation of NCKX2 protein expression in samples of ipsilateral cortex and striatum from ischemic rats at 6h, 24h and 72h from reperfusion. NCKX2 levels are expressed as percentage versus the sham-operated controls. Each column represents the mean ± S.E.M. Results of NCX1 expression were normalized with respect to β-actin. *p<0.05 vs. sham-operated controls. n=3 samples per each group. On the top, representative blots of NCKX2 and β-actin signals in the sham animals and ischemic rats sacrificed at 6h, 24h and 72h, respectively.
A) Protein expression levels of NCKX2 in cortex. (B) Protein expression levels of NCKX2 in striatum.

4.13 miR-223-5p Regulates NCKX2 Protein Levels in Cortical Neurons

Primary cortical neurons were transfected with rno-mir-223-5p Mimic, rno-miR-223-5p antimiRNA and respective negative controls, in order to verify that NCKX2 expression was actually modulated by miR-223 (figure 4.13). These results showed that the transient transfection with mimic of miR-223 was able to induce a remarkable downregulation of NCKX2 in cortical neurons of rat. In particular, a significant reduction in NCKX2 expression was obtained by exposing cells to 50 nM of miRNA mimic (46.64%±9.74%) compared to mimic negative control (100%±12.75%). On the other side, treatment with antimiR-223 did not induce significant changes in NCKX2 protein levels, confirming that the expression of this miRNA in normal conditions was very low and so antimiRNA has had no effect.

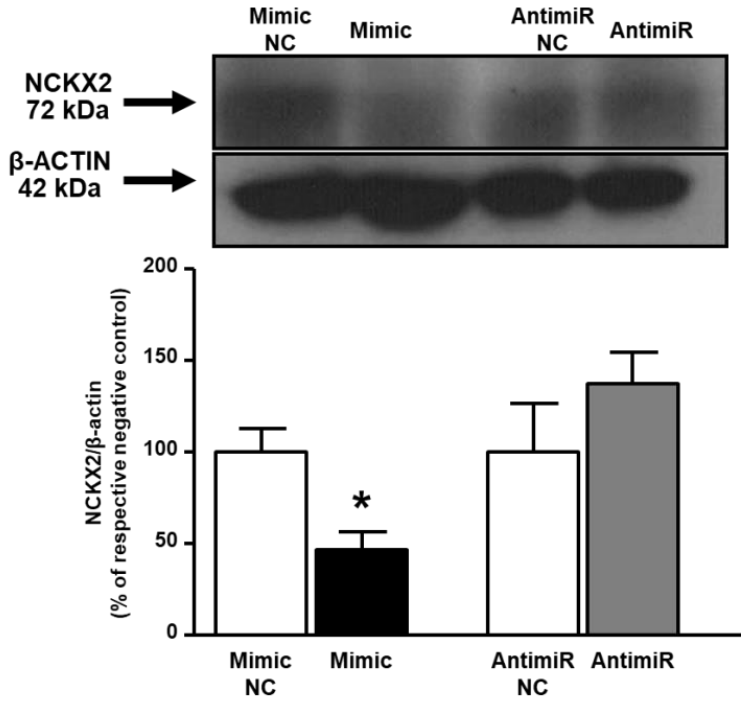

Figure 4.13: Assessment of NCKX2 protein levels on cortical neurons of rat transfected with mimic and antimiRNA of miR-223-5p. Rat cortical neurons were transfected with 50nM of miRNA mimic, miRNA antimiRNA and mimic and antimiR negative controls (NC). NCKX2 levels after mimic and antimiRNA treatment are expressed as percentage versus the respective negative controls. Each column represents the mean ± S.E.M. Results of NCKX2 expression were normalized with respect to β-actin. *p<0.05 vs. sham-operated controls. n=5 samples per mimic NC group; n=3 samples per mimic group; n=5 samples per antimiR NC group; n=5 samples per antimiR group. On the top, representative blots of NCKX2 and β-actin signals in mimic NC, mimic of miR-223-5p, antimiR NC and antimiR-223-5p, respectively.

4.14 *In Vivo* Administration of AntimiR-223-5p Ameliorates the Ischemic Damage

To investigate the possible therapeutic and protective role of miR-233 inhibition, rats were subjected to tMCAO and continuous infusion of miR-223 inhibitor or its negative control up to 72 hours from reperfusion; after this time antimiR-infusion was stopped and animals were sacrificed. Ischemic volume was significantly reduced by miR-223 inhibition (13.20±1.29) compared to negative control group (56.12±1.89) (figure 4.14 A). Furthermore, shortly before their sacrifice for ischemic volume measurement, animals were evaluated with general and focal scores that are representative of neurological deficits. As it's shown in figure 4.14 B and C, antimiR-223 treatment improved the neurologic state after 72h hours from reperfusion.

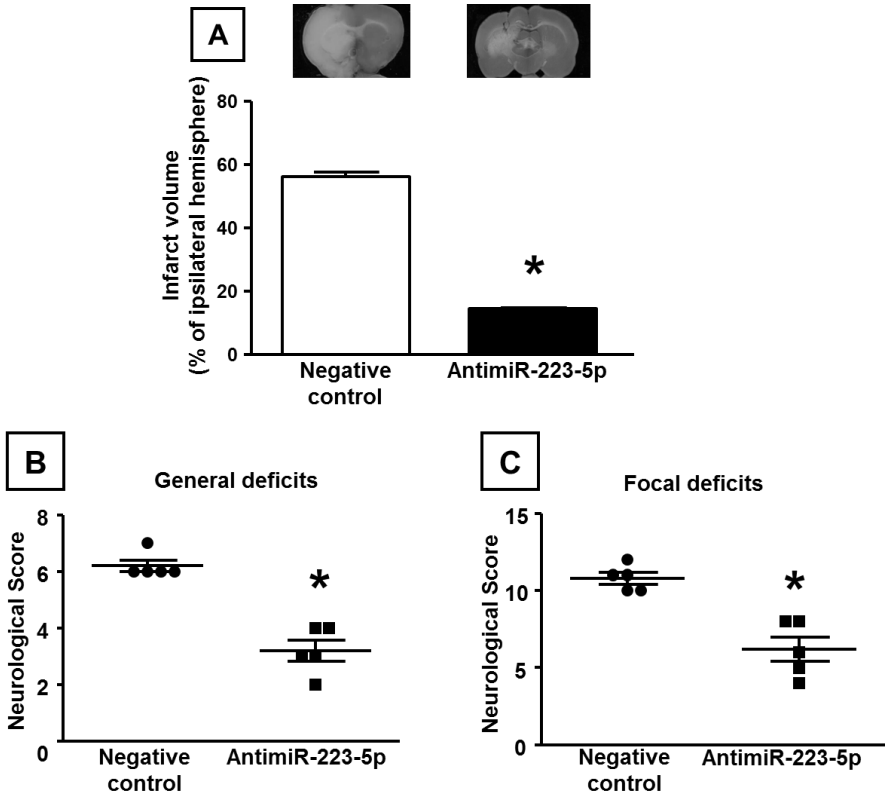

Figure 4.14: Evaluation of effect of antimiR-223-5p on infarct volume and neurological deficits in rats subjected to tMCAO. Ischemic damage was assessed on rats subjected to continuous icv infusion of antimiR-223-5p and antimiR negative control (10μM) 2 hours before tMCAO and up to 72 hours from reperfusion. Each column represents the mean ± S.E.M. *p<0.05 vs. negative control group.

A) Quantification of infarct volume after 100 minutes of MCAO and 72hours from reperfusion. n=4 samples per each group. On the top, representative figures of brain sections comprising cortex and striatum.
B) Measurement of general scores of neurological deficits after 100 minutes of MCAO and 72hours from reperfusion. n=5 samples per each group.
C) Measurement of focal scores of neurological deficits after 100 minutes of MCAO and 72hours from reperfusion. n=5 samples per each group.

4.15 *In Vivo* Administration of AntimiR-223-5p Prevents NCKX2 Downregulation in Striatum

To confirm the mechanism of regulation of NCKX2 expression by miR-223, western blot analysis was performed on samples of cortex and striatum of rats subjected to tMCAO and infused with miR-223 inhibitor; cerebral tissues were collected at 72 hours from reperfusion. The levels in cortex did not change after treatment with either antimiR negative control or antimiR-223 (figure 4.15 A). Conversely, in striatum, NCKX2 expression was strongly downregulated 72 hours after tMCAO induction and negative control administration (48.50%±12.90%), whereas miR-223 inhibitor caused an increase of protein levels (77.30%±15.40%), compared to sham-operated rats (100%±12.20%) (figure 4.15 B).

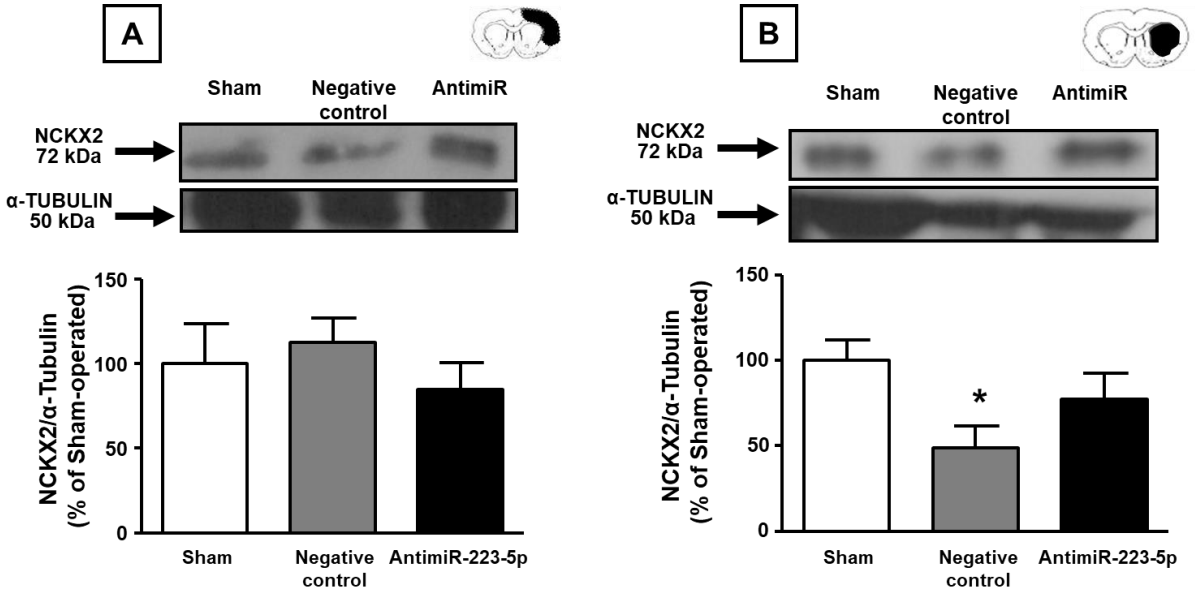

Figure 4.15: Assessment of NCKX2 protein levels on ipsilateral cortex and striatum of ischemic rats infused with antimiR-223. NCKX2 protein levels were evaluated by western blot analysis on rats subjected to continuous icv infusion of antimiR-223-5p and antimiR nevative control (10μM) 2 hours before tMCAO and up to 72 hours from reperfusion. NCKX2 levels after both negative control and antimiR-223 treatment are expressed as percentage versus the sham-operated animals. Each column represents the mean ± S.E.M. Results of NCKX2 expression were normalized with respect to α-tubulin. *p<0.05 vs. sham-operated controls. n=3 samples per each group. On the top, representative blots of NCKX2 and α-tubulin signals in sham-operated, antimiR negative control and antimiR-223 group, respectively.
A) Expression levels of NCKX2 in cortex; (B) Expression levels of NCKX2 in striatum.

5. DISCUSSION

The present study has examined the potential diagnostic and neuroprotective role of specific microRNAs against cerebral ischemia. In particular, four different aspects were dealt in this experimental thesis.

5.1 MicroRNA-103 as Diagnostic Marker of Cerebral Ischemia

Studies previously performed in our laboratory showed that miR-103 is an epigenetic regulator of NCX1 expression, and its levels, increased after tMCAO induction, correlated with ischemic injury (Vinciguerra et al., 2014). This evidence was the starting point of the present study, and the increased expression of miR-103 in animal models of ischemia suggested that also peripheral levels might be changed in tissues easily withdrawable, such as plasma. Nevertheless, contrary to what we expected, plasma miR-103 expression did not increase in ischemic animals at brief times from reperfusion, but decreased significantly at 7 days from reperfusion (figure 4.1). In addition, downregulation of miR-103 also occurred in human samples of ischemic patients, collected immediately after their hospitalization (figure 4.2 A, B and C). This timing discrepancy between human and animal model could be related to the surgical procedure for the induction of stroke and to the absence of a sham-operated control for the comparison, because we used plasma samples of the same rats withdrawn before and after ischemia. Moreover, to exclude the hypothesis that reperfusion in the animal model did not influence the results, miR-103 expression was assessed also in ischemic patients who have previously received thrombolytic treatment (figure 4.2 D, E and F) and the expression of miRNA was still strongly reduced in all patients regardless of their NIH Stroke Score. Interestingly, reduction on miR-103 expression levels in the plasma did not mirror the course that was shown by *Vinciguerra et al.* in brain after cerebral ischemia; however, also in other studies, differences of expression between damaged tissue and plasma (or serum) were already observed, especially in tumor tissues (Zhu et al., 2014). The explanation of this phenomenon might lie in a sort of compensation mechanism that organism activates to prevent overactivation of this miRNA and injurious consequences also in other tissues, for instance mediating inflammatory processes (Hartmann et al., 2016). By contrast, other studies revealed that miR-103 is a hypoxia-responsive microRNA

(HRM) (Kulshreshtha et al., 2007) and is strongly induced in vascular endothelial cells by HIF1α, resulting important to promote angiogenesis in hypoxic conditions, such as cerebral ischemia (Chen et al., 2013). In conclusion miR-103 function is tissue-specific, with both positive and negative effects, and regulation of blood levels is crucial to prevent systemic damage.

5.2 Evaluation of miR-103 - Mediated NCX1 Activation after Remote Postconditioning Induction

Little it's known in literature about the mechanisms that mediate the protection elicited by the phenomenon of remote postconditioning (RIPO). To date, we know that neural and humoral mechanisms have been proposed as ways required to transmit the peripheral signal from an organ as limb to a distant organ as the brain. Since it has been established that microRNAs can be released in the blood and transported by vesicles and other carriers, we supposed that also microRNAs might belong to humoral factors that mediate that communication between distant organs. Moreover, we wanted to investigate whether NCX1 expression was affected by RIPO induction, because we hypothesized that restoration of ionic homeostasis could be one of the neuronal mechanisms mediating RIPO protection, as it was previously demonstrated with other forms of conditioning. Therefore, in the second part of this study we have investigated whether miR-103 increase observed during ischemia is prevented by RIPO, and results shown in figure 4.3 indicated that RIPO application induced a reduction of miR-103 expression in the whole damaged area. However, to better characterize the course of miR-103 in the two tissues affected by ischemic damage, we have performed time course experiments in cortex and striatum, separately. No difference of expression was observed in striatum between tMCAO and RIPO groups, confirming that in both conditions the ischemic core is still damaged (figure 4.4 B). On the other side, experiments on cortex have evidenced a different expression pattern of miR-103 after ischemia and after RIPO. Nevertheless, expression profiles that we obtained were not exactly what we expected. In fact, at 24 and 72 hours from reperfusion, miR-103 levels in cortex were lower in the animals subjected to tMCAO compared to those subjected to RIPO (figure 4.4 A). To better clarify the regulatory role of miR-103 on NCX1 expression, by western blot analysis we have evaluated NCX1 protein levels at 24 hours from reperfusion, because at this

time both damage and protection are well obvious. As showed in figures 4.5 in both cortex and striatum NCX1 protein was reduced in tMCAO group, as we expected, and its level was restored after RIPO induction. These results confirm that NCX1 activation might be involved in the neuroprotection conferred by RIPO, with a miR-103-independent regulation mechanism. Interestingly, NCX1 mRNA levels did not mirror those of protein. Indeed, while in cortex no expression change was found after tMCAO and after RIPO (figure 4.6 A), in striatum mRNA significantly increased after both ischemia and neuroprotection (figure 4.6 B). Our theory to explain these results it's that transcriptional mechanisms activated by ischemic event work to synthetize RNA, whose translation is regulated by other posttranscriptional processes. Since this mRNA increase is still overexpressed after RIPO, we suppose that mRNA synthesis may be triggered by the same hypoxic damage, maybe involving factors such as HIFα (Valsecchi et al., 2011). It should be underlined that the increase of mRNA levels was observed only in the striatum that in our model of ischemia represents the ischemic core, the area directly affected by the infarct.

Even if with the data in our hands miR-103 does not appear to be involved in NCX1 regulation occurring after RIPO, its different expression levels after RIPO suggested a possible involvement in the neuroprotection elicited by RIPO. Therefore, in order to verify if miR-103 may be released in blood after a short occlusion of a peripheral vessel, we investigated its plasma levels after only FAO and RIPO application. Results demonstrated that the only occlusion of femoral artery did not induce changes in miR-103 release in blood compared to sham-operated animals, whereas the occlusion of femoral artery 20 minutes from tMCAO induced a significant increase of plasma miR-103, just after a few hours from RIPO induction (figure 4.7). We have supposed that probably this miRNA is released in blood to contact other tissues, such as the brain, and to regulate gene expression of proteins related to death mechanisms.

5.3 Expression Profile of MicroRNAs Modulated by Remote Postconditioning

In order to set up a gene therapy approach for stroke, based on the manipulation of miRNA, we have performed a miRNA profiling to identify a miRNA signature for brain protection specifically modulated by remote limb postconditioning. Results of general miRNA expression profile (figure 4.8) were validated by Real-time PCR (figure 4.9)

for 13 miRNAs whose expression resulted significantly modulated after stroke and after the neuroprotective strategy of RIPO. Among these we have further selected 4 miRNAs whose expression changes were more significant compared to the others: let-7a-5p, miR-143-3p, miR-451-5p and miR-485-3p. In particular their levels strongly increased 24 hours after ischemia, but were almost restored after RIPO induction. It's usually very difficult to define the role of miRNA, because of their ability to target several genes; however, comparing bioinformatic analysis of predicted targets and experimental evidence collected in scientific literature, it has been possible to clarify that they mediate a harmful action in ischemia, whereas their inhibition is neuroprotective. Indeed, let-7a-5p appears to regulate the expression of proteins involved in inflammation and neurogenesis, and many genes regulating cell cycle and cell proliferation are responsive to alterations of let-7a levels. Moreover, let-7a gene knockdown was demonstrated to protect against cerebral ischemia/reperfusion injury (Wang et al., 2016). Similarly, miR-451 regulates autophagy and cell proliferation (Pan et al., 2013); thus changes of its expression are often related to some types of cancer. MiR-485 is predicted to manage expression of genes that are involved in apoptosis, but also cell growth and migration, that are two crucial phases for tumor development. As described above, atherosclerosis is affected by miR-143, and it would be therefore interesting to investigate its potential diagnostic role for stroke (Quintavalle et al., 2011). Furthermore, predicted analysis indicated as probable targets of miR-143-3p some membrane proteins that work in transduction pathways and solute carriers of different families. Hence, its role seems to be associated with cell responses to outer stimuli. In addition, miR-143 is involved in the regulation of mitochondrial proteins, and studies conducted in ischemia showed that it can promote mitochondria damage in cardiac ischemia (Hong et al., 2017).

Our studies have demonstrated that changes of let-7a-5p, miR-143-3p, miR-451-5p and miR-485-3p expression are related to ischemic damage, whereas the restoration of their basal levels mirrors the neuroprotective condition elicited by the strategy or RIPO. However, it would be appropriate to investigate about the origin of the expression changes of these miRNA: it's important, in particular, to clarify if it's just the RIPO to cause the decrease of their levels, necessary event to mediate the protection; or to investigate whether these expression changes are only a consequence of the neuroprotective state caused by RIPO. In this latter case, the manipulation of these miRNAs could be ineffective for stroke protection.

Moreover, to confirm that the expression of these miRNAs correlate with the ischemic damage, our perspective is to repeat gene expression analysis on cortex and striatum areas, in order to demonstrate that reduction of miRNA expression is limited to the penumbra region, keeping higher levels in the striatum.

5.4 MicroRNA-223 as Potential Regulator of NCKX2

In the last part of this thesis work the attention was moved on NCKX2 and its regulation by miR-223. Our preliminary data showed that miR-223 levels significantly increased after stroke in both cortex and striatum (figure 4.11), and these data were in agreement with data produced by other authors (Jeyaseelan et al., 2008; Dharap et al., 2009). Interestingly, levels of this miRNA in the sham-operated controls were very low, almost absent; so, rather than an increase, it appears more as a process of transcription activation of miR-223. This is also the reason for which the error standard of miRNA expression results are so high: when threshold cycles (Ct) are higher than 35, Real-Time PCR does not discriminate in precise way the signals of expression, and so these courses indicate an increase, but the percentage of increase is unclear (figure 4.11).

In addition, our results suggest that miR-223 is a possible regulator of NCKX2, as demonstrated by western blot analysis on neurons transfected with miR-223 mimic and inhibitor (figure 4.13), and confirmed in *in vivo* experiments with icv administration of antimiRNA (figure 4.15). Interestingly, antimiR-223 treatment on cortical neurons did not induce changes of NCKX2 protein levels, probably due to low levels of miR-223 in basal conditions as suggested by Real-Time PCR analysis. However, to confirm that miR-223 directly regulates NCKX2 gene expression other analyses are required, such as luciferase assay.

6. CONCLUSIONS

Overall, our data support the thesis that microRNA can be considered potential targets for stroke therapy and may represent mediators of protection induced by Remote Postconditioning. In order to confirm that protection elicited by Remote Postconditioning is really mediated by the four miRNAs identified (let-7a-5p, miR-143-3p, miR-451-5p and miR-485-3p), other experiments are required. In particular, we will test whether *in vivo* overexpression of these miRNA by specific mimics, intracerebroventricularly infused, can prevent the amelioration of ischemic damage.

Moreover, our results suggest a potential role for miR-103 as peripheral marker of stroke. As future perspective, it would be appropriate to create a multicentre study, in order to confirm clinical trials in a large number of patients with a more heterogeneous clinical picture.

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