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MicroRNA 103-1 EXERTS A NEUROPROTECTIVE EFFECT IN STROKE BY ENHANCING NCX1 EXPRESSION IN THE BRAIN

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ABSTRACT

Background and purpose: MicroRNAs (miRNA) are single-stranded short RNA molecules that regulate gene expression by either degradation or translational repression of mRNA. Recent studies showed that several neurodegenerative disorders including cerebral ischemia significantly alter cerebral miRNA profiles, mediating profound effect on the disease outcome. In that scenario the Na^+/Ca^{2+} exchanger, by mediating Ca^{2+} and Na^+ fluxes in a bidirectional way across the synaptic plasma membrane, may play a pivotal role in the events leading to anoxic damage. The objective of this study was to set up a valid therapeutical strategy able to contrast the role of specific miRNAs that downregulate NCX expression under experimental conditions mimicking stroke.

Methods: NCX protein expression was evaluated after miRNA cell trasfection in PC12, BHK cell lines and neuronal cultures from rat embryons. Then, it has been tested the capability of AntimiRNA 103-1 to target mir-103-1 and to block its detrimental action on NCX1 RNA messanger. This second part has been conducted on a rat model of transient cerebral ischemia.

Results: The results showed that NCX1 physiological expression was dramatically reduced when cells were treated with mir-103-1. This tight regulation of NCX by a specific microRNA represents the in vitro confirmation of a perfect complementarity existing between 3'UTR of NCX and seed sequence of miRNA 103-1 already evidenced by in silico analysis. Conversely, the *in vivo* approach consisted in administering, by intracerebroventricular infusion, miRNA 103-1 silencing (AntimiRNA) in a specific temporal delay from transient ischemia in which ischemic damage was at the highest level and NCX1 protein was strongly downregulated. Results showed that antimiRNA-103-1 protected brain from ischemia and sustained high level of neurobeneficial protein NCX1.

Conclusions: The present findings support the idea that blocking mir-103-1 by microRNA inhibitor is a reasonable strategy to stop neurodetrimental downregulation of NCX occurring during ischemic conditions.

1. PREMISE

Acute stroke is one of the leading factors of morbidity and mortality worldwide (Donnan et al., 2008). After cardiovascular disease and cancer, stroke ranks as third most common cause of death in industrialised countries (European Stroke Organization). Stroke as the most important cause of morbidity and it imposes an enormous economic burden. It is estimated that on average a person suffering from stroke in the acute phase of hospitalization, diagnosis and care, costs 10/15 thousand euro. Permanent disability for people that exceed the acute phase determines an expense for years of about 80/120 thousand euro. Psychological consequences and family costs are incalculable. By the year 2020 the mortality from stroke will be duplicated because of the elderly and the persistence of smoking cigarettes. Stroke is caused by an interruption of blood flow to the brain prolonged in time. The CNS is more vulnerable to ischemic events than any other organ or system in aerobic metabolism. The loss of blood supply to the brain may be the result of two separate events: ischemic or hemorrhagic stroke. The first, more frequent, is due to the formation of a thrombus or an embolus responsible for obstruction of a vessel that supplies the brain tissue, the second, more frequently fatal, is due to rupture of a cerebral vessel. Interruption of cerebral vasculature for only 5 minutes produces neuronal death in the corresponding areas of the brain, whereas, for example, 20-40 minutes of ischemia are necessary to cause myocyte death (Ogawa et al., 2007). In recent years, stroke has been increasingly recognised as a medical emergency. Current available treatments, particularly thrombolytic strategy, have a much greater chance of improving stroke outcome if they are started as soon as possible after the onset of ischemia. Educating the public and training health professionals to treat

stroke as a true emergency is likely to have real benefits for the outcome of patients with stroke. The key element of acute stroke management summarises the procedures and therapies currently available for use in acute stroke that may help to improve outcomes for stroke patients. It includes general stroke therapies, treatment of secondary complications, and thrombolytic therapy (ESO Annual Report, 2011). In stroke prevention, primary prevention aims to reduce the risk of stroke in asymptomatic people and secondary stroke prevention focuses on therapies that inhibit the ability of platelets to form thromboemboli. This objective can be achieved either through direct antiplatelet action or by regulating mechanisms that affect platelet aggregation.

2. INTRODUCTION

2.1 STROKE PATHOPHYSIOLOGY

Stroke originates from rupture or blockade of a blood vessel in the brain that causes rapid cell death in the core of the injured region and triggers mechanisms in the surrounding area – the penumbra – that leads among several mediators to changes in the concentrations of several ions such as intracellular Ca^{2+}, Na^{+}, H^{+}, K^{+}, and radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). All these transductional factors might initiate cell death. In particular, it is widely accepted that a critical factor in determining neuronal death during cerebral ischemia is the progressive accumulation of intracellular Na^{+} ions, which can precipitate necrosis and apoptosis of vulnerable neurons. Whereas the detrimental action of $[Na^{+}]_i$ increase is attributable to both cell swelling and microtubular disorganization – phenomena that lead to cell necrosis – a change in Ca^{2+},Na^{+}, K^{+}, H^{+} ions has been shown to be a key factor in ischemic brain damage, for it modulates several death pathways, including oxidative and nitrosative stress, mitochondrial dysfunction, protease activation, and apoptosis (Annunziato et al., 2009, Springer). A large amount of work has been accumulated showing that glutamate extracellular concentrations briskly rise during acute brain injury, thus triggering an influx of Ca^{2+} and Na^{+} ions into neurons through ionotropic glutamate receptor subtypes. This evidence has led to the elaboration of the paradigm of glutamate excitotoxicity that explains ischemic neuronal cell death as a mere consequence of Na^{+} and Ca^{2+} influx through glutamate receptors (Olney et al., 1973). Although this theory has been guiding basic research in the field of neurodegeneration for almost three decades, more recently it has become the object of serious criticism and reassessment. What

has aroused such skepticism among researchers has been the fact that although first, second, and third generation glutamate receptor antagonists have long yielded promising results in animal models of brain ischemia, they have failed to elicit a neuroprotective action in stroke and traumatic brain injury in humans. Therefore, the theory of excitotoxicity can only explain some of the events occurring in the acute phase of anoxic insult but cannot be seen as a major target for developing new therapeutic avenues for brain ischemia. In the last decade, several seminal experimental works are markedly changing the scenario of research of principal actors of an ischemic event. In fact, it has been shown that some integral plasma-membrane proteins, involved in the control of Ca^{2+}, Na^+, K^+, H^+ ions influx or efflux and, therefore, responsible for maintaining the homeostasis of these four cations, might function as crucial players in the brain ischemic process. Indeed, these proteins, by regulating Ca^{2+}, Na^+, K^+, H^+ homeostasis, may provide the molecular basis underlying glutamate-independent Ca^{2+} overload mechanisms in neuronal ischemic cell death and, most importantly, may represent more suitable molecular targets for therapeutic intervention (Annunziato et al., 2009). What happens when brain hypoxia or ischemia occurs is that tissue energy demands can not be met, so ATP levels fall. Loss of ATP results in decreased function of active ion pumps, such as the Na^+/K^+-ATPase, the most important transporter for maintaining high intracellular concentrations of K^+ (~155 mM) and low intracellular concentrations of Na^+ (~12 mM). Loss of ion pump function allows rundown of transmembrane ion gradients, leading to membrane depolarization, the opening of voltagesensitive ion channels and a cascade of subsequent events, which, if sustained, lead ultimately to cell death. Depending on the circumstances, this death may be restricted to selectively vulnerable neuronal populations or may involve all cells (tissue infarction). Within seconds of an ischemic insult, normal brain electrical activity ceases due to

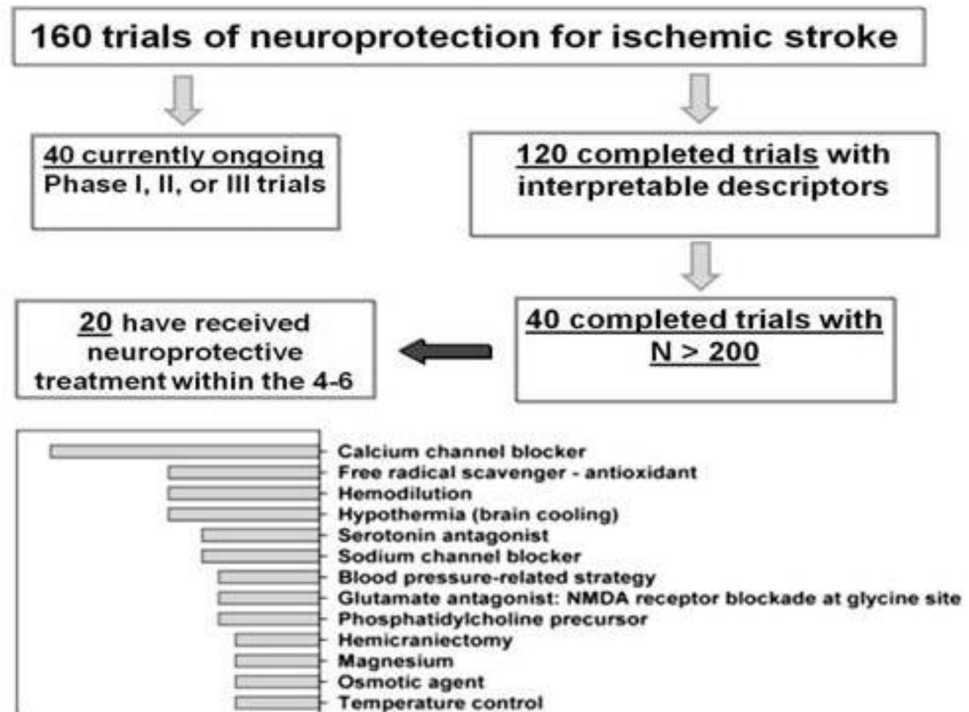
the activation of membrane K^+ channels and widespread neuronal hyperpolarization. The hyperpolarization, presumably protective, however fails to preserve high-energy phosphate levels in tissue as concentrations of phosphocreatine (PCr) and ATP fall within minutes after ischemia onset. The fall in pO_2 during ischemia leads to enhanced lactic acid production so there is a shift from aerobic metabolism to a dependence on glycolysis. The resulting lactic acidosis decreases the pH of the ischemic tissue from the normal 7.3 to intra-ischemic values ranging from 6.8 to 6.2. In addition, efflux of K^+ from depolarizing neurons results in prolonged elevations in extracellular [K^+] and massive cellular depolarization, a state known as spreading depression, which can propagate in the brain tissue. Rapid inactivation of O_2-sensitive K^+ channels by decreased pO_2 may represent one mechanism whereby neurons put a brake on this ongoing K^+ efflux (Haddad and Jiang 1997). Other cellular ion gradients are also lost; thus, intracellular Na^+ and Ca^{2+} rise and intracellular Mg^{2+} falls. Recently a great deal of interest has been devoted to clarify mechanisms of modulation of dangerous cascade actived by cerebral ischemia. Within them an important role seems to be played by the non-coding RNA called microRNA (miRNA). Indeed, miRNA are small RNA able to modulate protein expression at post-trascriptionale level. As reported previously, cerebral ischemia triggers a multifaceted cascade of physiologic and biochemical events. It is believed that these events are mediated in part by alterations in molecular processes such as transcription and translation. To date, no exhaustive reports are available on the miRNA microarray profiling of the ischemic brain. Nevertheless, several reports have demonstrated the roles of specific miRNAs in neuronal differentiation, neurogenesis, neural cell specification, and neurodevelopmental function (Kim et al., 2004; Osada et al., 2007; Schratt et al., 2006; Jeyaseelan et al., 2008; Lim et al., 2008), therefore miRNa can be included in the list of potential druggable targets for stroke treatment.

2.1.1 CURRENT STRATEGIES FOR ISCHEMIC STROKE TREATMENT

In acute stroke management, current evidence demonstrates that the most important declines in stroke incidence and mortality in developed countries have evolved from primary and secondary prevention measures aimed to better control of risk factors, including either pharmacological treatments or other procedures that decrease blood pressure, prevent consequences of atrial fibrillation, and reduce hyperglycemia and hyperlipidemia, among others. Although less successful, there have also been interventions with proven benefit for acute stroke treatment. One of the most significant advances has been the management of patients in stroke units, which has been shown to reduce mortality and to improve functional outcome by approximately 20% (Donnan et al., 2008) after acute ischemic stroke (Langhorne et al., 1993). In addition, recanalization of the occluded blood vessel with thrombolytics, mainly recombinant tissue plasminogen activator and, more recently, by mechanical clot removal or disruption, appears to be one of the most effective treatments for acute ischemic stroke. However, there have been major disappointments in the area of pharmacological neuroprotection, where many clinical trials have so far failed. Some of these failures might be due to deficiencies in trial design rather than absence of efficacy of the agents tested. In this sense, many current efforts are now devoted to develop methods for better patient selection and for analysis of outcomes, a way in which recent trials have begun to provide some hope. Still, acute stroke treatment guidelines until now do not encourage treatment with any neuroprotectant. Lastly, there is a field for which there are hopes that some interventions could be useful for patients with stroke, which is the phase of recovery. Indeed, in this setting, strategies including rehabilitation programs or pharmacological/cell therapies may serve to promote repair in late phases and to decrease stroke-associated disability. A way to embark on the search for new drugs for the chronic phase of stroke is by boosting

endogenous plasticity mechanisms. Interestingly, many of these endogenous active recovery processes initiate very early, in the acute phase of ischemia. In this line, a very elegant perspective by Eng Lo has recently proposed that, because most molecular targets for therapy have biphasic roles in stroke pathophysiology, failure in neuroprotection may partly be due to the fact that many neuroprotectants inhibit not only mechanisms of damage, but also those mechanisms needed for repair (Lo EH, 2008). This interesting hypothesis leads to propose that new drugs for acute stroke treatment should be able to promote a “safe” neuroprotection with the ability to preserve those mediators required for neural repair. One successful treatment strategy for salvaging ischemic tissue and improving functional outcome after ischemic stroke is reperfusion by trombolytic drugs. Thrombolytic therapy with rtPA (0.9 mg/kg body weight, maximum dose 90 mg) given within 4.5 h after stroke onset significantly improves outcome in patients with acute ischemic stroke (Hacke et al., 1995). The importance of the correct time window is underlined by the ECASS (European Cooperative Acute Stroke Study) and ECASS II studies that did not show statistically significant superiority of rtPA for the primary endpoints when treatment was given within 6 h (Hacke et al., 1995, 1998). The recently published trial ECASS III has shown that intravenous alteplase administered between 3 and 4.5 hours (median 3 h 59 min) after the onset of symptoms significantly improves clinical outcomes in patients with acute ischemic stroke compared to placebo (Hacke W. et al., 1998); the absolute improvement was 7.2% and the adjusted Odds Ratio of favourable outcome expressed in modified Ranking scale (mRS 0-1) was 1.42 (1.02-1.98 CI of OR). Treatment benefit is time-dependent. The number needed to treat to get one more favourable outcome drops from two during the first 90 minutes through seven within 3 hours and towards 14 between 3 and 4.5 hours (Hacke et al., 1998). Moreover, trombolitic drug category comprises beyond just mentioned thrombolytic

agents (e.g., tissue plasminogen activator,urokinase, mechanical devices), anti-thrombotic agents (e.g.,heparin, low molecular weight heparin), anti-platelet drugs (e.g., aspirin, dipyridamole, abciximab), and fibrinogen depleting agents (e.g., Ancrod). Admittedly, these agents protect the brain but do so primarily via hemodynamic rather than metabolic mechanisms. Clinical trials of neuroprotection for ischemic stroke have been analyzed in recent reviews (Cheng et al., 2004; Labiche and Grotta, 2004). The Internet Stroke Center website provides a comprehensive clinical stroke-trial database that summarize the current universe of neuroprotection trials in acute ischemic stroke. This Internet Site provides patients, their families, and caregivers with valuable resources about how to recognize the symptoms of a stroke and understand how strokes are diagnosed and treated. Of the ca. 160 such trials, one-quarter are currently ongoing and three-quarters have been completed (or prematurely terminated). It is instructive to consider the completed trials in greater detail. Eighty completed studies are, in fact, merely early-phase safety or feasibility trials with 200 or fewer subjects (Figure 1). Only 40 completed trials have enrolled over 200 subjects, and of these fewer than one-half have instituted neuroprotective treatment within the 4-6 h therapeutic window within which efficacious neuroprotection is considered possible. This fact alone is sufficient to explain the abundance of non-successful (‘‘failed’’) clinical trials (Ginsberg et al., 2008).



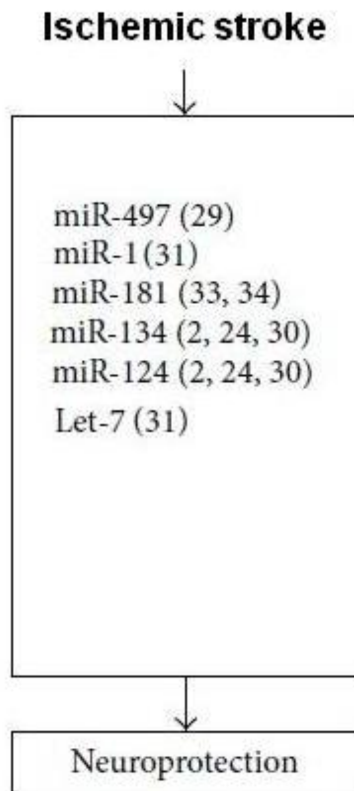


Figure 1. State of the art in stroke neuroprotection *(modified from Ginsberg et al., 2008 and Wang et al., 2013).*

2.2 MICRORNA PATHWAYS

2.2.1 BIOGENESIS OF MICRORNAS

A microRNA (miRNA) is a small non-coding RNA molecule (ca. 22 nucleotides) found in plants and animals, which functions in transcriptional and post-transcriptional regulation of gene expression (Chen et al., 2007). MicroRNAs were discovered in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum during a study of the gene lin-14 in C. elegans development (Lim et al., 2005). These short RNA molecules control the expression levels of their target genes acting as potent modulators of gene expression (He and Hannon, 2004). They have been found to play critical roles in a growing number of biological functions and human diseases (Thai et al., 2010; Dalmay and Edwards, 2006; Chang and Mendell, 2007; Zhang, 2008; Meola et al., 2009). MicroRNAs are produced from either their own genes or from introns but, often , a microRNA gene is transcribed together with its host gene as part of an unique transcription unit. The pathway of miRNAs starts from the cellular nucleus where genes coding for miRNAs are transcribed by RNA polymerase II (Pol II). RNA polymerase II generates long primary transcripts named "pri-miRNA" which have a stem-loop structure that in turn forms from a part of a several hundred nucleotides long miRNA precursor. A single pri-miRNA may contain from one to six miRNA precursors. These hairpin loop structures are composed of about 70 nucleotides each. Each hairpin is flanked by sequences necessary for efficient processing. The double-stranded RNA structure of the hairpins is recognized by the enzyme Drosha a protein that cuts RNA, to form the "Microprocessor" complex (Gregory et al., 2006). In this complex, DGCR8 orients the catalytic RNase III domain of Drosha to release hairpins from pri-miRNAs by cleaving RNA about eleven nucleotides from the hairpin base (two helical RNA turns into the

stem). The product resulting has a two-nucleotide overhang at its 3’ end; it has 3' hydroxyl and 5' phosphate groups. It is often termed as a pre-miRNA (precursor-miRNA). pre-miRNA hairpins are exported from the nucleus in a process involving the nucleocytoplasmic shuttle Exportin-5. In cytoplasm, the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer (Lund et al., 2007) This endoribonuclease interacts with the 3' end of the hairpin and cuts away the loop joining the 3' and 5' arms, yielding an imperfect miRNA : miRNA* duplex about 22 nucleotides in length (Lund et al., 2007). Overall hairpin length and loop size influence the efficiency of Dicer processing, and the imperfect nature of the miRNA:miRNA* pairing also affects cleavage (Lund et al., 2007; Lelandais-Brière et al., 2009). Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact.

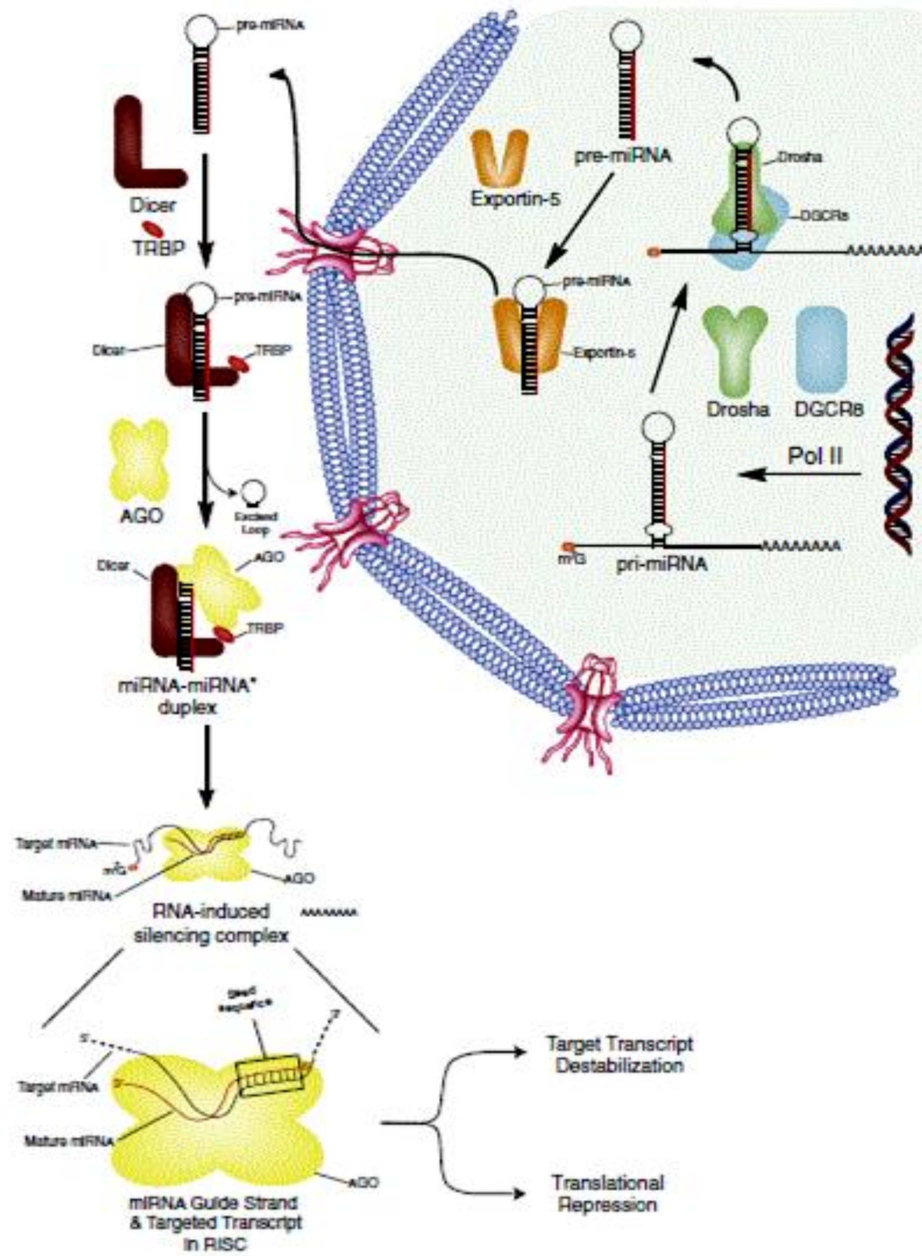


Figure 2. miRNA pathway.

2.2.2 RISC OR RNA INDUCING SILENCING COMPLEX

The mature miRNA is part of an active RNA-induced silencing complex (RISC) containing Dicer and many associated proteins (Rana et al., 2007). RISC is also known as a microRNA ribonucleoprotein complex (miRNP)(Schwarz et al., 2002). RISC with incorporated miRNA is sometimes referred to as "miRISC." Dicer processing of the pre-miRNA is coupled with the incorporation of one strand of immature miRNA into the miRISC. The choise between two sequences is made on the basis of its thermodynamic instability and weaker base-pairing relative to the other strand (Krol et al., 2004; Khvorova et al., 2003; Schwarz et al., 2003). The position of the stem-loop may also influence strand choice (Lin et al., 2003). The other strand, called the passenger strand due to its lower levels in the steady state, is indicated with an asterisk (*) and is normally degraded. In some cases, both strands of the duplex are viable and become functional miRNAs that target different mRNA populations (Okamura et al., 2008). Members of the Argonaute (Ago) protein family are central to RISC function. Argonautes are needed for miRNA-induced silencing and contain two conserved RNA binding domains: a PAZ domain that can bind the single stranded 3' end of the mature miRNA and a PIWI domain that structurally resembles ribonuclease-H and functions to interact with the 5' end of the guide strand. They bind the mature miRNA and orient it for interaction with a target mRNA. Some argonautes, for example human Ago2, cleave target transcripts directly; argonautes may also recruit additional proteins to achieve translational repression (Pratt et al., 2009). Additional RISC components include TRBP (human immunodeficiency virus transactivating response RNA binding protein)(MacRae et al., 2008), PACT (protein activator of the interferon induced protein kinase), SMN complex (Survival of Motor Neurons gene), FMRP (fragile X mental retardation protein), Tudor-SN (Tudor staphylococcal nuclease-domain-containing protein),

MOV10 (putative DNA helicase) and the RNA recognition motif containing protein TNRC6B (Murchison et al., 2004; Mourelatos et al., 2002; Meister et al., 2005).

2.2.3 PROCESSING BODIES

Once microRNAs and their associated RISCs are bound to an mRNA target, the whole complex can be sequestered into processing bodies (Pillai et al., 2005, 2007; Sheth and Parker, 2003). This action involves phosphorylation of Ago2 in the RISCs by p38 mitogen-activated protein kinase (Zeng et al, 2008). Processing bodies (P-bodies) are distinct foci within the cytoplasm of the eukaryotic cell consisting of many enzymes involved in mRNA turnover. When a cellular stress signal releases the RISC bound mRNAs from processing bodies, the free mRNAs are recruited to ribosomes and translation can occur (Bhattacharyya et al., 2006). Sequestration of RISC-bound mRNAs in processing bodies also occurs in synapses (Konecna et al., 2009), suggesting that this mechanism is an important regulator of mRNA translation in response to synaptic activity (Saugstad, 2010).

2.2.4 MICRORNA TURNOVER

During miRNA maturation in the cytoplasm, uptake by the Argonaute protein is a mechanism by which the guide strand is stabilized, while the opposite strand (* or "passenger") strand is preferentially destroyed. Several miRNA modifications affect miRNA stability. Mature miRNAs in plants appear to be stabilized by the addition of methyls at the 3' end. The 2'-O-conjugated methyl groups block the addition of uracil (U) residues by uridyltransferase enzymes, a modification that may be associated

with miRNA degradation. However, uridylation may also protect some miRNAs; the consequences of this modification are incompletely understood. Uridylation of some animal miRNAs has also been reported. Both plant and animal miRNAs may be altered by addition of adenine (A) residues to the 3' end of the miRNA. An extra A added to the end of mammalian miR-122, a liver-enriched miRNA important in Hepatitis C, stabilizes the molecule, and plant miRNAs ending with an adenine residue have slower decay rates. It is obvious to consider that a shorter mRNA half-life generally correlates with reduced miRNA efficacy (Larsson et al., 2010). When miRNA terminals are exposed and appear to interact with AGO proteins (Wang et al., 2008b) miRNA is stabilized against exonucleolytic degradation (Winter and Diederichs, 2011). Moreover recent studies suggest that while miRNAs are generally very stable *in vivo* they appear to be under a regulatory control in neurons that promotes rapid turnover (Saugstad, 2010).

2.2.5 MECHANISM OF ACTION OF MICRORNAS

Encoded by eukaryotic nuclear DNA, miRNAs function via base-pairing with complementary sequences with mRNA molecules, usually resulting in gene silencing via translational repression or target degradation (Bartel et al., 2009; Kusenda et al., 2009). miRNAs cause translational repression and mRNA degradation by binding, with an imperfect pairing, to the 3′ untranslated region (3′ UTR) of their target genes (Kim et al., 2006; Giraldez et al., 2006). miRNA : mRNA base-pairing usually includes a ‘‘nucleus’’ (or ‘‘seed’’), typically a perfect Watson-Crick base-paired stretch of approximately seven nucleotides with a key role both in target site recognition and repression of the target transcript. The nucleus is located at the 5’ end of the miRNA, typically between nucleotides 2 and 8 (Lewis et al.,

2005). Initiation of translation requires a direct interaction between eukaryotic initiation factor 4E and a 7-methylguanosine sequence on mature mRNAs (Gebauer and Hentze, 2004; Merrick, 2004; Richter and Sonenberg, 2005). When microRNAs are bound to the mRNA 3'-UTR, Ago proteins in the RISC interact with the 7-methylguanosine cap of the mRNA, which blocks eukaryotic initiation factor 4E binding to the mRNA and initiation of translation (Kiriakidou et al., 2007). This is the earliest event known to be regulated by microRNAs, but further mRNA degradation may serve to strengthen mRNA silencing (Mathonnet et al., 2007). Studies on miR-124 mRNA targets support that: (1) microRNAs reduce both translation and abundance of mRNA targets, (2) translation is blocked at beginning or ribosomes preferentially collapse near the translation start site, and (3) regulation of translation and mRNA decay are strictly correlated (Hendrickson et al., 2009). Thus, most mRNAs are not differentially targeted for either translational repression or mRNA decay. Estimated number of human miRNAs suggest (miRBase database, http://microrna.sanger.ac.uk/sequences/)(Griffiths-Jones, 2004), that their actual number may exceed 1000 (Bentwich et al., 2005). Current findings indicate that each miRNA may regulate, on average, the expression of 100–200 mRNAs (Ambros, 2004; Lim et al., 2005). Currently, there are 718 annotated miRNAs in human (Griffiths-Jones et al., 2004), localized in intragenic (60% of cases) or in intergenic (40%) regions. Intragenic miRNAs are contained within transcriptional units termed “host genes” (Kim et al., 2009), which are generally protein-coding. Recent works demonstrated that several intronic miRNAs and their host genes are co-transcribed from a common promoter (Rodriguez et al., 2004; Kim and Kim, 2007). The large impact of miRNAs in the regulation of biological processes has generated a strong interest in novel technologies for the detection/prediction of miRNA target genes

(Thomas et al., 2010) to dissect their regulatory gene networks and understand their function.

2.2.6 CIRCULATING MIRNAS

The presence of endogenous miRNAs circulating in human plasma has been recently demonstrated (Mitchell et al., 2008). Unlike mRNAs, circulating miRNAs displayed remarkable stability (Weber et al., 2010) and resistance to degradation from endogenous RNAse activity (Tsui et al., 2002; Wang et al., 2009) (see section 2.2.4). In contrast, rapid degradation was observed within minutes when synthetic miRNAs were spiked into human plasma. Indeed, while naked miRNAs are susceptible to rapid degradation in plasma, circulating miRNAs are protected and resistant to RNase activity. The possible explanation is that circulating miRNAs can reside in microvesicles [exosomes, microparticles (MPs), and apoptotic bodies (ABs)], which account for shedding of miRNAs into the circulation and offer protection from RNase activity. Exosomes are small vesicles (50–90 nm) of endocytic origin (Camussi et al., 2010; Thery et al., 2009). Exosomes contain substantial amounts of RNA (Ratajczak et al., 2006; Skog et al., 2008) including both mRNA and miRNAs.(Wang et al., 2010; Skog et al., 2008; Valadi, 2007). A total of 121 miRNAs were identified in exosomes from mast cells and the expression of certain miRNAs was higher in microvesicles than in the parent cells (Valadi, 2007). In contrast, selective retention of specific miRNAs that are not released into the extracellular milieu was reported in cancer, implying that miRNAs can be selectively packaged (Pigati et al., 2010). Interaction of exosomes with recipient cells is thought to arise through receptor-ligand interactions,48 although fusion to the plasma membrane of target cells or endocytosis-like internalization of exosomes has also been observed. In an *in vivo* model that enabled close monitoring of miR-16 activity, nude mice were

implanted with cells engineered to express a reporter for the 3′ UTR of B-cell lymphoma 2, a validated target of miR-16. Subsequent intratumour injection of exosomes derived from HEK293 cells overexpressing miR-16 led to suppression of luciferase activity. Control exosomes had no effect on bioluminescence. These data indicate that exosomal miR-16 delivered its inhibitory ability on its target gene to the recipient cells *in vivo* (Iguchi et al., 2010).

2.2.7 MicroRNA in Physiological Processes

Accumulating evidence have demonstrated that microRNAs play a major role in a wide range of developmental processes including cell proliferation, cell differentiation, cell cycle, metabolism, apoptosis, developmental timing, neuronal cell fate, neuronal gene expression, brain morphogenesis, muscle differentiation and stem cell division. miRNA expression can be induced by a variety of stimuli and mechanisms. These stimuli include direct transcriptional activation or repression from transcriptional enhancers, epigenetic modifications of the genome, genomic amplification or deletion, cellular stress and inflammatory stimuli (Tili et al., 2008; Dai et al., 2011; Jopling et al., 2005). The biological effect of a specific miRNA will depend on the cellular environment in which it is expressed, on its turnover rate and on the target sequence that the miRNA can bind (Tili et al., 2008, Dai et al., 2011).

2.2.7.1 MicroRNA in Neurodegenerative Diseases

The number of known miRNAs has sharply increased in recent years; the latest estimate is about 1000 different miRNAs in human cells. Each miRNA has the potential to target a large number of mRNAs (200–500 mRNAs for each miRNA), suggesting that a large fraction of the protein coding genes may be somehow

regulated by miRNAs (Friedman et al., 2009). Frequently, a single mRNA is regulated by different miRNAs that act on its 3′UTR, indicating that miRNAs may act in a cooperative and/ or combinatorial mode in the regulation of a target mRNA (Krek et al., 2005). Altogether, these features show that miRNAs are emerging as a class of master regulatory molecules that may add another level of complexity to the canonical regulatory networks necessary to govern complex cell processes. Given the complex architecture of the brain, it is not surprising that miRNAs are abundantly expressed in the brain, where they have been found to play important roles in the regulation of brain function (Saba and Schratt, 2010). A number of mechanisms are employed to maintain the integrity of nerve cell networks and to facilitate responses to external and internal environmental stimuli and maintain neuron integrity and functional capability after damage (Persengiev et al., 2012). The accumulation of toxic proteins transcribed from mutated genes causes inherited forms of Alzheimer’s disease (amyloid precursor protein and presenilins), Parkinson’s disease (*α*-synuclein and Parkin), and trinucleotide repeat disorders (huntingtin, androgen receptor, ataxin, and others) by overcoming the endogenous neuroprotective mechanisms. Specific miRNAs have been shown and in some cases predicted with high confidence to be involved in Alzheimer’s disease, spinocerebellar ataxia type 1, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS), in addition to the general dysregulation of miRNA expression observed in neurodegenerative disorders (Villardo, et al., 2010; Doxakis, et al., 2010; Lee, et al., 2008; Persengiev, et al., 2011; Williams et al., 2009; Grimson et al., 2007; Lewis et al., 2003; Cogswell et al., 2008; Lukiw, et al., 2007; Nelson et al., 2008; Johnson et al., 2009). Alzheimer’s Disease (AD) is an incurable, degenerative disease leading to dementia. The basis of the impairment in synaptic functions observed in that pathology consists in the accumulation, in brain hippocampus, of soluble oligomers of b-amyloid peptide (Ab),

cleaved from the amyloid precursor protein (APP) (Ma et al., 2010). It is conceivable that initial events causing toxicity in the neurons, for example the appearance of toxic Ab oligomers in incipient AD, could lead to changes in miRNA expression, which could then feed into an amplification loop, for example affecting the expression of the proteases responsible for the generation of the Ab peptides. Interestingly, miR-29a and miR-29b-1, which regulate BACE1 expression, are under control of transcription factor RE1-silencing transcription factor (REST) (Kim et al., 2007; Donmez et al., 2010), which has a fundamental role in regulating neuronal gene expression and promoting neuronal fate. In addition, miR-29 is predicted to target the 3'UTR of REST and a substantial number of neuronal genes (Menghini et al., 2009). Such a regulatory pathway could become hypothetically compromised in the AD brain and would act in parallel with the increased BACE1 expression (Herbert et al., 2009) and possible Ab production in such patients. Notably, increased miR-146a levels observed in the AD brain (Lukiw et al., 2008) might be linked to a detrimental feedback loop between NFkB signaling, Ab generation and inflammation. Another important neurodegenerative pathology is Parkinson's disease (PD). This disease is a neurodegenerative pathology that affects specific populations of central and peripheral neurons, including those in the Substantia Nigra pars compacta (SNpc) and sympathetic ganglia (Marras and Lang, 2008). The pathology of the disease is due to the accumulation of a protein called alpha-synuclein into inclusions called Lewy bodies in dopaminergic neurons. Also for Parkinson Disease, a neurodegenerative disorder caused by environmental and genetic factors, there is evidence for a role of miRNAs (Saugstad, 2010). For example, miR-133b is specifically expressed in midbrain dopaminergic neurons and regulates the maturation and function of midbrain dopaminergic neurons, but miR-133b is deficient in patients with PD (Kim et al., 2007). In addition, fibroblast growth factor 20 is a risk

factor for PD, and genetic analysis of single-nucleotide polymorphisms within the fibroblast growth factor 20 gene revealed association with a risk allele (rs12720208) in the 3'-UTR (Wang et al., 2008). Furthermore, miR-7 mainly expressed in neurons binds to the a-synuclein mRNA 3'-UTR to repress protein expression, which protects cells against oxidative stress (Junn et al., 2009). Huntington's disease (HD) is a devastating neurodegenerative disorder characterized by progressive motor dysfunction, dementia and emotional disturbances. It is inherited in an autosomal dominant manner and its prevalence is 5–10 cases per 100,000. The HD gene codes for a large highly conserved protein named huntingtin (Sarkar et al., 2008). The transcription factor REST silences neuronal gene expression in nonneuronal cells, and REST is sequestered in the cytoplasm in part through binding to the Huntingtin protein. However, Huntingtin proteins that contain polyglutamine expansions cannot bind to REST, which frees REST to translocate to the nucleus where it represses neuronal gene expression. Recent studies revealed dysregulated expression of several neuronal- specific microRNAs in mouse models of HD and in human HD that likely result from REST repression (Johnson et al., 2008). The loss of microRNA expression correlates with increased expression of several mRNA targets, supporting that HD reflects a loss of neuronal identity caused in part by dysregulation of both transcriptional and posttranscriptional gene expression (Johnson et al., 2008). Moreover microRNA expression studies suggest a role for postinjury microRNAs in traumatic brain injury processes. In fact, in rat cortex and hippocampus, traumatic brain injury induced expression of several micro-RNAs and caused global upregulation of miR-21 after injury (Lei et al., 2009). Similarly, in rat and mouse hippocampus, controlled cortical impact injury decreased expression of 50 microRNAs and increased expression of 35 microRNAs (Redell et al., 2009). Predicted targets of validated microRNAs regulated by impact (miR-107, miR-130a,

miR-223, miR-292-5p, miR-433-3p, miR-451, miR-541, and miR-711) include several proteins and pathways known to be initiated after injury, including signal transduction, transcriptional regulation, proliferation, and differentiation (Redell et al., 2009; Saugstad, 2010) The use of genetically modified animals that develop AD- or PD-like pathology and viceversa, which contain a specific knockout of candidate miRNA genes, provide good models to address the problem of clarifying specific miRNA function. miRNA expression profiles were recently investigated in mouse models of Huntington's disease (HD) (Johnson et al., 2008) and PD (Gillardon et al., 2008) and compared with human patient profiles with some overlap. In addition to lower miR-133b expression in PD, a decrease in miR-132, for instance, is observed in the HD mouse R6/2 model and in human HD patients. Recent studies performed in humans support the idea that changes in miRNA expression profiles or miRNA target sequences could contribute significantly to risk for major neurodegenerative diseases such as AD and PD. Of particular interest, miRNAs seem to participate directly in the regulation of expression of AD-related genes involved in Ab production. In this regard, miRNA research seems to be particular promising for the understanding of the very prevalent and poorly understood sporadic forms of AD and possibly PD. The challenge would be to address the role of specific miRNAs in biological models and expand the clinical studies. The search for disease-associated SNPs influencing miRNA function is also under way. Thus, apart from a direct role in regulating the expression of disease-related genes, it is clear that miRNAs might contribute in many additional aspects to the neurodegenerative process. Several studies have now addressed directly the role of miRNAs in neurodegeneration. Complete loss of miRNA expression in the brain leads to neurodegeneration in several animal models. Evidence from patient material is emerging that miRNA dysregulation could, indeed, contribute to neurodegenerative disorders (Hebert et al., 2009).

2.2.7.1.1 MICRORNA IN BRAIN ISCHEMIA

Several reports have demonstrated the effects of specific miRNAs in neuronal differentiation, neurogenesis, neural cell specification, and neurodevelopmental function (Kosik et al., 2006; Schratt et al., 2006). In stroke etiology, miRNAs have distinct expression patterns that modulate pathogenic processes, including atherosclerosis (miR- 21 and miR-126), hyperlipidemia (miR-33 and miR-125a-5p), hypertension (miR-155), and plaque rupture (miR-222 and miR-210)(Rink and Khanna, 2011). Furthermore, transient focal ischemia in adult rat brain regulates the expression of microRNAs predicted to target proteins known to mediate inflammation, transcription, neuroprotection, receptor function, and ionic homeostasis in the brain (Dharap et al., 2009). The mRNA levels for proteins important to microRNA biogenesis pathways, including Drosha, Dicer, the cofactor Pasha, and the precursor microRNA transporter Exportin 5, are not altered after transient ischemia. In a recent paper it has been demonstrated that ischemia repressed miR-145 expression, which resulted in increased translation of its mRNA target, superoxide dismutase-2, in postischemic adult rat brain (Dharap et al., 2009). In his study Dharap et al., found that mir-145 is one of the miRNAs upregulated significantly in the postischemic brain from 3 h to 3 days after transient MCAO. This information was confirmed by bioinformatics search showing that SOD2 is a major target mRNA of mir-145. Another evidence for miRNA involvement in stroke derives from a work by XiaHeng Deng et al., where it has been shown that Matrix metallinoprotease-9 (MMP9) plays a key role in the pathogenesis of post-ischemic blood brain barrier (BBB) disruption and the formation of lesions after cerebral ischemia. This study revealed that significantly upregulated of miR-21 occurred in the hippocampus after stroke so, by silencing this miRNA (antagomiR strategy), levels of MMP9 protein after cerebral ischemia decreased with a strong amelioration of ischemia outcome in rats. The

study suggested that cerebral ischemia up-regulates expression level of miR-21, which is involved in ERK-stimulated upregulation of MMP9 following cerebral ischemia via a calcium-dependent mechanism. Furthermore, in another recent publication it has been shown that in a female rats model of endothelin (ET)-1 induced Middle Cerebral Artery occlusion (Biernaskie et al., 2001; Selvamani et al., 2010, 2010b) IGF-1 (Insulin-like Growth Factor) infusion following stroke, prevents estrogen neurotoxicity. Authors have demonstrated that IGF-1 is negatively regulated by two microRNAs, miR1 and the Let7. So, when after four hours of ET-1 injection, animals received an intracerebroventricular (ICV) injection of antimiRNA agains two miRNAs it was observed a significant reduction in infarct volume compared to control female rats group subjected to icv administration of a scrambled miRNA. More recently in a paper by Maged M. Harraz and collegues it has been proven that in rat brain overexpression of miR-223 lowering the levels of a subunit of glutamate receptor by targeting 3′-UTR in GluR2 and NR2B, inhibits NMDA-induced calcium influx in hippocampal neurons, and protects the brain from neuronal cell death following transient global ischemia and excitotoxic injury (Harraz et al., 2012). Stroke determines alteration in expression profiles of multiple miRNAs in SVZ neural progenitor cells and introduction in cerebral ventricle of rats of miR-124a inhibited ischemic neural progenitor cell proliferation and promoted the neuronal differentiation of the progenitor cells. This mechanism provide new insights into the molecular mechanisms underlying stroke-induced neurogenesis (Liu et al., 2011). Studies also support the potential for microRNAs as novel biomarkers for vascular injury and diseases. Expression profiling of microRNAs in ischemic rat brains revealed significant changes in several microRNAs, and some of the microRNAs highly expressed in ischemic brain were detected in blood samples (Jeyaseelan et al., 2008). Peripheral blood examined in ischemic stroke patients revealed differential

expression of microRNAs implicated in endothelial cell and vascular function, erythropoiesis, angiogenesis, neural function, and hypoxia, and altered microRNAs were detectable even several months after the onset of stroke (Tan et al., 2009). Rat models of ischemia, brain hemorrhage, and kainate-induced seizures also revealed regulated expression of microRNAs in hippocampus and blood in each treatment group, many of which changed > 1.5-fold in both tissues (Liu et al., 2010).

2.2.7.2 MICRORNA IN OTHER DISEASES

Over the past several years it has become clear that alteration in the expression of miRNA genes contribute to the pathogenesis of most human malignancies (Tsujimoto et al., 1985; Chang et al., 2007). These alteration can be caused by various mechanisms, including deletions, amplifications or mutations involving miRNA loci, epigenetic silencing or the dysregulation of transcription factors that targets specific miRNAs (Schetter et al., 2010; Calin et al., 2006). Malignant cells show dependence on the dysregulated expression of miRNA genes, which in turn control or are controlled by the dysregulation of multiple protein-coding oncogenes or tumor suppressor. Importantly, miRNA deficiencies or excess have been correlated with a number of clinically important diseases ranging from myocardial infarction to cancers (Soifer et al., 2007). The loss or gain of miRNA function can be caused by a single point mutation in either the miRNA or its target or by epigenetic silencing of pri-miRNA transcription units (Soifer et al., 2007). Interestingly, a highthroughput analysis of miRNA expression in cancer demonstrated that some miRNA are over-expressed in cancer, while others are markedly reduced in malignant tissue (Soifer et al., 2007). These correlative data suggest that miRNAs function as both oncogenes and tumor suppressors (Soifer et al., 2007; Lynam-Lennon et al., 2009; Zhang et al., 2007; Hernando et al., 2007; Osaki et al., 2008).

Numerous studies in cancer cell lines show a direct functional link between aberrant miRNA expression and particular tumor types (Calin et al., 2006; Soifer et al., 2007; Zhang, et al., 2007; Jazbutyte et al., 2010). As before mentioned, recent studies also show that some miRNAs regulate cell proliferation and apoptosis processes that are important in cancer formation (Zhang et al., 2007). Furthermore accumulating experimental results clearly show that miRNAs play a significant role in cardiovascular development and disease, and that miRNAs are important for regulating cardiomyocytes self-renewal and differentiation, as well as for normal cardiac structural integrity (Small et al., 2010; Barringhaus et al., 2009; Latronico et al., 2009). In particular in acute myocardial infarction that is the world's leading cause of morbidity and mortality there is a report showing that after infarction in humans and mice, muscle-enriched miRNAs, such as miR-1, miR-133a, miR-133b, and miR-499-5p, are increased in plasma and that miR-499 and miR-133a are highly expressed in heart, whereas miR-1 and miR-133a are highly expressed both in heart and in skeletal muscel (Wang et al., 2010; Contu et al., 2010). This evidence indicate that circulating miRNAs may become helpful and reliable tools for the diagnosis ad prognosis of patients with cardiovascular diseases.

2.2.8 IDENTIFYING PUTATIVE MIRNA TARGET SITES

The first step in the workflow for elucidating miRNA networks that physiologically regulate gene products implicated in CNS disorders is to identify putative miRNA target sites in the transcript of interest. There are two general approaches to identifying these putative target sites: i) computational predictions, and ii) experimental methods for detecting physical interactions between miRNA and mRNA in a regulatory context. Computational predictions utilize algorithms to scan transcript

sequences (generally limited to the 3′-UTR) for putative sites of interaction with miRNA, whereas the experimental methods utilize pull-down assays to detect physical interactions between nmiRNA, AGO proteins and specific transcript sites. Since neither of these approaches assesses the functionality of miRNA interactions with putative target sites, validation experiments must be pursued to confirm regulatory effects mediated by miRNA and the specificity of the putative target sites.

2.2.8.1 BIOINFORMATIC PREDICTIONS OF MIRNA TARGET SITES

Current efforts are dedicated to both experimental (Baek et al., 2008; Selbach et al., 2008) and bioinformatic (Krek et al., 2005; Betel et al., 2008; Friedman et al., 2009; Gennarino et al., 2009) approaches to address miRNA target identification. However, giving the laborious nature of the experiments needed for target validation, and considering that most ad-hoc developed high-throughput techniques (e.g. p-Silac) are costly and only validate a few target genes (Lim et al., 2005), it is imperative to improve in silico approaches to identify miRNA target genes. In order to identify true miRNA targets, it is essential to improve the efficiency of their in silico prediction by means of computational techniques (Maziere and Enright, 2007). Several computational approaches have recently been developed for the prediction of miRNA targets including,among the most popular ones, the miRanda, TargetScan, and PicTar softwares (Lewis et al., 2003; John et al., 2004; Krek et al., 2005; Rajewsky 2006; Kuhn et al., 2008), which mainly rely on the identification of the seed region between the miRNA and the corresponding target genes. Unfortunately, the presence of a seed region, although conserved across evolution, is not in itself a reliable way to identify functional miRNA targets. It has been shown that a significant proportion of predicted miRNA–mRNA target pairs, in spite of the presence of an

appropriate seed region, are false positives (Lewis et al., 2005; Didiano and Hobert, 2006), thus rendering the in silico preselection of miRNA targets very complex and laborious. The first open-source software for target prediction, miRanda 2005, revealed that overrepresented groups of microRNA targets include mRNAs for transcription factors, components of the microRNA machinery, and other proteins involved in translational regulation (John et al., 2004). In contrast, a large number of genes for proteins involved in basic cellular processes have very short 3'-UTRs and are specifically depleted of microRNA-binding sites (Stark et al., 2005). Vertebrate microRNAs target an average of 200 mRNA transcripts each (Krek et al., 2005), but the number of predicted targets per microRNA can vary from a few to > 800 transcripts. MiRanda 2005 and 2008 (Betel et al., 2008; John et al., 2004), and PicTar (Krek et al., 2005), allow combinatorial analysis of microRNAs for common targets, which is important because mRNAs targeted by multiple microRNAs show enhanced translational repression (Doench and Sharp, 2004). Currently, the first type of approach to target prediction utilizes sequence alignments between the seed region of the microRNA and sequences within the mRNA target. However, specificity can be increased by analyzing the evolutionary conservation and structural accessibility of mRNA binding sites, as well as the nucleotide composition or location of binding sites within the mRNA 3'-UTR (Alexiou et al., 2009). Databases of targets with experimental validation include Tarbase (Sethupathyet al., 2006), Ago (Shahi et al., 2006), and miRNAMAP (Hsu et al., 2006, 2008). At the present time, there is no universal standard for establishing a causal relationship between microRNAs and predicted mRNA targets, such as Koch's postulates for the relationship between a microbe and a disease. However, Kuhn et al. (2008) proposed that four criteria should be met before microRNA target validation is considered confirmed: (1) the micro- RNA/mRNA interaction must be experimentally verified, (2) the microRNA and

mRNA target must be coexpressed, (3) a given microRNA must have a predictable effect on target protein expression, and (4) microRNA-mediated regulation of target gene expression should equate to altered biological function (Kuhn et al., 2008). Thus, rapid and continued evolution of prediction tools and criteria for experimental validation are essential to establish the functional effects of microRNAs on their mRNA targets.

2.2.8.1.1 MIRANDA

The software Miranda was initially designed to predict miRNA targets in D. melanogaster (Enright et al., 2003: John, et al., 2004) and consisted of three basic steps: (i) the identification of a sequence that can be linked by a miRNA; (ii) the calculation of the free energy for the formation of 'heteroduplex miRNA-mRNA; (iii) the identification of evolutionary conservation between D. melanogaster, D. pseudoobscura, and Anopheles gambiae. This method has identified 9 of 10 miRNA-target interactions published and the false positive rate was estimated by 24%. As TargetScan, all the functions of the target genes predicted is enriched transcription factors, emphasizing the possible importance of miRNAs in the development, morphogenesis and function of the nervous system. The same algorithm was applied to the prediction of human miRNA targets. About 2000 human putative target genes of miRNAs have been identified, which suggests that 10% or more of all human genes are regulated by miRNAs. Even in this case the target overrepresented included transcription factors, proteins involved in the translation, components of the complex miRNA / ubiquitin, representing a new feedback circuit in gene regulation.

2.2.8.1.2 DIANA

This algorithm is specifically trained on a positive and a negative set of miRNA Recognition Elements (MREs) located in both the 3'-UTR and CDS (Coding DNA Sequence) regions. DIANA-microT-CDS provides a significant increase in sensitivity when compared with experimental proteomics data. It exhibited the highest sensitivity at any level of specificity, when compared against other state of the art implementations. Furthermore, microT-CDS users can examine the species where each binding site is conserved, filter results using score thresholds or by restricting the algorithm on genes belonging to specific pathways. DIANA-mirExTra is an algorithm that can identify microRNA effects on the expression levels of protein-coding transcripts, based on the frequency of six nucleotide long motifs (hexamers) in the 3'UTR sequences of genes. Additional features include the combination of multiple hexamers corresponding to the same microRNA sequence, use of evolutionary conservation between human and mouse to increase robustness and correction of microarray data for single nucleotide compositional bias (Bernstein et al., 2001; Kiriakidou et al., 2004).

2.2.8.1.3 MICROCOSM

MicroCosm is a web resource that contains computationally predicted targets for microRNAs across many species. The miRNA sequences are obtained from the miRNA Registry and most genomic sequence from EnsEMBL. This resource uses the miRanda algorithm to identify potential binding sites for a given miRNA in genomic sequences. The current version uses dynamic programming alignment to identify highly complementary sites which are scored between 0 and 100, where 0 represents no complementarity and 100 complete complementary. The algorithm

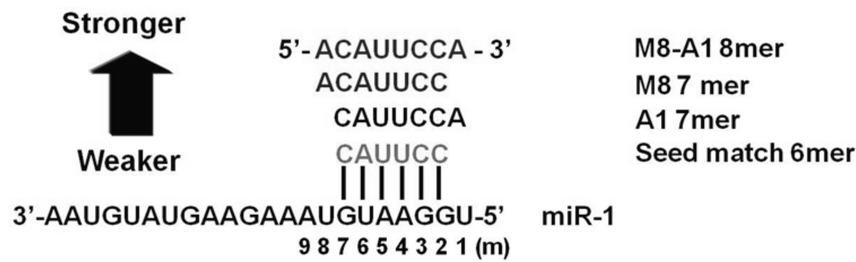
uses a weighted scoring system and rewards complementarity at the 5' end of the microRNA. Every potential target site in a 3'UTR detected is checked to see whether the site is conserved in orthologous transcripts from other species. It is necessary for a site to be conserved in order to be detected at the same position in a cross-species orthologous UTR alignment by an miRNA of the same family. Each target must be conserved in at least two species for inclusion in the database (with the exception of Human and Chimp whose sequences are too similar). The entire process of assembling miRNAs, genomic sequence, cross species UTR alignments and miRanda analysis is performed in parallel on a high-performance compute cluster.

2.2.8.1.4 PICTAR

An important property of miRNAs is represented by their high conservation between species. This characteristic has been exploited by those software as PicTar (Krek et al., 2005), who base their prediction on comparative data for any species to identify common targets for specific miRNAs. Furthermore, PicTar calculates the probability that a given sequence in the target is linked by one or more miRNAs. The target mRNAs are first predicted based on common criteria, such as an optimal binding free energy, and are then tested statistically using an alignment of the genome of eight vertebrates to filter out false positives. The false positive rate for PicTar has been estimated to be approximately 30%. However, sequences, already known as miRNA targets were correctly identified using this software. Krek et al. have used this algorithm to the prediction of miRNA targets in vertebrates and have suggested that, on average, about 200 transcripts are regulated by a single miRNA (John et al., 2004; Krek et al., 2005).

2.2.8.1.5 TARGETSCAN

The software TargetScan combines the prediction of structure (modeling) of miRNA-mRNA heteroduplexes, based on thermodynamics, with a comparative analysis of sequence, based on conservation between species. It has been observed that the bases 2-8 at extremity 5' end of miRNA, the well known seed sequence, could pair with Watson-Crick complementarity to its target. Based on that, was developed an algorithm looking for the pairing between the sequences "seed" of the miRNA sequences and 3' UTR of mRNAs, calculating the thermodynamic properties with software RNAFold. Eleven of the 15 predicted targets obtained by this software were validated experimentally. In addition, the false positive rate was estimated to be between 22% and 31% for the targets of mammal, and for the first time, the software was used to predict potential targets of miRNA 451. Even if the predicted target included a wide range of functions, was an enrichment for genes involved in transcriptional regulation. Subsequently, the algorithm was improved and was proposed TargetScanS, which requires the annealing of a sequence more short, of only six nucleotides, independent from thermodynamic stability or by the presence of multiple target sites and an adenosine base preserved in seed sequence (Lewis et al., 2005). The authors have also added two species in more for the analysis of conservation between species, dog, and chicken. These changes have reduced the estimation of false positive rate of the algorithm to 22% in mammals. In addition, the algorithm has been able to successfully predict all known miRNA-target interactions and a total of more than 5,300 human genes as potential mRNA targets of miRNAs. Thus, these analyzes indicate that more than one third of human genes are conserved miRNA targets and therefore potentially regulated by them (Hake et al., 2003; Jidong et al., 2005; Lewis et al., 2003).



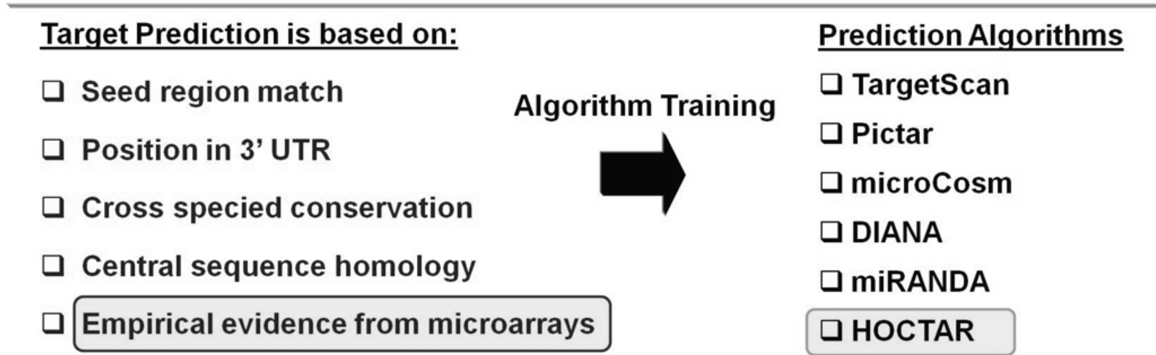


Figure 3. Comparison between different prediction software of miRNA-mRNA interactions.

2.2.9 PROBLEMS WITH IN SILICO PREDICTIONS OF INTERACTIONS

New studies have been conducted to compare different prediction software, and suggested that in reality no program is superior to others (RAjewsky et al., 2006; Sethupathy et al., 2006). A common practice among researchers is to use different programs for the prediction of the target and focus on the outcome of their intersection (Sonkoly et al., 2007; Megraw et al., 2007). The bioinformatic prediction of miRNA targets is certainly an important first approach in the study of the functional role of miRNAs themselves, but the predicted hypothetical target must always be validated by specific experimental methodologies. This is the reason why there are designed database that collect the miRNA interactions: target experimentally validated, such miRecords and TarBase (Feifei et al., 2009; Sethupathy et al., 2006).

In particular, miRecords that is an integrated software for the detection of miRNA-target interactions in animals. MiRecords is a useful resource not only at an experimental level for students miRNAs, but also for computer scientists who are working on developing next-generation programs for the prediction of target. Often to associate miRNAs and their expression to specific biological processes or pathways is needed to use different computational analyses, complex and time-consuming. One method consists in identify the target mRNAs of microRNAs and classify them according to their molecular function. On this approach is based on software and miRGator MAMI, which categorize the targets of miRNAs according to the terms of Gene Ontology and the associations of disease (Nam et al., 2008; Mami Site web). Instead, a different approach to identify which biological processes are affected by miRNAs, is to study their expression and relate it to that of their target. In fact, one of the first studies, carried out by microarray, demonstrated that the overexpression of a miRNA induces downregulation of a large number of transcripts (Lim et al., 2005). More recent work has confirmed this inverse correlation between the levels of expression of miRNAs and their target mRNA, despite the biological effect to occur primarily on the levels of the protein (Wang et al., 2006; Linsley et al., 2007; Stark et al., 2005; Sood et al., 2006; Creighton et al., 2008). Therefore have been developed a series of programs that bioinformatics be used to correlate the expression levels of miRNAs and their predicted targets, taking into account their own inverse correlation. One of the first methods developed named SigTerms use Microsoft Excel to compare experimental data on gene expression publicly available with the predictions of miRNA targets (Xie et al., 2005). SigTerms allows to make an enrichment analysis of gene expression data for the targets of miRNAs. In this way, before they are identified miRNAs and mRNAs differentially expressed significantly, in the same experimental conditions. The results are compared with the targets of deregulated

miRNAs, predicted at least by three different algorithms (TargetScan, PITA and PicTar).

2.2.9.1 Transcriptome profiling approach

Some of the earliest experimental approaches for identifying putative miRNA target sites relied on transfection of specific miRNAs into cell types followed by high-throughput mRNA expression analysis by microarray (Grimson et al., 2007; Lim et al., 2005; Linsley et al., 2007). Transcripts with reduced expression were then scanned for putative target sites by identifying sequences complementary to the miRNA seed sequence. These studies demonstrated that mRNA transcripts deregulated following miRNA transfection were significantly enriched for matches to the seed sequence of the transfected miRNA. Since delivery of supraphysiological levels of an exogenous miRNA may mediate non-specific effects, knockdown of miRNA using antisense oligonucleotides followed by microarray analysis of global transcript expression has been employed (Elmén et al., 2008; Krützfeldt et al., 2005). Transcript destabilization is not the exclusive mechanism by which miRNA inhibit gene expression; translational repression is another. Therefore, similar studies have been performed utilizing proteomic approaches to detect repressed protein expression following miRNA transfection (Baek et al., 2008; Selbach et al., 2008). Similar scanning of deregulated transcripts revealed enrichment for sequences complementary to the seed sequence of the transfected miRNA (Long et al., 2012).

2.2.10 HOCTAR DATABASE

Recently, the Tigem Research Team lead by Professor Sandro Banfi has developed a new and efficient approach to perform miRNA target prediction, the HOCTAR (Host Gene Oppositely Correlated Targets) procedure (Gennarino et al., 2009). Current estimates indicate that each miRNA may regulate, on average, the expression of 100–200 mRNAs (Ambros, 2004; Lim et al., 2005). Currently, there are 718 annotated miRNAs in human (Griffiths-Jones, 2004), localized in intragenic (60% of cases) or in intergenic (40%) regions. Intragenic miRNAs are contained within transcriptional units termed “host genes” (Kim et al., 2009), which are generally protein-coding. Recent work demonstrated that many intronic miRNAs and their host genes are co-transcribed from a common promoter (Rodriguez et al., 2004; Kim and Kim, 2007).

Figure 4. HOCTAR database homepage.

2.2.10.1 THE HOCTAR PROCEDURE

Based on the evidence that it is possible to use a miRNA host gene as a proxy for the expression of the miRNA itself (Tsang et al. 2007), authors have hypothesized that the expression behavior of a miRNA host gene may be inversely correlated to that of the targets of the embedded miRNA. As a result, an increase in the expression levels of the host gene should correspond to a decrease in the expression levels of the targets of its embedded miRNA, at least in some tissues or cellular conditions. In this study, have been tested whether such an inverse correlation can be exploited to improve the prediction of miRNA targets. To achieve this goal, we devised a novel strategy that we termed HOCTAR. For each intragenic miRNA authors compiled a non-redundant list of predicted mRNA targets by pooling all corresponding miRanda, TargetScan, and PicTar predictions. Expression correlation analysis of miRNA host genes and putative targets was based on total of 217 microarray data sets (3583 microarray experiments) downloaded from GEO repository (http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2007). All experiments were performed on the same microarray platform, the HG-U133A GeneChip array (GPL96, Feb 19, 2002) and each dataset was normalized and pre-processed independently. For each probe targeting a miRNA host gene, the 3% most anti-correlated probes (using Pearson correlation coefficient) were selected in each data set and all the lists were combined and ranked according to the number of occurrences and to the average rank in the single lists. As “host gene” have been considered those genes that a) had a sequence overlapping that of the precursor miRNA either in introns, exons, or UTRs, b) were transcribed from the same strand as the miRNA, and c) were represented in the HG-U133A Affymetrix platform, which mostly includes known genes. By using HOCTAR have been analyzed 230 host genes and 377 Affymetrix probes corresponding to 290 human miRNAs. 265 miRNA

target predictions are present in HOCTARdb corresponding to a non-redundant total of 9963 genes. All host gene intragenic miRNA relationships were manually verified by using the UCSCGenome Browser (release 2006/March; http://genome.ucsc.edu/). The analysis of dozens of validated miRNA targets showed that the HOCTAR procedure is generally applicable to all intragenic miRNAs and that its performance overruns that of first-generation prediction softwares, which are based on sequence analysis alone (Gennarino et al., 2009).

2.2.11 IDENTIFICATION OF MIRNA

Under a standard nomenclature system, names are assigned to experimentally confirmed miRNAs before publication of their discovery (Ambros et al., 2003; Griffiths-Jones et al., 2006). The prefix "mir" is followed by a dash and a number, the latter often indicating order of naming. For example, mir-123 was named and likely discovered prior to mir-456. The uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-" refers to the mature form. Often there is a great rate of conservation in nucleotides of 18-25 usual sequence of miRNA between species. For example it has been found that the sequence of the mature form of human miRNA has-mir-103-1 (accession number in mirBase MIMAT0000101) is the same of rattus norvegicus rno-mir-103-1 whose accession number is MIMAT0000824 (mirBase). This gave the rationale to choose an human database of miRNA (HOCTAR) to infer on putative homologous miRNAs in rattus norvegicus species useful for our purpose to identify what miRNAs are modulated during transient cerebral ischemia in rats.

2.2.12 IBM 22; VALIDATION OF MIRNA-MRNA INTERACTION

rna22 (http://cbcsrv.watson.ibm.com/rna22.html) is a method for identifying microRNA binding sites on 3'UTR of target genes based on thermodynamic stability

of corresponding heteroduplexes. *Rna22* does not rely upon cross-species conservation, is resilient to noise, and, unlike previous methods, it first finds putative microRNA binding sites in the sequence of interest, then identifies the targeting microRNA. Computationally, it has been demonstrated that *rna22* identifies most of the currently known heteroduplexes. On the experimental point of view, this resource, with luciferase assays, have evidence of an average repression of 30% or more for 168 out of 226 tested targets. The analysis suggested that: (1) some microRNAs may have as many as few thousand targets, and (2) between 74% and 92% of the gene transcripts are likely under microRNA control through their untranslated and aminoacid coding regions. The method's key idea was also extended to a low-error microRNA-precursor-discovery scheme; thus suggesting that the number of microRNA precursors in mammalian genomes likely ranges in the tens of thousands (Lewis et al., 2005; Miranda et al., 2006).

2.2.13 STRATEGIES OF MIRNA MODULATION

2.2.13.1 DELIVERY OF MIRNA MODULATORS

Experiments utilizing either miRNA inhibitors or target protectors require that they are transfected or otherwise delivered into cell cultures or other model systems and that gene expression is measured thereafter. *In vivo* delivery of miRNA modulators to the CNS is still difficult but direct cortical or intraventricular injection of modified synthetic molecules (Krützfeldt et al., 2007) or transgene expressing viral particles is an option. The exosome strategy previously mentioned may also represent a facile method for systemic administration of miRNA modulators to the CNS and warrants further testing (Alvarez-Erviti et al., 2011).

2.2.13.2 ANTIMIRNA STRATEGY TO BLOCK AN ENDOGENOUS MIRNA

Antisense oligonucletides (ASOs) have been widely used to target specific mRNAs to study gene function. Several independent chemical modifications of ASOs that improve affinity and stability have been used to inhibit miRNA function both *in vitro* and *in vivo*. In some cases, miRNA ASO inhibition leads to target miRNA degradation. Indeed, recent studies have shown that the stability of miRNAs is defined by the Argonaute protein with which it binds, and the degree of complementarity between the miRNA and its target (Ameres et al., 2010). “AntagomiRs” were the first miRNA inhibitors demonstrated to work in mammals (Krutzfeldt et al., 2005). These ASOs harbor various modifications for RNAse protection and pharmacological properties,such as enhanced tissue and cellular uptake. They contain 2′-O-Methyl- modified ribose sugars (2′-OMe), a terminal phosphorothioate linkage instead of a natural phosphate linkag, and a cholesterol group at the 3′ end. Although AntagomiRs have been found to inhibit a specific miRNA in several tissues, they require higher doses to achieve the same efficacy as other ASO strategies. Locked Nucleic Acid (LNA) antisense nucleotides introduce a 2′, 4′ methylene bridge in the ribose to form a bicyclic nucleotide. LNA modification increases the RNA:RNA melting temperature by 2–4 °C per modification and confers resistance to many endonucleases. These features make LNA-antimiR more specific and permit the use lower levels of antisense nucleotides. LNA ASOs have been used succesfully in several *in vitro* studies to inhibit specific miRNAs. Studies in mice have demonstrated that the delivery of LNA-antimiRwith phosphorothioate modifications may inhibit miR-122, a miRNA that binds the hepatitis C virus (HCV) and stimulates its replication.

2.2.13.3 MIRNA DELIVERY *IN VIVO*

The delivery of miRNA mimic and miRNA antagonists oligonucleotides *in vivo* is possible but there are limiting factors to overcome, including the low stability of RNA nucleotides *in vivo*, the lack of regulated expression, and the inefficient uptake of oligonucleotides by neurons. Tools based on recombinant viral vectors derived from lentivirus, adeno-associated virus (rAAV), and retrovirus (Papale et al., 2009) reach a high efficency rate in neuronal cells and have been used to study miRNA function in neurons *in vitro* and *in vivo*. However, even if we bypass the problem of overcoming the BBB, the toxicity of therapeutic small oligonucleotides would remain a problem. Viral delivery of miRNA-based molecules, as reported in viral-shRNA studies, might lead to toxicity and tissue damage (Boudreau et al., 2009; McBride et al., 2008).

2.3 SODIUM/CALCIUM EXCHANGER

The Na^{+}/Ca^{2+} exchanger (NCX) is one of the major membrane proteins involved in Ca^{2+} extrusion at the plasma membrane. The regulation of Ca^{2+} and Na^{+} homeostasis is a crucial physiological phenomenon in neurons. In fact, Ca^{2+} ions play a key role as a second messenger in the cytosol and in the nucleus (Choi, 1988), while the Na^{+} ion regulates the cellular osmolarity, inducing action potentials (Lipton, 1999), and it is involved in the signal translation (Yu et al., 1997). The control of this regulation is delegated to ionic channels selective for Ca^{2+} and Na^{+}, to Na^{+} pumps, Ca^{2+} ATP-dependent and to NCX (Blaustein and Lederer, 1999). The NCX family, which exchanges three Na^{+} ions for one Ca^{2+} ion or four Na+ ions for one Ca^{2+} ion depending on $[Na^{+}]_i$ and $[Ca^{2+}]_i$ (Reeves and Hale, 1984; Fujioka et al., 2000; Hang and Hilgemann, 2004) consists of three dominant genes coding for the three different isoforms of the exchanger: NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996) proteins. These three genes appear to be dispersed, since NCX1, NCX2, and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al., 1996). At the post-transcriptional level, at least 12 NCX1 and 3 NCX3 proteins are generated through alternative splicing of the primary nuclear transcripts. These variants arise from a region of the large intracellular f-loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner. To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive (Quednau et al. 1997). NCX1 is composed of 938 amino acids in the canine heart and has a molecular mass of 120 kDa and contains nine transmembrane segments (TMS). NCX1 amino terminus (N-terminal) is located in the extracellular space, whereas the carboxyl terminus (C-terminal) is located intracellularly. The nine transmembrane segments can be divided into an N-terminal hydrophobic domain,

composed of the first five TMS (1–5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1–5) TMS are separated from the last four (6–9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f-loop (Nicoll et al., 1999). Although the f-loop is not implicated in Na^{+} and Ca^{2+} translocation, it is responsible for the regulation of NCX activity.

2.3.1 Mechanism of action of sodium/calcium exchanger

NCX can facilitate both Ca^{2+} and Na^{+} flow in a bidirectional way through the plasmamembrane (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000) with a stoichiometry of 3 Na^{+} ions versus 1 Ca^{2+} ion. Depending on the intracellular levels of Na^{+} and Ca^{2+}, NCX can operate in the forward mode by extruding one Ca^{2+} against three entering Na^{+}, using the Na+ gradient across the plasma membrane as a source of energy (Blaustein and Lederer, 1999; Annunziato et al., 2004). Alternatively, in the reverse mode, NCX can function as Na^{+} efflux–Ca^{2+} influx. Because of its high exchange capacity, NCX is well-suited for rapid recovery from high intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and may play an important role in maintaining Ca^{2+} homeostasis and protecting cells from Ca^{2+} overload and eventually death (Blaustein and Lederer, 1999; Annunziato et al., 2004).

2.3.2 Sodium/calcium exchanger isoform 1 distribution in brain

The NCX1 gene displays an ubiquitous expression and therefore is present in several tissues, including brain, heart, skeletal muscle, smooth muscle, kidney, eye, secretory, and blood cells whereas NCX2 and NCX3 gene products have been found exclusively in neuronal and skeletal muscle tissues (Lee, 2004). NCX1 has several

splicing variants that appear to be selectively expressed in different regions and cellular populations of the brain (Quednau et al., 1997; Yu et al., 1997). In cerebral cortex, NCX1 is intensively expressed in the pyramidal neurons of layers III and V within the molecular layer of the cerebral motor cortex. This area, which contains the terminal dendritic field of the pyramidal cells, displays an intense NCX1 immunoreactivity. NCX1 protein expression is particularly intense in the granule cell layer and in the hilum of the dentate gyrus, which constitutes the terminal field of the perforant pathway, the major excitatory input to the hippocampus originating from the enthorinal cortex. NCX1 mRNA can be detected in the substantia nigra pars compacta, in which dopaminergic cell bodies are localized; the NCX1 protein isoform is present in the striatum, in which the terminal projection fields of dopaminergic nigrostriatal neurons are found. Interestingly, the transcript and the protein, encoded by NCX genes, is abundantly expressed in the nucleus accumbens (Canitano et al., 2002; Papa et al., 2003), a brain region involved in the motivational control of motor coordination and damaged following middle cerebral artery occlusion.

2.3.3 Regulation of sodium/calcium exchanger

During brain ischemia there is a dramatic impairment of mechanisms regulating the homeostasis of sodium and calcium ions between the internal neurons and their extracellular melieu. One of the crucial actor of these biochemical deleterious events leading to neuronal cell pain is represented by the sodium/calcium exchanger. The activity of this membrane antiporter protein has been focused for several years of experimental work of our research group. The exchanger acts by modulating in excitable cells the intracellular concentrations of Na^+ and Ca^{2+} ions, thus providing for the maintenance of cell ions homeostasis. NCX mediates ions fluxes of Ca^{2+} and Na^+

ions across the plasma membrane in a bidirectional way (Blaustein et al., 1999; Philipson et al., 2000; Annunziato et al., 2004). There are several factors that regulate the activity of the exchanger sodium calcium, among which: (i) the concentration of the two cations transported, Na^+ and $Ca^{2+;}$ (ii) the intracellular pH; (III) compounds related to metabolism, ATP, PIP2, PKA and PKC, and (iv) reactive oxygen species (ROS) and reactive nitrogen species (RNS). The concentration of calcium regulates NCX through CBD (Ca^{2+-}binding domain) in the same way the Na^+ ion plays a regulatory function. In particular when the sodium concentration increases, it binds to the site of transport of the heat exchanger, and after this influx of sodium, is an inactivation of the same. This process of inactivation, is very similar to the phenomenon that occurs in the voltage-gated ion channels, and is called sodium-dependent inactivation. The exchanger can also be regulated by the intracellular pH. A strong acidity inhibits the activity of NCX leaving it under a constant steady state, in fact, reductions in pH value below 0.4, can induce an inhibition of NCX than 90%. The ATP, which acts as a donor of phosphate groups, can increase the activity of the exchanger in different ways. First, activating the G protein-coupled receptors for endogenous and exogenous ligands. As a second, ATP can stimulate the activity of NCX through the pathway involving PKC or PKA; each isoform of NCX is presumed to have several phosphorylation sites. Finally, another mechanism by which NCX can be activated requires the production of lipid PIP2. In fact, this lipid binds the XIP region of the loop "f" eliminating the inactivation of NCX, thus stimulating its function. Interestingly, the depletion of ATP within the cell act differently on the three isoforms of the exchanger by inactivating both NCX1 and NCX2 but not by influencing the activity of NCX3 (Secondo et al., 2007) . The sodium-calcium exchanger is sensitive to reactive oxygen species, in fact by altered redox cell can result increases the activity of NCX (Annunziato et al., 2004). Among

the several factors that regulate the activity of NCX can be probably included miRNAs as it has been already demonstrated to fine-tune numerous target genes at the post-transcriptional level.

2.3.4 SODIUM/CALCIUM EXCHANGER ROLE IN ISCHEMIC STROKE

In an *in vivo* model of cerebral ischemia, reproduced in our laboratories, based on permanent middle cerebral artery occlusion (pMCAO) it has been observed a downregulation of about 90% of the levels of expression of NCX1 and NCX3 in ischemic core and in the peri-ischemic regions. In other brain districts, belonging to the ischemic penumbra, after cerebral ischemia, there was an increase of the levels of RNA messenger of NCX3 and NCX1. In contrast, in the same regions, the pMCAO causes a decrease in mRNA expression of NCX2. The up-regulation of NCX3 in peri-infarct tissue has been interpreted as a compensatory mechanism to offset the reduced activity of NCX2, that in the course of ischemia is down-regulated and to keep at a proper homeostasis ions Na^+ and Ca^{2+}. In essence, the expression of NCX1 and NCX3 after permanent middle cerebral artery occlusion (pMCAO) in rats is regulated in a differential manner, depending on the region involved in the insult (Boscia et al., 2006; Pignataro et al., 2004a). Furthermore, antisense-induced downregulation of NCX1 and NCX3 or genetic ablation of NCX3 worsens the experimentally-induced ischemic damage in mice and rats (Molinaro et al., 2008; Pignataro et al., 2004a). Evidence for NCX3 neuroprotective role relies in the remarkable broadening of the infarct volume occurring when NCX3 protein is knocked down with a selective antisense oligonucleotide, thereby worsening the neurologic deficits (Pignataro et al., 2004). Accordingly, it has been recently showed in ischemic NCX3–/– mice that NCX3 exerts a neuroprotective effect (Molinaro et al.,

2008). For instance, in homozygous ncx3−/− mice subjected to MCAO, an increased brain damage occurs (Molinaro et al., 2008). In addition, the silencing of NCX1 and NCX3 expression by RNA interference increases cerebellar granule neurons vulnerability to Ca^{2+} overload and excitotoxicity (Bano et al., 2005; Secondo et al., 2007). Moreover, the vulnerability to chemical hypoxia of BHK cells overexpressing NCX1 or NCX3 considerably increases when either NCX1 or NCX3 is silenced (Bano et al., 2005; Secondo et al., 2007). Finally, ischemic rats treated with NCX1 or NCX3 antisense display a remarkable enlargement of the infarct volume (Pignataro et al., 2004a). In a recent paper published by Pignataro et al., it has been shown that among the three NCX brain isoforms, NCX1 represent a new molecular effector involved in a neuroprotective mechanism named "ischemic preconditioning" (Pignataro et al., 2011, 2013). In effect, the brain possesses internal defense mechanisms that can be triggered by several stimuli. Among these mechanisms, preconditioning has recently attracted a great deal of interest. Preconditioning is a phenomenon whereby a subliminal injurious stimulus applied before a longer harmful ischemia (Dirnagl et al., 2003; Gidday, 2006; Kirino, 2002) is able to exert a remarkable neuroprotection, thus establishing a state of tolerance to anoxic conditions. Pignataro has demonstrated that among the three NCX brain isoforms, NCX1 and NCX3 represent two molecular effectors involved in the neuroprotective mechanisms of ischemic preconditioning. So, results of Pignataro work support the importance of NCX1 and NCX3 in the pathogenesis of ischemic lesion and, most important, offer a new possible interpretation of the neuroprotective mechanism elicited by ischemic preconditioning. Furthermore the overexpression of NCX1 and NCX3 observed during preconditioning may be related to their ability to counteract the dysregulation of intracellular Na^{+}, ($[Na^{+}]_{I}$) and Ca^{2+}, ($[Ca^{2+}]_{I}$) homeostasis occurring in the brain under anoxic conditions corresponding to an harmful ischemia.

In a rat model of cerebral transient ischemia the reduction in NCX1 expression induced by the ischemic insult alone (Boscia et al., 2009; Pignataro et al., 2004b) was completely prevented when the animals were exposed to preconditioning alone. Indeed, since NCX1 and NCX3 silencing partially prevented ischemic neuroprotection mediated by preconditioning these results have shown that it would be reasonable to tune up a pharmacological strategy able to modulate NCX1. This approach has been derived from the evidence that is possible to hypothesize that the increased expression of certain proteins induced by a neuroprotective strategy like preconditioning could render the brain tissue ready to withstand subsequent, more severe brain conditions. Interestingly, the activation of these mechanisms has appeared to be longlasting, as relatively to sodium/calcium exchanger protein the upregulation of NCX1 and NCX3 was still present even after 72h after preconditioning induction, thus suggesting that both NCX1 and NCX3 might be considered as two possible effectors of delayed preconditioning. More important, the increased expression of NCX1 and NCX3 observed at early time points does not necessarily implicated that the neuroprotection might have occured at the same time points. The results mentioned in this study have suggester that, in order to reduce the extension of the infarct volume after a harmful ischemic insult an enhancement of NCX1 and NCX3 expression and/or activity might be desirable. Subsequent studies in spontaneously hypertensive rats also observed that multiple microRNAs downregulated after preconditioning are predicted to target MeCP2 mRNAs (Dharap et al. 2009, and Vemuganti et al., 2010). MeCP2 is a potent transcriptional repressor (Nan et al, 1998) and transcriptional activator (Chahrour et al., 2008). Mutations in the MeCP2 gene cause Rett syndrome (Hite et al., 2009) and several other CNS disorders including mental retardation, Angelman syndrome, and autism (Gonzales and LaSalle, 2010). Given that repressed gene expression is a feature of tolerance, it

was significant hat expression of MeCP2 rapidly increased in preconditioned mouse cortex with no correlating changes in mRNA expression and MeCP2 knockout mice showed increased susceptibility to ischemia (Lusardi et al., 2010), suggesting that MeCP2 may be an effector of preconditioning-induced tolerance.

3. AIM OF THE STUDY

The role of miRNAs in cerebral ischemia has been largely unexplored. Giving that one miRNA regulates more than one hundred gene targets, and one gene can be regulated by a great number of miRNAs, it is mandatory to improve the knowledge of rules that govern miRNA-mRNA interaction and functional outcome when that interaction is modulated. In the last years robust biological validation of miRNA targets is moving us ever closer to better understand the complex molecular mechanisms associated with pathological outcome. In the light of these premises the present thesis work has been focused on the identification of microRNA pathways able to modulate sodium/calcium exchanger during an ischemic event in the brain. The choice of NCX as putative target of miRNA involved in stroke pathophysiology derives from previous work produced in our Department. Indeed, in the last 20 years our results gave a strong contribution in demonstrating that NCX activation represents an important mechanism of neuroprotection. In fact, brain ischemia determines a permanent decrease in NCX protein expression that is paralleled to the maximum evolution of ischemic damage. More interestingly, NCX activation is able to induce a remarkable neuroprotection (Pignataro et al., 2011, 2012, 2013). Furthermore, NCX1 has been recently included in the list of effectors recruited in the course of the well known neuroprotective phenomenon called ischemic preconditioning (Pignataro et al., 2011, 2012). In this regard, due to the difficulty to translate preconditioning into a valid clinical perspective, my purpose has been to tune up a reasonable pharmacological strategy able to reduce the extension of the infarct volume after an harmful ischemic insult by the enhancement of NCX1 mediated by miRNA. To achieve this aim we used a strategy comprising the following

steps: (1) identification of a candidate miRNA; (2) overexpression experiments with miRNA Mimic; (3) loss of function experiments with miRNA inhibitors; (4) *in vivo* block of miRNA pathway. In fact, two main experimental strategies have been used during identification of candidate microRNA: overexpression by adding exogenous miRNAs, and loss-of-function approaches. Overexpression strategies are frequently criticized because artificially increasing the intracellular concentration of miRNAs may result in the repression of mRNAs that are not physiological targets. By contrast, loss of-function approaches, if carefully designed to avoid off-target effects, may reveal miRNA functions that rely on physiological miRNA levels. Another problem has concerned the delivery of miRNA mimic and miRNA antagonists oligonucleotides *in vivo* that is possible as operative strategy, but there have been some limiting factors, including the low stability of RNA nucleotides *in vivo*, the lack of regulated expression, and the inefficient uptake of oligonucleotides by neurons that have been exceeded by using chemical modified miRNAs directly infused intracerebroventricularly.

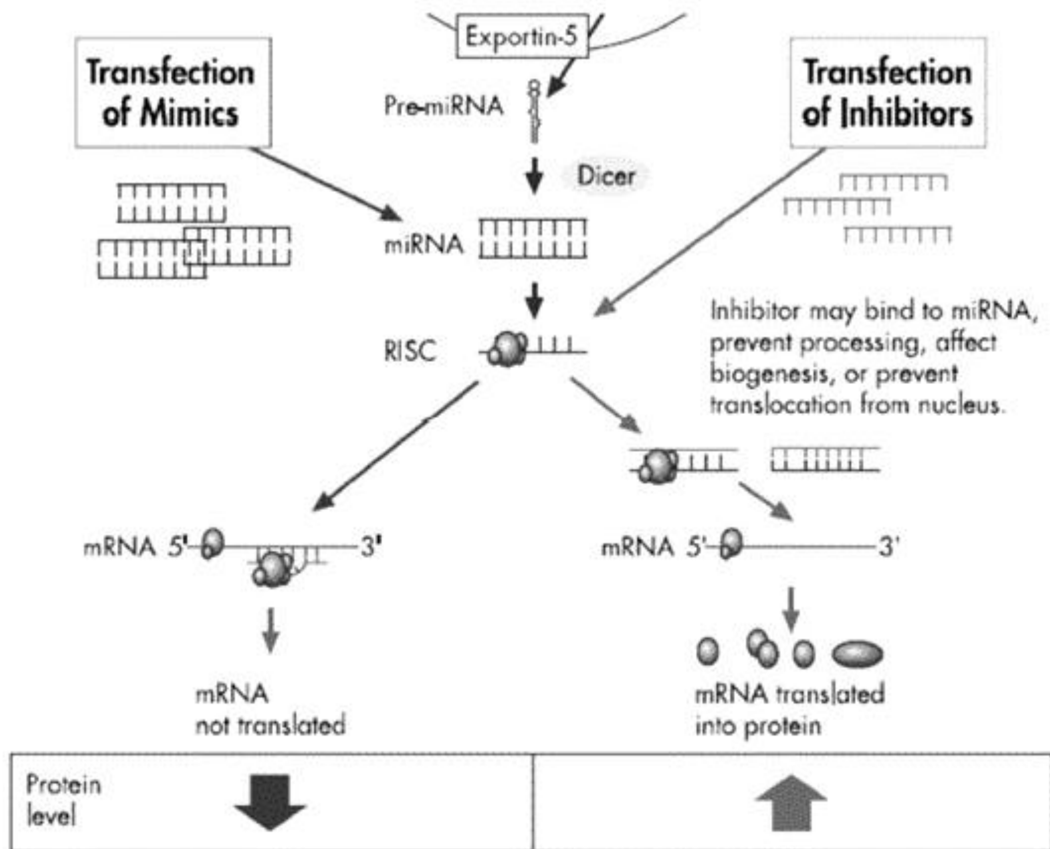


Figure 5. A schematic approach to miRNA functional studies.

4. MATERIALS AND METHODS

4.1 SPECIFIC MIRNA IDENTIFICATION

The database HOCTAR is an opensource resource that allows to obtain a so-called "ranking list", a list miRNAs that includes miRNAs most likely able to base-pair the region at 3 'UTR of RNA messenger of the protein of interest, in our case the sodium/calcium exchanger isoform 1. The HOCTAR analysis aims to create a lists of miRNAs organized on the basis of the possibility of NCX1 gene transcript modulation based on the number of times that a given host gene (in which a specific miRNA is contained) is inversely correlated to the gene of interest (NCX1). In this way, considering that most of miRNAs are part of a single transcriptional unit together with the confining host genes, it is conceivable that by monitoring thes genes is possible to obtain indirect information on the embedded miRNA. Inverse correlation information between all host gene and target genes derives from experimental data on miRNA-mRNA interactions validated by microarray experiments. This procedure allows to believe that miRNA, contained in its host gene, directly modulates target gene inversely correlated. The list shows in descending order miRNAs that have progressively lower probability to bind NCX RNA messenger. Then, the order of decreasing probability of this matching has to be confirmed by the existence of a more or less strict complementarity, based on Watson-Crick annealing between the "seed sequence" of the miRNA of interest (usually a region of 6-8 nucleotides at the 5 'end of miRNA) and the sequence targeted on NCX1 mRNA, usually on 3’ UTR of gene. For this purpose has been used the pairing software RNA 22.

4.2 DRUGS AND CHEMICALS

For gain of function studies, miRIDIAN microRNA hsa-miR-103a-3p (C-300522-03-0005 5 nmol) mimic corresponding to mirbase accession: MIMAT0000101 and miRIDIAN Mimic Trasfection Control with Dy547 (Cp-004500-01-05 5nmol) were purchased from Thermo Fisher Scientific Inc.

For loss of function experiments, Locked nucleic acids (LNA) AntimiRNA 103-1 (414336-00 Pre-designed miRCURY LNA ™ microRNA Inhibitor, 5nmol) and Negative Control A miRNA (199004-00, microRNA miRCURY LNA ™ Power Antisense Control A, 5nmol) were purchased from Exiqon, Denmark.

4.3 CELL CULTURES

4.3.1 BHK CELLS

Baby hamster kidney (BHK) cells, stably transfected with canine cardiac NCX1, were grown on plastic dishes in a mix of DMEM and Ham's F12 media (1:1) (Gibco, Invitrogen, MI, Italy) supplemented with 5% fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin. Cells were cultured in a humidified 5% CO_2 atmosphere; the culture medium was changed every 2 days (Secondo et al., 2007).

4.3.2 PC-12 CELLS

Rat pheochromocytoma cells (PC-12 cells) were grown in 85% RPMI 1640, 10% horse serum and 5% heat-inactivated fetal bovine serum, containing 5 U/mL penicillin and 5 Ig/mL streptomycin, at 37 °C with 5% CO_2 (Pannaccione et al. 2005). For all the experiments, cells were seeded at low density on glass cover-slips coated with

poly-L-lysine (50ng/ml). Differentiation of PC-12 cells was achieved by NGF 2.5S treatment (50ng/ml) for 7-9 days (Greene and Tischler, 1976).

4.3.3 RAT CORTICAL NEURONS

Cortical neurons were prepared from brains of 14-d-old mouse embryos (Charles River), plated on coverslips, and cultured in MEM/F1(Invitrogen) containing glucose, 5% of deactivated fetal bovine serum and 5% horse serum (Invitrogen), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 g/ml). Cytosine--D-arabinofuranoside (10 M) was added within 5 d of plating to prevent them growth of non-neuronal cells. Neurons were cultured at 37°C in a humidified 5% CO2 atmosphere and used after 7–10 d *in vitro* (DIV) (Scorziello et al., 2007).

4.3.4 TRANSFECTION OF BHK CELLS, PC12 AND RAT CORTICAL NEURONS

PC12 cells and BHK were transfected with 10, 50, 100 and 150 nM of hsa-mir-103-1 Mimic and with 10, 50, 100 and 150 nM of microRNA Mimic Trasfection Control. As transfection agent, use is made of 10 ul of HiPerFect Trasfection Reagent, according to the manufacturer’s protocol. After an incubation period of 5 hours, the medium was replaced and respectively to 24, 48 and 72 hours after the transfection, the cells were harvested and used for western blot or PCR Real Time analysis. Conversely rat cortical neurons were transfected with 150 nM of hsa-mir-103-1 for a period of 72 hours.

4.3.4.1 *In vitro* Experimental groups

Group 1. BHK-NCX1 cell clones transiently transfected with miRNA Mimic 103-1 at a concentration of 10 nmol; the cells were harvested at 24 and 48 hours and analyzed by western blot for the protein NCX1.

Group 2. BHK-NCX1 cells clones transiently transfected with miRNA Mimic 103-1 at a concentration of 50 nmol; the cells were harvested at 24 and 48 hours of temporal delay from the beginning of transfection and analyzed by western blot for the protein NCX1.

Group 3. BHK-NCX1 cells transiently transfected with miRNA Mimic 103-1 at a concentration of 100 nmol; the cells were harvested at 24 and 48 hours hours of temporal delay from the beginning transfection and analyzed by western blot for the protein NCX1.

Group 4. PC12 cells transiently transfected with miRNA Mimic 103-1 at a concentration of 50 nmol; cells were harvested at 24 and 48h of distance and analyzed by western blot for the protein NCX1.

Group 5. PC12 cells transiently transfected with miRNA Mimic 103-1 at a concentration of 100 nmol; the cells were harvested at 24 and 48 and 72 hours apart and analyzed by western blot for the protein NCX1.

Group 6. PC12 cells transiently transfected with miRNA Mimic 103-1 at a concentration of 150 nmol; the cells were harvested at 48 and 72 hours apart and analyzed by western blot for the protein NCX1.

Group 7. Embryonic cortical neurons transiently transfected with miRNA Mimic 103-1 at a concentration of 150 nmol; neurons were collected at 72 hours of temporal

delay from the beginning of the trasfection and analyzed by western blot for the protein NCX1.

4.4 Induction of cerebral ischemia

Male Sprague–Dawley rats (Charles River) weighing 250 to 300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the "Federico II" University of Naples. Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized using 2% sevofluorane, 60% N_2O, and 38% O_2 (Pignataro et al., 2008). Under an operating stereomicroscope (Nikon SMZ800, Nikon Instruments, Florence, Italy) the right carotid bifurcation was carefully exposed and the external carotid artery (ECA) coagulated distal to the bifurcation. A silicon-coated nylon filament (Doccol, Ca, USA) was inserted through the ECA stump and gently advanced (19 mm) into the right internal carotid artery until it blocked the origin of the MCA. The surgical wound was closed and the filament left in place. After 100-minutes MCA occlusion, the filament was gently withdrawn in order to restore blood flow. Animals were allowed to recover from anesthesia at room temperature. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA. Cerebral blood flow was monitored 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).

4.4.1 *IN VIVO* EXPERIMENTAL GROUPS (I)

To assess both endogenous levels of expression of mir-103-1 (by means of TaqMan probes) and protein NCX1 (antibody Swant) after ischemic event up to 6, 24 and 72 hours after MCA occlusion three different experimental groups have been planned;

Group 1. 8 Rats subjected to tMCAO procedure and sacrificed after 6 hours from MCA occlusion. Samples from temporoparietal ipsilateral cortex and striatum caudato putamen have been harvested.

Group 2. 8 Rats subjected to tMCAO procedure and sacrificed after 24 hours from MCA occlusion. Samples from temporoparietal ipsilateral cortex and striatum caudato putamen have been harvested.

Group 3. 8 Rats subjected to intervention of tMCAO and sacrificed after 72 hours from MCA occlusion. Samples from temporoparietal ipsilateral cortex and striatum caudato putamen have been harvested.

Group 4. 5 sham-operated rats. Samples from temporoparietal ipsilateral cortex and striatum caudato putamen were harvested.

Brain specimens obtained in the above described way were used both to compare mir-103-1 level of expression in rats underwent to tMCAO to control levels observed in sham-operated rats (Real Time PCR) and to analyze the levels of expression of proteins NCX1, NCX2 and NCX3 in specific brain regions (Western Blot).

4.5 INTRACEREBROVENTRICULAR ADMINISTRATION OF ANTIMIR-103-1

The continuous release of AntimiRNA by infusion into brain lateral ventricle has been achieved using osmotic pumps (Alza Co., Palo Alto, CA, USA). In rats positioned on a stereotaxic frame implantation of the osmotic pump in brain was carried out 24 hours before the induction of transient ischemia (Vemuganti et al, 2004; Satriotomo et al, 2006). The osmotic pump was connected to a brain infusion kit (Alzet, n° 0004760) made of a stainless steel cannula that was implanted into the right lateral ventricle using the stereotaxic coordinates from the bregma: 0.4 mm caudal, 2 mm lateral and 2 mm below the dura and secured to the skull with dental cement. (Paxinos and Watson, 1997; Pignataro et al., 2004b). The pump was placed in the skin fold on the neck of the rat (Dharap et al. 2009). Infusion of antimiRNA lasting 48 hours has allowed to overcome problems relatively to the short half-life of miRNA (ca. 1-3,5 h). AntimiRNA 103-1 and the Negative Control A were diluted to the final concentration in saline solution (0,9% NaCl g/l) previously filtered (Microglass filters). The initial concentration used to test the efficacy of antimiRNA in modulating endogenous miRNA 103-1 levels was increased by 40 times (considering CSF volume as factor of dilution) according to the concentration of corresponding mir-103-1 Mimic capable, *in vitro*, to exert the maximal effect of reduction in the NCX protein levels of expression respectively both in cortical neurons and in PC12 cells. The release of antimiRNA by the osmotic pump within rat cerebral ventricle was set up at a speed of 1ul/hr. AntimiRNA has been used at two different concentrations on rats: 20uM and 60 uM.

4.5.1 *IN VIVO* EXPERIMENTAL GROUPS (II)

Group 1. 7 Rats treated with antimiRNA 103-1 at a concentration of 20uM subjected to tMCAO surgery and sacrificed 24 hours after the beginning of reperfusion. Samples from ischemic brain were obtained.

Group 2. 7 Rats treated with antimiRNA 103-1 at a concentration of 60 uM subjected to tMCAO surgery and sacrificed 24 hours after the beginning of reperfusion. Samples from ischemic brain were obtained.

Group 3. 7 Rats treated with LNA Negative Control subjected to tMCAO surgery and sacrificed 24 hours after the beginning of reperfusion. Samples from ischemic brain were obtained.

Group 4. 3 Sham-operated rats. Samples from ischemic brain were obtained.

4.6 WESTERN BLOT ANALYSIS

PC12 cells, BHK cells, rat cortical neurons and rat brain samples were homogenized in a lysis buffer (50 mmol/L Tris–HCl, pH 7.5, 100 mmol/L NaCl, 1% Triton X-100) containing protease and the phosphatase inhibitor. After centrifugation at 12,000 g at 4 °C for 5 min, the supernatants were collected. Protein concentration was estimated using the Bradford reagent. Then, 50 μg 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 polyvinylidene difluoride membranes. Blots were probed with antibodies to NCX1 (1:1000 Swant), NCX2 (1:1000, Alpha Diagnostic), NCX3 (1:2000, a kind gift from Prof. Philipson and Prof. Nicoll), and α-tubulin (1:2000; Abcam, MA, USA) diluted in tris buffered saline (TBS-T) 1% bovine serum albumin overnight (4 °C).

4.7 ANALYSIS BY REAL-TIME PCR

Total RNA was extracted from cells with TRIZOL according to the manufacturer's protocol (Invitrogen). The cDNA was synthesized from 5 grams of total RNA extracted from cells using reverse transcriptase MultiScribe for the retro-transcription polymerase reaction. The semi-quantitative polymerase reaction was performed according to the following conditions: 95 ° C for 3 hours, 30 or 35 cycles of reaction (95 ° C for one hour, 48 ° C for one hour, 72 ° C for one hour) and 72 ° C for 10 minutes. The pairs of oligonucleotides used were: 5'-ACCACCAAGACTACAGTGCG-3 and 5'-TTGGAAGCTGGTCTGTCTCC-3 'and 5'-CCTGCTGGATTACATTAAAGCACTG-3’ for NCX1 and 5'-CCTGAAGTACTCATTATAGTCAAG-3 'for the HPRT gene. The Real Time PCR reaction was carried out with Universal Master Mix No UNG provided by TaqMan kit (Life Technologies) in a 7500 Fast Real-time polymerase chain reaction system apparatus (Applied Biosystems). Normalization of data was performed using HPRT gene (Hypoxanthine-guanine phosphoribosyltransferase) as an internal control. The differences in mRNA content between groups were calculated as described by Formisano et al., 2007. The microRNA extraction from brain samples of cerebral cortex and striatum was achieved using Mirvana miRNA Isolation Kit (Applied Biosystems). Samples have been obtained from rodents after sacrifice by euthanization up to 6, 24 and 72 hours. The amplification and the normalization of the microRNA of interest was performed by real-time PCR (qRT-PCR). The probes used were TaqMan types (Life Technologies Co.). miRNA assay for rno-mir-103-1 (batch ID 4427975 miRNA mature sequence detection) and miRNA assay for rno-mir 4.5S (H) as endogenous control (batch ID 001716). For both miRNA of interest it was provided a primer for reverse transcription of cDNA from RNA extracted (TaqMan ® MicroRNA Reverse Transcription Kit, Applied Biosystems) and a pair of PCR primers

(forward and reverse) optimized for the detection and sensitive amplification of specific miRNAs by RT-PCR.

4.8 ANALYSIS OF ISCHEMIC VOLUME

To assess the effects of neuro protective treatment based on AntimiRNA 103-1 in rats subjected to ischemia after rats euthanization by an overdose sevoflurane to brains were removed from the skull to analyze them in order to determine the extent of ischemic damage. The brains were sectioned coronally at 1 mm intervals, and stained by immersion in the vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC). The infarct volume was calculated by summing infarction areas of all sections and by multiplying the total by slice thickness. The percentage of the infarct was calculated by dividing the infarct volume by the total ipsilateral hemispheric volume (Pignataro et al., 2008). Edema was calculated as follows: (volume of hemisphere ipsilateral to the lesion) – (volume of hemisphere contralateral to the lesion). This value was expressed as percentage of the volume of the hemisphere ipsilateral to the lesion (volume of edema: volume of hemisphere ipsilateral to the lesion = x: 100). This percentage was subtracted from the volume of the infarct. The person who did the image analysis was blinded to the study groups (Pignataro, Meller et al. 2008).

4.8.1 *IN VIVO* EXPERIMENTAL GROUPS (III)

Group 1. 7 Rats treated with antimiRNA 103-1 at a concentration of 60uM subjected to tMCAO surgery and sacrificed at 24 hours after MCAO occlusion.

Group 2. 7 Rats treated with negative control underwent tMCAO surgery and sacrificed 24 hours after MCAO occlusion.

Group 3. 7 Rats treated with vehicle alone, subjected tMCAO and sacrificed 24 hours after MCA occlusion.

4.9 Evaluation on general and focal deficits

Neurological scores were evaluated after 24 hours of reperfusion according to 2 scales: a general neurologic scale and a focal neurologic scale (Clark et al., 1991) . 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. In the focal score, the folllowing 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 at total focal score.

4.10 Statistical Analysis

Data obtained in cultured cells were expressed as mean±standard error and statistical significance of differences between groups was valuated by two-way ANOVA. $p<0.05$ was considered to be significant. For the evaluation of the protective

effect of antimiRNA 103-1 *in vivo*, on the infarct volume, data were expressed as mean±standard error and statistical analysis was performed by 2-way ANOVA followed by Newman Keuls test. Data relative to focal and general neurological deficits, being ordinal data, were analyzed using the non-parametric Kruskal–Wallis test, followed by the Nemenyi test for the non-parametric multiple comparison. Statistical significance was accepted at the 95% confidence level ($p<0.05$).

5. RESULTS

5.1 Identification of miR-103-1 by HOCTAR database and in silico validation of its interaction on 3'UTR of NCX1 RNA messenger.

By using as “Target Gene Name” query in HOCTAR screen database the word Slc8a1 for isoform 1 of sodium/calcium exchanger, (Figure 6), it has been obtained a list of all intragenic miRNAs predicted to bind the selected target gene. Intragenic miRNAs inversely correlates with target NCX1 transcripts have been ordered using two different colours to indicate miRNAs whose predicted targeting fall, respectively, above or below the set threshold corresponding to the 50th percentile of the HOCTAR prediction ranked lists as seen in (Figure 6A). By analyzing miRNAs affinity for 3'UTR of NCX1 messenger of first 50 percentile of ranking list it has been possible to identify mir-103-1 that has shown to pair up perfectly with 7 nucleotides of its sequence (seed nucleus) to the 3 'UTR of rat NCX1 with a release of free interaction energy equal to -28.5 kCal / mol by heteroduplex formation (Figure 6B). Thanks to these interaction data it has been supposed that miR-103-1 is a likely interactor of NCX1.

A

Results for the Target Gene: NCX1

MicroRNA	Host Probe	Host Gene Symbol	Target position
hsa-miR-1255a	202429_s_at	PPP3CA	145/1764
hsa-miR-1255a	202457_s_at	PPP3CA	145/1750
hsa-miR-548l	205395_s_at	MRE11A	235/3416
hsa-miR-576-3p	202798_at	SEC24B	136/2060
hsa-miR-576-5p	202798_at	SEC24B	136/1946
hsa-miR-620	212209_at	MED13L	90/1806
hsa-miR-628-3p	214152_at	CCPG1	102/1142
hsa-miR-103-1	218433_at	PANK3	735/2860

B

IBM Research

RNA22 microRNA target detection

Input *single* microRNA sequence (fasta format): Sample microRNA CLEAR

```
>hsa-miR-103a-3p MIMAT0000101
AGCAGCAUUGUACAGGGCUAUGA
```

Input *single* target sequence (fasta format): Sample target sequence CLEAR

```
>rn4_knownGene_X68813 range=chr6:4420108-4421221 5'pad=0 3'pad=0
strand=- repeatMasking=none
AGGAACAATCAAGATATAATAAATTTATATATATATGTATACATATATAT
ATACATAAAAATTATGTATAATGAACAGAGGAAACTGACATTTGTCATGT
TCACTTAACCTGCTgatggaatccagcttcaagaacgtactctgtactag
gccggaagtcagaaaccatcatctcccatcatcgagttgaacaaggcatg
gaggcggggccatcttgcagctcgtcctagaaagagttatattctctcca
agtgcataaatggttaaggcttttatttgttttcttgttttgttttgttt
```

Maximum number of allowed UN-paired bases 0 **in seed/nucleus of** 7 **nucleotides**
Minimum number of paired-up bases in heteroduplex: 14
Maximum folding energy for heteroduplex (Kcal/mol): -25

C

Figure 6. (A) A screen-shot of HOCTAR during research for putative interactors of NCX1. (B) IBM RNA 22 showing sequences (FASTA format) of mir-103-1 and RNA messenger of Slc8a1 (NCX1) before annealing. (C) Representation of 3' UTR region of NCX1 that interacts, by Watson-Crick rules, to miRNA.

5.2 TRANSIENT TRANSFECTION OF MIR-103-1 IN BHK-NCX1 CELLS DETERMINES A REDUCTION IN NCX1 EXPRESSION.

In order to verify whether NCX1 was actually a target for miRNA 103-1, BHK cells transfected with NCX1 were treated with increasing concentration of miRNA 103-1. These results showed that the transient transfection with miRNA 103-1 was able to induce a remarkable downregulation of NCX1 in BHK-NCX1 cells. In particular, a significant reduction in NCX1 expression was obtained by exposing cells to 10 nM of miRNA 103-1 (69,6 ± 7,8) and 100 nM (42,4 ± 3,1) compared to control (100 ± 1,9)(Figure 7A).This effect was also present at 48 hours of distance from transient transfection with 10nM (79,5 ± 2,2) and 100 nM (51 ± 1,7) miRNA 103-1 compared to control (100 ± 1,6)(Figure 7B). Interestingly, the effect of NCX1 downregulation mediated by miRNA 103-1 was not time dependent and was present at the two used concentrations of 10 nM and 100nM both at 24 and at 48 hours from transfection**.**

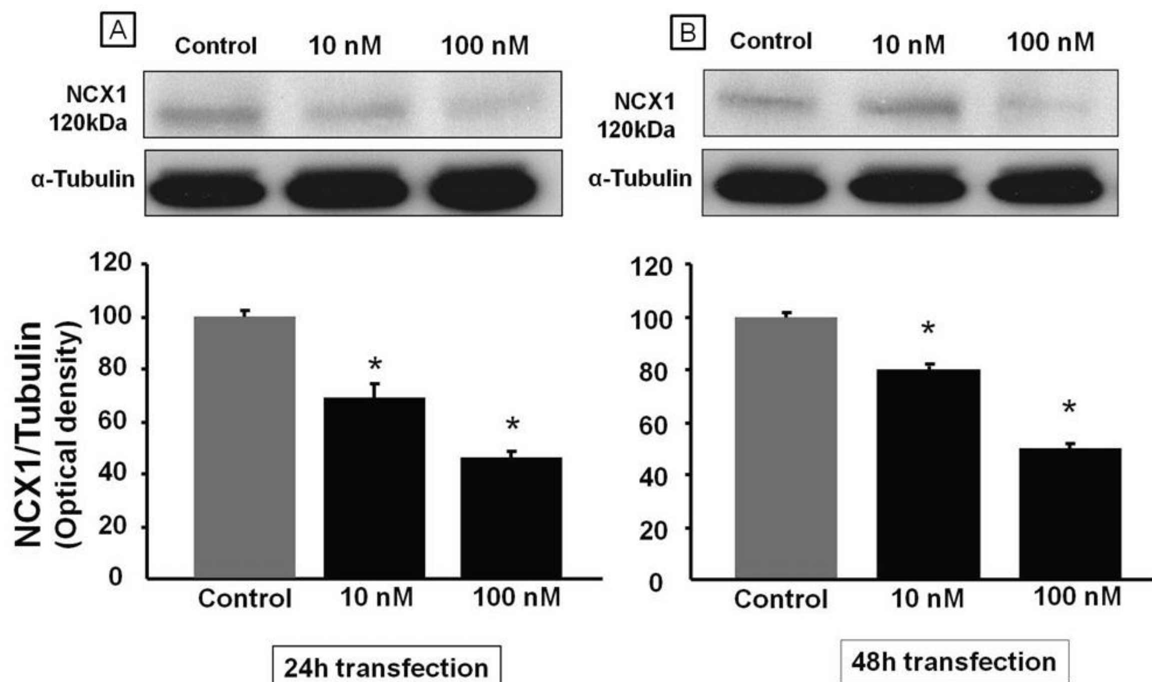


Figure 7. Representative Western blot of NCX1 protein levels and densitometric quantification after BHK-NCX1 cell transfection with mir-103-1. (A) MiRNA 103-1 mimic transfection in BHK cell clones determins a dose dependent modulation on NCX1 protein levels at 24h. (B) A parallel trend of downregulation of NCX1 protein at 48h after transfection. The given quantization of NCX1 is

expressed normalized with respect to α-tubulin. The experiment has been made in triplicates. The values are expressed as mean±SEM of 3 independent experimental sessions. *p<0.05 versus their respective controls.

5.3 MIR-103-1 CAUSES A DOSE AND TIME DEPENDENT REDUCTION IN NCX1 EXPRESSION IN PC12 CELLS.

In order to prove the species specificity, miRNA 103-1 was transfected in PC12 cells. Data indicated that increasing concentration of miRNA 103-1 reduced NCX1 expression levels at all time points considered. Notably, at 10 nM concentration there was no significant modulation on NCX1 both at 24 hours of transfection (97 ± 5,2) and at 48 hours (105 ± 8,3) compared to controls (100 ± 4 ,8 at 24h and 100 ± 3,5 at 48 h respectively) (Figure 8A) (Figure 8B). By increasing doses of miRNA-103-1 Mimic it was observed no significant modulation on NCX1 at 24h (104 ± 8,9) compared to control. Conversly 100nM at 48 hours downregulated significantly levels of NCX1 expression (59,5 ± 7,3); this effect was more impressive at 72h (12 ± 3,4) compared to control 72h (100 ± 11,5). To the highest dose of miRNA mimic tested, 150 nM, corresponded a stronger reduction in NCX1 expression levels both at 48h (51 ± 8,1) and at 72h (6,2 ± 5,3) compared to respective controls (Figure 8)

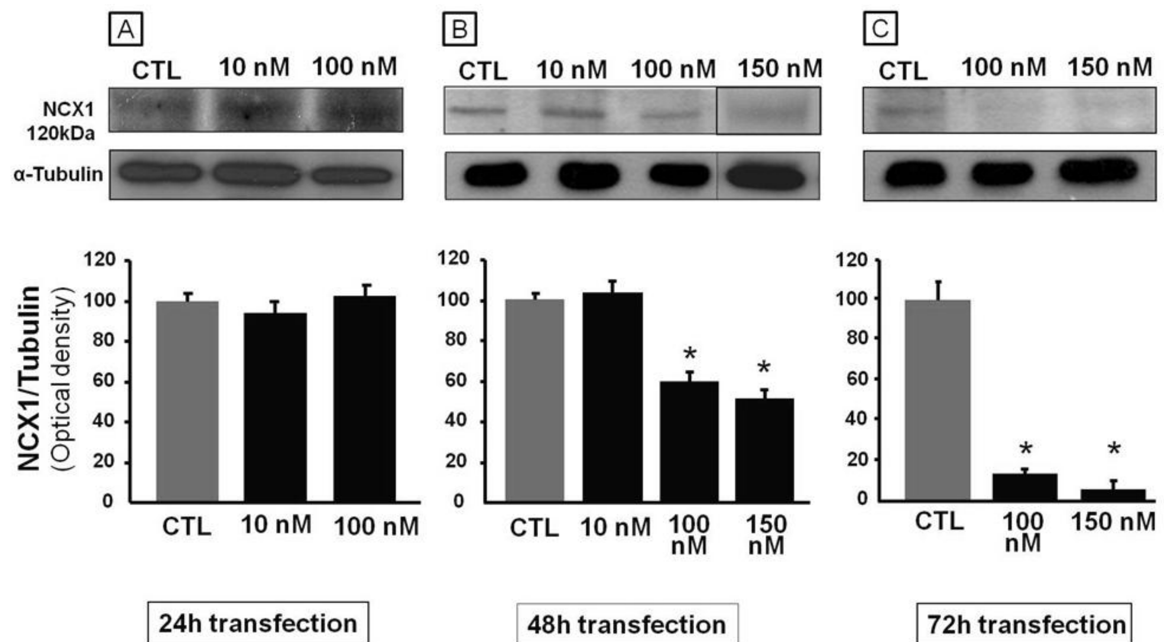


Figure 7. Representative Western blot of NCX1 protein levels in PC12 cell transfected with increasing doses of miRNA mimic 103-1 at several time-points. (A), (B) and (C) MiRNA mimic tested at several time from transient trasfection acts as a potent repressor of NCX1 protein expression in particular by 100nM concentration, 48h transfection. Each of the experimental groups has been made in triplicates. The values are expressed as mean±SEM of 3 independent experimental sessions. *p<0.05 versus control group; each column represents the mean ± S.E.M.

5.4 MiRNA mimic 103-1 reduces NCX1 expression when transiently transfected in embryonic rat neurons.

In order to verify whether miRNA 103-1 was able to induce NCX1 reduction also in neurons, cortical neurons were exposed to 150nM miRNA 103-1. Notably, at 72h from transient transfection, 150 nM miRNA Mimic 103-1 significantly downregulated NCX1 levels (50 ± 6,0) compared to neurons treated with vehicle alone (100 ± 9,8).

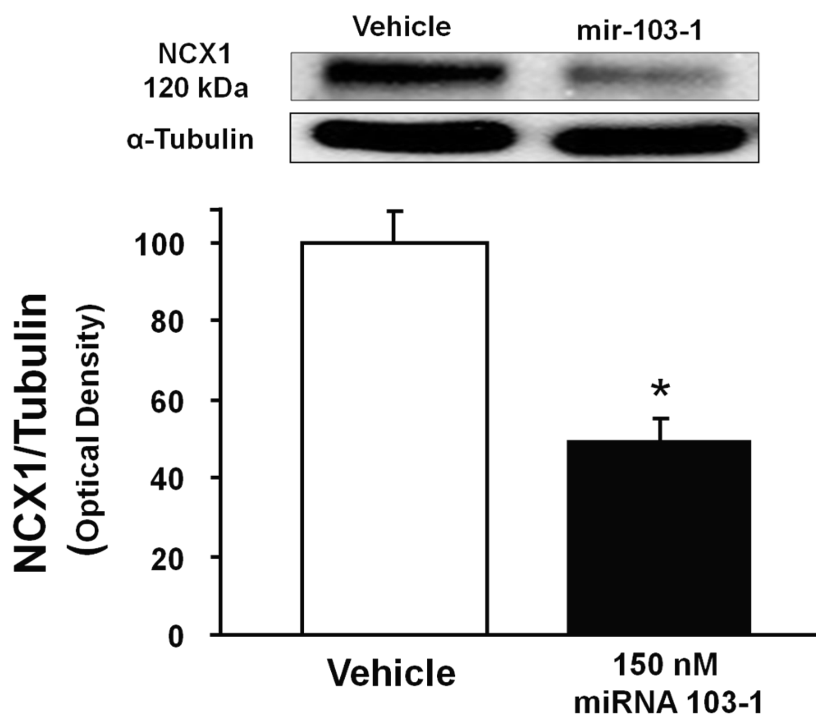


Figure 9. Western blot of NCX1 protein expression in cortical neurons transfected with 150 nM of miRNA mimic 103-1. Once identified the maximum dosage and the best time of transfection able to modulate significantly NCX1 in PC12 and BHK cells, this dosage downregulated significantly NCX1 also in neurons. NCX1 levels of expression have been normalized to α-tubulin. Each of the experimental groups has been made in triplicates. *p<0.05 versus vehicle group; each column represents the mean ± S.E.M.

5.5 CORRELATION BETWEEN MIR-103-1 LEVELS OF EXPRESSION OF AND CONCOMITANT LEVELS OF NCX1 PROTEIN EXPRESSION IN RAT BRAIN.

A time-course analisys of miRNA 103-1 levels and NCX1 expression after stroke revealed that miR-103-1 inversely correlates with NCX1. Indeed, in rat 6h after transient ischemia endogenous levels of mir-103-1 both in cerebral cortex (1,23 ± 0,33) and in striatum (1,14 ± 0,22) were similar to those observed in cortex (1 ± 0,14) and in striatum (1 ± 0,156) of sham-operated animals (Figure 10A) . At 24h mir-103-1 expression levels were dramatically increased both in cerebral cortex (2,8 ± 0,22) and in striatum (2,97 ± 0,47). Finally at 72 hours, levels of endogenous mir-103-1 fell down both in cortex samples (0,14 ± 0,32) and in striatum (0,7 ± 0,59) compared to

control levels. For the three time points considered (6, 24 and 72h), NCX1 protein levels of expression significantly decreased both in cerebral cortex (80,2 ± 5,8, 67,8 ± 5,4 and 72,8 ±1,8 respectively at 6, 24 and 72h) and in cerebral striatum (76,2 ± 0,2, 64,7 ± 6,8 and 68,9 ± 7,6 respectively at 6, 24 and 72h from tMCAO) compared to control sham-operated levels observed in cerebral cortex (100 ± 3,1) and striatum (100 ± 6,1). (Figure 10 B).

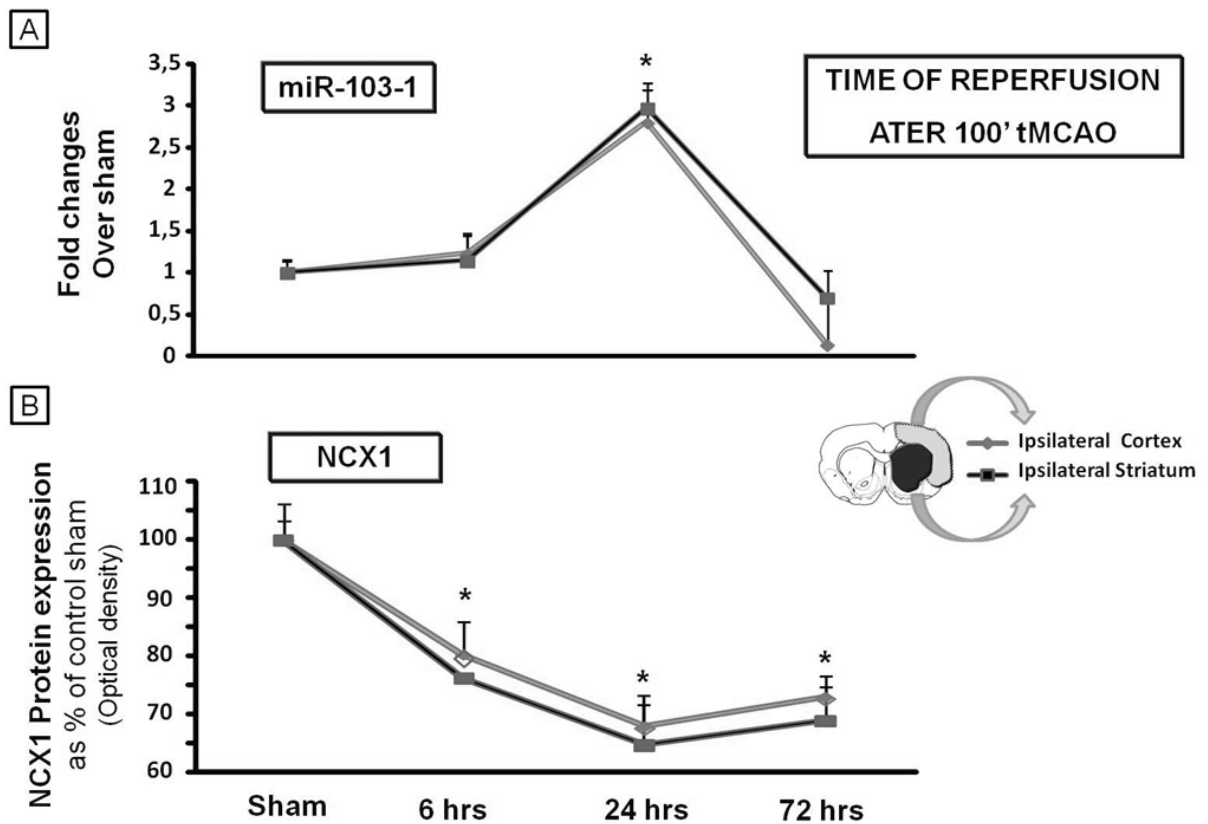


Figure 10. Inverse correlation between the levels of expression of mir-103-1 and the protein NCX1 in samples from rat cerebral ischemic cortex and striatum dissected from animals at various time-points. (A) Time-course of levels of expression of miRNA 103-1 in cerebral cortex and brain striatum samples dissected from rats subjected to 100 'tMCAO and subsequently sacrificed at 6, 24 and 72 hours. Results are expressed as fold changes of expression of miRNA 103-1 compared to sham. (B) Time course of level of protein NCX1. Expression levels have been normalized to α-tubulin and compared to sham. On the right side is depicted a representation of the brain area in which the analysis has been carried out. n=3 animals per group. *$p<0.05$ versus sham operated group in both expression profiles. Each point of the graphic line represents the mean ± S.E.M.

5.6 INTRACEREBROVENTRICULAR INFUSION OF ANTIMIRNA 103-1 REVERTS ISCHEMIA-INDUCED NCX1 DOWNREGULATION.

In order to prove whether AntimiRNA 103-1 upregulated NCX1 in brain ischemic area, an experiment in which ischemic rats were continuously injected with AntimiRNA was carried out. This experiment revealed that 20 μM AntimiRNA 103-1 did not affect NCX1 expression levels (102 ± 18) compared to levels of sham-operated animals (100 ± 9,9). More interestingly, 60 uM AntimiRNA caused a strong elevation in NCX1 protein levels (124 ± 9,5) compared both to ischemic animals treated with Negative Control miRNA (68 ± 5,3) and to sham.

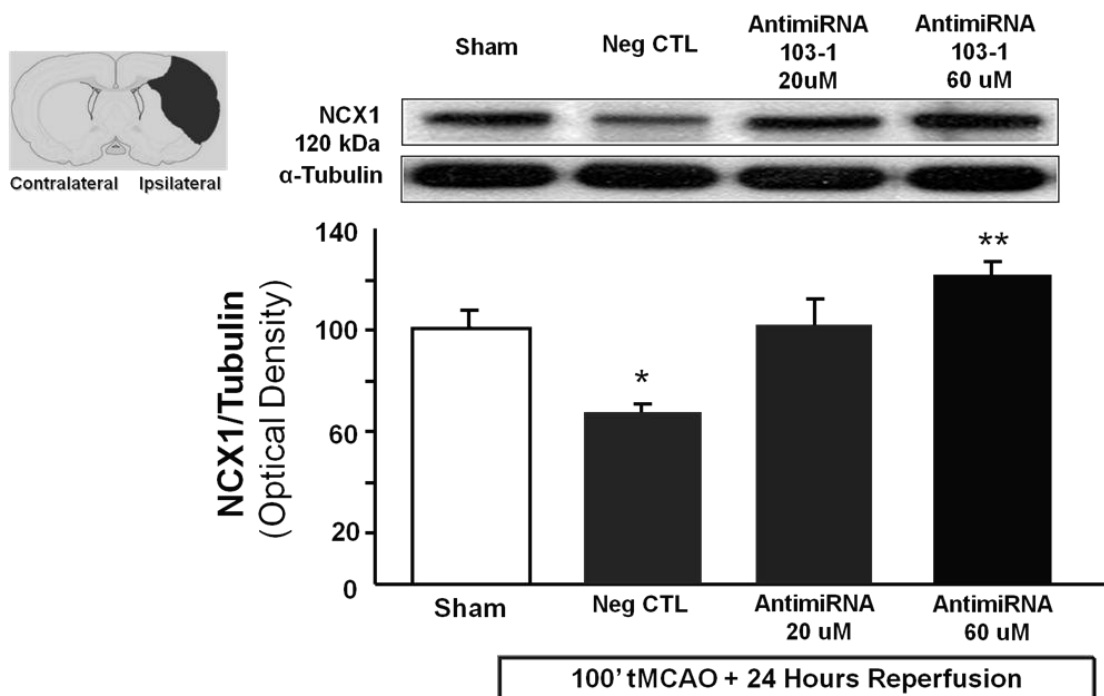


Figure 11. Quantitative analysis of NCX1 protein expression levels from ipsilateral damaged brain area of rats subjected to tMCAO and treated respectively with Negative control, AntimiRNA 20 uM and AntimiRNA 60 uM. n=5 animals per group. Data were normalized on the basis of α-tubulin levels and expressed as percentage of sham-operated controls (CTL). *p<0.05 versus sham operated group; **p<0.05 vs Negative Control group and vs. sham operated group group. Each column represents the mean ± S.E.M.

5.7 ANTIMIRNA 103-1 PREVENTS ISCHEMIA-INDUCED REDUCTION IN NCX1 PROTEIN BY PRESERVING NCX1 GENE TRANSCRIPTS.

AntimiRNA-103-1 increased RNA messenger levels of NCX1 targeting endogenous elevation of mir-103-1. In presence of Negative Control NCX1 transcript levels were highly degraded in cerebral cortex of ischemic rats (45 ± 8,9) compared to sham-operated animals (100 ± 5,4) while AntimiRNA caused an increase of NCX1 mRNA (143 ± 12,1). In the striatum AntimiRNA preserved NCX1 mRNA levels from endogenous miRNA elevation (156 ± 19,4) compared to sham-operated animals (100 ± 12,8) and Negative Control treated ischemic rats (36 ± 15,1).

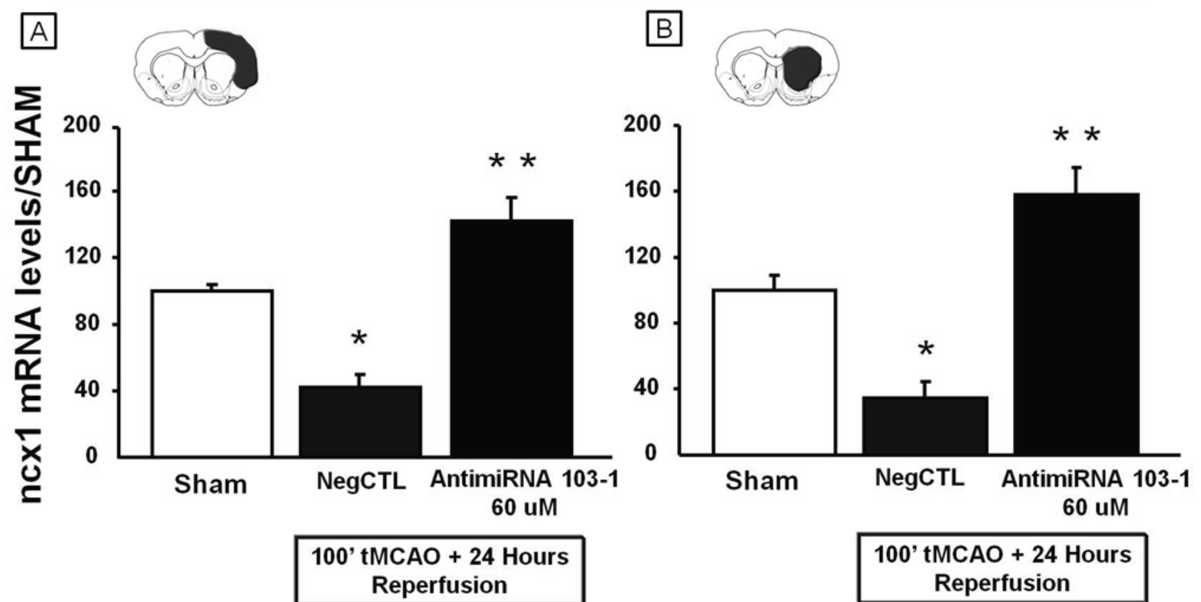
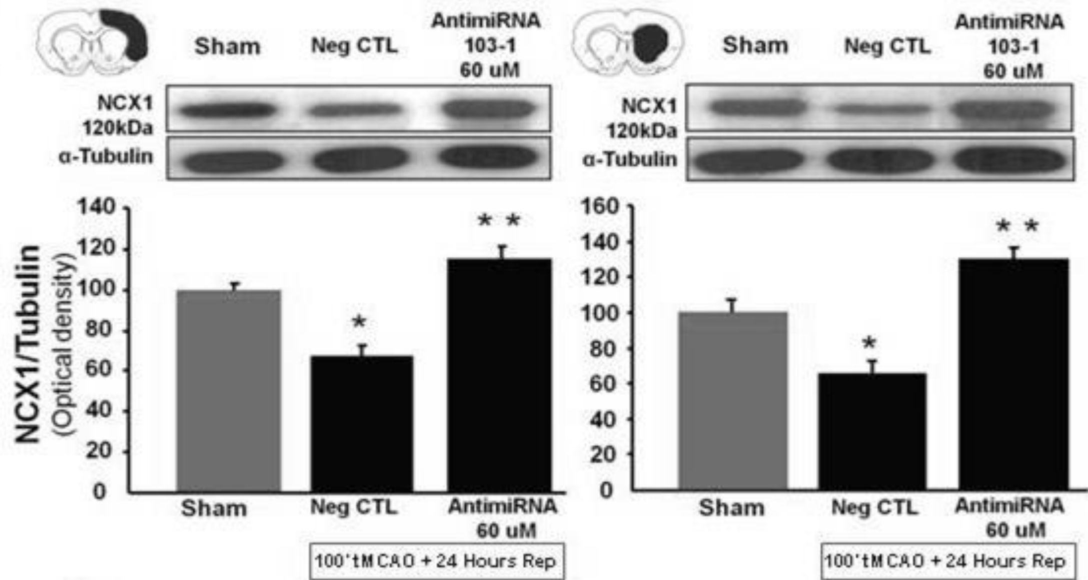
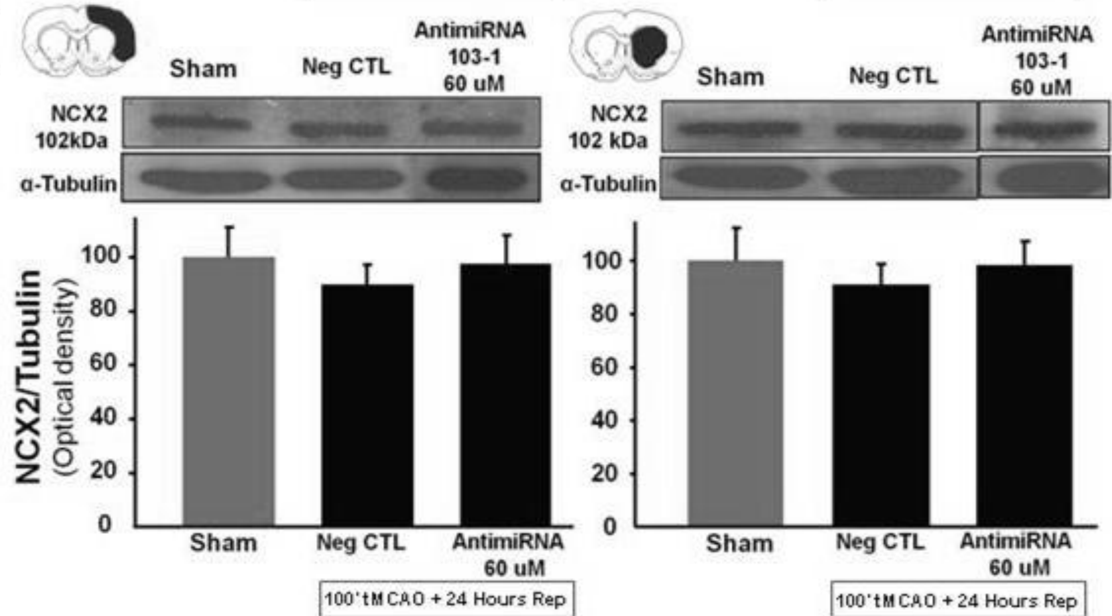


Figure 12. Changes in expression levels of ncx1 mRNA in ischemic brain cortex (panel A) and striatum (panel B) of animals sham-operated (Sham), subjected to tMCAO and treated with Negative Control (NegCTL) and subjected to tMCAo and treated with AntimiRNA (AntimiRNA 103-1 60 μM). *p<0.05 vs. sham-operated animals. mRNA levels are expressed as percentage of sham-operated controls (CTL). **p<0.05 vs both Negative Control groups and Sham-operated groups. n=3 animals per group. Each column represents the mean ± S.E.M.

5.8 AntimiRNA 103-1 significantly and selectively upregulates NCX1 protein after ischemia both in cortex and in striatum of rat brain.

In order to test the specificity of AntimiRNA 103-1 for NCX1 the possible effects of AntimiRNA treatment on other two brain isoforms of NCX, NCX2 and NCX3, was evaluated. AntimiRNA 103-1 induced an upregulation of NCX1 both in cerebral cortex (114,2 ± 6,2) and in striatum (130,4 ± 6,2) in rats subjected to ischemic stroke compared to Negative Control ischemic rats (67,6 ± 5,8 and 65,3 ± 5,2 respectively in cerebral cortex and in striatum) and to sham-operated animals (100 ± 3,1 and 100 ± 6,1 respectively in cerebral cortex and in striatum) (Figure11 A). AntimiRNA 103-1 administrated in ischemic rats did not regulate NCX2 protein expression (98,6 ± 11,5 and 97,8 ± 10,8 respectively in cerebral cortex and in striatum) compared to Negative control ischemic rats (91,22 ± 8,5 and 90,7 ± 8,8 respectively in cerebral cortex and striatum) and to sham-operated rats (100 ± 12, 3 and 100 ± 12,8 respectively in cerebral cortex and in striatum). Similarly to NCX2, AntimiRNA 103-1 administrated in ischemic rats did not regulate significantly NCX3 protein expression (94,5 ± 18,1 and 75,3 ± 4,8 respectively in cerebral cortex and in striatum) compared to Negative control ischemic rats (74,1 ± 6,9 and 69,5 ± 5,8 respectively in cerebral cortex and striatum) and to sham-operated rats (100 ± 3,9 and 100 ± 4,5 respectively in cerebral cortex and in striatum).


A
Sham
Neg CTL
AntimiRNA 103-1 60 uM
NCX1 120kDa
α-Tubulin
NCX1/Tubulin (Optical density)
Sham
Neg CTL
AntimiRNA 60 uM
100'tMCAO + 24 Hours Rep


B
Sham
Neg CTL
AntimiRNA 103-1 60 uM
NCX2 102kDa
α-Tubulin
NCX2/Tubulin (Optical density)
Sham
Neg CTL
AntimiRNA 60 uM
100'tMCAO + 24 Hours Rep

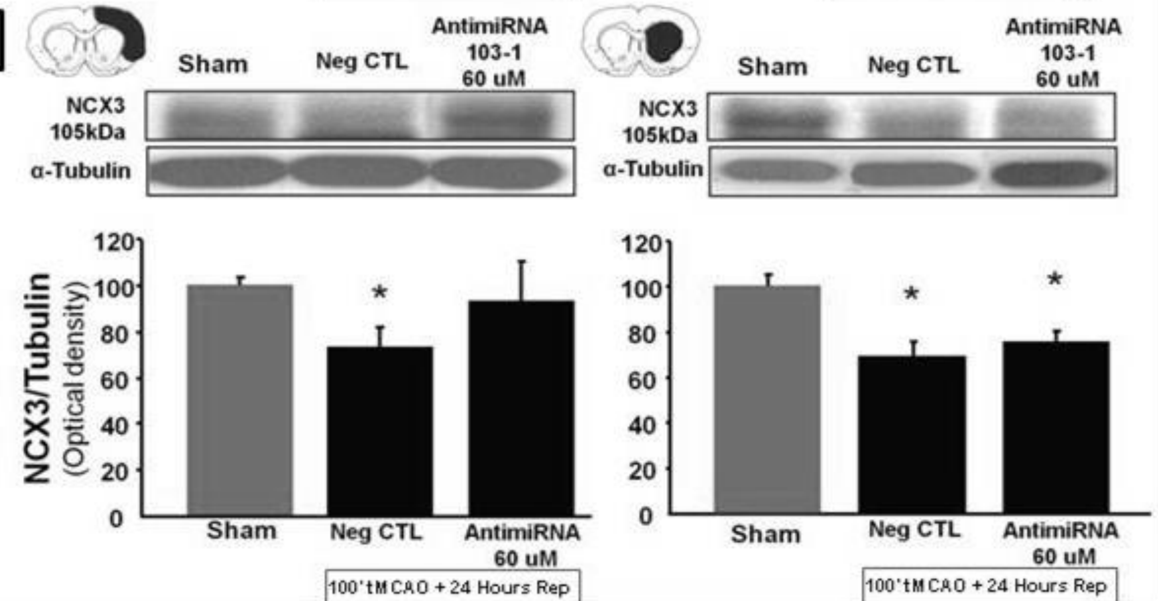

C
Sham
Neg CTL
AntimiRNA 103-1 60 uM
NCX3 105kDa
α-Tubulin
NCX3/Tubulin (Optical density)
Sham
Neg CTL
AntimiRNA 60 uM
100'tMCAO + 24 Hours Rep

Figure 13. Isoform-specificity of drug treatment based on antimiRNA 103-1 evaluated by western blotting at 24 hours of reperfusion after ischemic event. (A), (B) and (C), expression levels of three different isoforms of NCX in brain cortex and striatum at 24h after ischemia. n=3 animals per group. All left panels are referred to NCX expression in the cortex, while right panels are referred to NCX expression in striatum. *p<0.05 versus sham operated group; **p<0.05 vs sham operated group for each isoform. Each column represents the mean ± S.E.M. Data were normalized on the basis of α-tubulin levels and expressed as percentage of sham-operated controls (CTL).

5.9 ANTIMIRNA 103-1 IS ABLE TO EXERT A STRONG NEUROPROTECTIVE EFFECT ON ISCHEMIC DAMAGE.

In order to demonstrate whether AntimiRNA 103-1 was able to induce a reduction in the ischemic volume, it was administered in rats subjected to ischemia. Results show that AntimiRNA 103-1 reduced the extent of brain ischemia by ~ 60% (19 ± 3,4) compared to rats subjected to 100 min of tMCAO alone treated with vehicle (51 ± 6,3) or with negative control (59,5 ± 7,9).

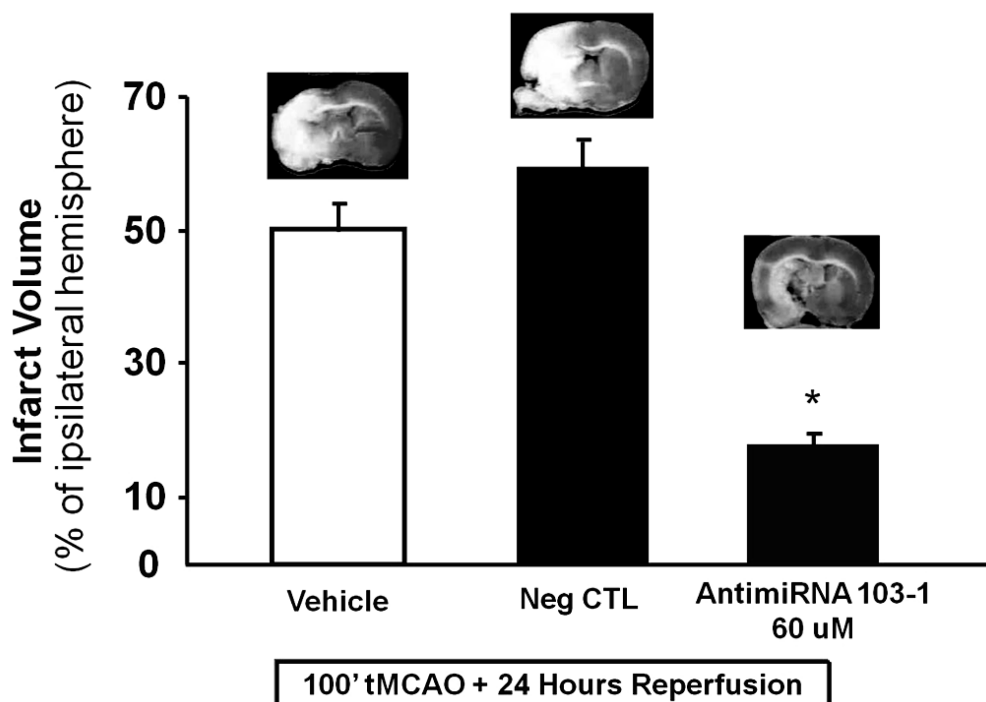


Figure 14. Quantification of infarct volume after 100 minutes of MCAO and administration of AntimiRNA 103-1. *p<0.05 versus 100 minutes of MCAO (vehicle) and vs. ischemic rats treated with Negative Control. n=5-6 animals per group. Each column represents the mean ± S.E.M.

5.10 ANTIMIRNA 103-1 TREATMENT IMPROVES GENERAL AND FOCAL DEFICITS

To verify whether the reduction in the infarct volume observed after AntimiRNA administration was accompanied by an amelioration in the neurological deficits, the animals were scored for general and focal deficits immediately before they are killed. Obtained results demonstrated that antimiRNA is able to induce a dramatic reduction in the neurological scores when evaluated 24 hours later (Figure 14).

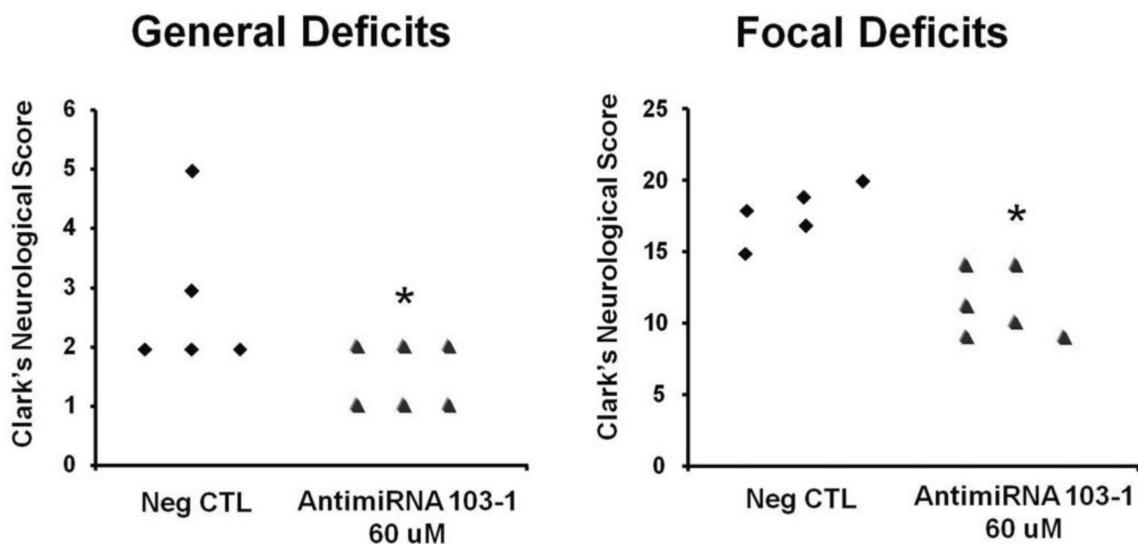


Figure 15. Performance of general and focal neurological deficits after AntimiRNA drug treatment in ischemic rats. Effect of AntimiRNA 60 uM on general and focal scores when rats were euthanized 24 hours after tMCAO plus Negative control or tMCAo plus AntimiRNA.*p<0.05 versus ischemic rats treated with Negative Control. *p<0.05 versus rats subjected to 100 minutes of MCAO and treated with Negative Control.

6. DISCUSSION

The present study demonstrates that AntimiRNA 103-1, a locked nucleic acid able to block endogenous mir-103-1 is able to enhance NCX1 expression thus exerting a remarkable neuroprotective effect in stroke. This action translates in a marked improvement of neurological conditions of animals which have received an ischemic insult. It is notable that AntimiRNA displayed a potent upregulation effect on NCX1 24h following brain ischemia, counteracting brain damage caused by stroke when it reaches the maximum stage of temporal evolution. By constrast, the neuroprotective effect exerted by AntimiRNA 103-1 due to its action on NCX1 expression, is demonstrated by the fact that it has no effect on the expression of the other two brain isoforms of NCX, NCX2 and NCX3. A possible explanation of the neuroprotective effect exerted by AntimiRNA 103-1 derives from the fact that the specific knocking down or knocking out of the three NCX isoforms worsens ischemic brain damage in *in vitro* and *in vivo* models of cerebral ischemia (Pignataro et al., 2004a; Jeon et al., 2008; Molinaro et al., 2008) whereas its activation reduces infarct volume (Pignataro et al., 2004a; Molinaro et al., 2008). Indeed, the stimulation of the antiporter, by modifying the dysregulation of intracellular Na^+ and Ca^{2+} ion homeostasis, could help the rescue of injured neurons in the ischemic and peri-ischemic areas of the brain. However, to date, only non selective NCX activators have been reported to stimulate NCX activity, including lithium (Iwamoto et al., 1999), redox agents (Reeves et al., 1986; Secondo et al., 2011), agonists of G-protein-coupled receptors (Stengl et al., 1998; Eriksson et al., 2001; Woo and Morad, 2001; Annunziato et al., 2004), diethylpyrocarbonate (Ottolia et al., 2002), concanavalin A, nerve growth factor, and insulin (Gupta et al., 1986; Makino et al., 1988; Formisano et al., 2008). The

identification of microRNA able to regulate sodium/calcium exchanger isoform 1 at brain level paves the road for a future therapeutic treatment in stroke and sheds light on an important molecular mechanism of protein regulation. Interestingly, by a time course analysis of endogenous miRNA 103-1 after ischemia a marked increase occurs at 24h while corresponding levels of protein NCX1 targeted remain elevated both at 24h and at 72h from ischemia. These findings might corroborate the hypothesis that an early elevation of miRNA 103-1 levels (24 hours) needs a complete turnover of NCX1 bound RNA messenger in order to show its modulatory effects. The use of miRNAs as potential therapeutic targets remains more controversial with regard to methods of delivery and target specificity. Several siRNA delivery systems for *in vivo* purposes are currently being developed, including vector-based, chemically modified and ‘packaged’ RNA oligonucleotides (Kim et al., 2007) and progress in the latter area will immediately translate into progress in the miRNA area because both are based on the same principles. They both operate at the post-transcriptional level and miRNAs and siRNAs are chemically identical. With the present work we have demonstrated, following the path already traced by Dharap et al. in a work appeared in 2009 on Journal of Cerebral Blood Flow & Metabolism, that is possible by using specific designed miRNA Inhibitors, such as Locked Nucleic Acids or Antagomirs filled into osmotic minipumps for continuous infusion in cerebral ventricle, to overcome problem of delivering *in vivo* such “packaged” small RNA modulators. However the big question is whether these different approaches will crystallize in clinically feasible therapies because bioavailability and toxicity issues are inherent to all these approaches and the blood–brain barrier constitutes an enormous hurdle for the effective delivery of these experimental drugs in the brain (Hebért et al., 2008). miR-103 and miR-107 are intronic miRNAs contained in three PANK (Pantothenate kinase) loci of the human genome, in particular for what

concerns mir-103-1 is contained into PANK3 (i.e., PANK1, 2, and 3 correspond to pri-miR-107, pri-miR-103-2, and pri-miR-103-1, respectively). Recently, Martello et al. indicated that miR-103/107 family is able to target Dicer, a key component of the miRNA processing machinery. Furthermore, in human breast cancer, high levels of miR-103/107 have been associated with metastasis and poor outcome. Therefore, mir-103 in human malignancies by reducing the expression of Dicer, causes a global reduction of miRNA abundance in cell cytoplasm, thus playing a causal role in the transformed phenotype (Kumar et al., 2007; Lu et al., 2005; Ozen et al., 2008; Martello et al. 2010). Conversely, in another recent paper by Harraz and collegues, it has been demonstrated that overexpression of a specific miRNA, miR-223, lowers the levels of GluR2 and NR2B by targeting their 3′-UTR target sites (TSs) thus inducing an inhibition of NMDA-induced calcium influx in hippocampal neurons (Harraz et al., 2008). This effect protects the brain from neuronal cell death following transient global ischemia and excitotoxic injury (Harraz et al., 2008). The conclusion of the authors is that the blockade of this miRNA pathway in a model of global ischemia might be deleterious. In the light of the above and according to the recent findings by Martello et al., our working hypothesis is that an elevation of endogenous levels of miRNA 103-1 might occur also after brain damage, thus causing a general reduction of miRNA global aboundance. This effect might be deleterious in the course of brain ischemic damage evolution since it might cause an increased expression of those proteins, such as specific subunits of glutamate receptors, normally downregulated, by those miRNAs that are no more synthesized, this effect precipitating brain damage caused by glutamatergic excitotoxicity. Furthermore, there are other evidences for neurobeneficial effect of downregulated miRNA 103-1 levels on stroke outcome (Lee et al., 2010). These researchers demonstrated that in normoxic conditions elevated levels of miR-200 family by binding 3’UTR of PHDs

hydroxylate (f prolyl hydroxylase 2) determine hydroxylation of HIF-1α allowing its proteosomal degradation. HIF-1α is a well established transcription factor that is rapidly induced by hypoxia and accounts for the transcription of 89% of upregulated and 17% of downregulated genes during hypoxia and ischemia (Lee et al., 2010). Since blocking mir-103-1 action (AntimiRNA) causes miRNA global increased levels in neuroblast cells, this effect may reduce activity of PHD (targeted at its 3'UTR by mir-200) and consequently the HiF-α degradation mechanism is turned off. Resulting increased levels of HIF-α might be responsible for protective induced upregulation of those neuroprotective genes regulated at a trascriprional level by this transcriptional factor (Greijer et al., 2005).

7. FUTURE DIRECTIONS

Starting from the evidence that different patterns of miRNA expression occur in brain and blood 24h after brain ischemia, brain hemorrhage, kainate seizures, compared with sham operated animals (Da-Zhi et al., 2010), our purpose would be to analyze specific miRNAs modulated in our model of brain ischemia. In that scenario a pioneristic work by Liu and collegues has been conducted in order to compare miRNA levels found in blood samples obtained from rat tail vein after middle cerebal arthery occlusion to those present in hippocampus samples of rats subjected to the same experimental procedure. Results have shown that a great number of miRNAs were significantly regulated ($P < 0.05$) more than 1.5-fold in brain and blood after each brain injury. Several miRNAs were upregulated or downregulated in both brain and blood after the injury; and a few miRNAs, including miR-298, mir-155, mir-362-3p, etc., were upregulated or downregulated in both brain and blood after several different injuries. These results pave the road for the possible use of blood miRNAs as biomarkers for selected brain miRNAs after one or more specific types of brain injuries. They also provide a partial mechanism for specific miRNA induced mRNA expression profiles in brain and blood after different types of brain injury.

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