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Circadium rhythm effects remote ischemic postconditioning neuroprotection

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Despite the decreasing numbers in stroke risk and post-stroke disability, many neuroprotectants have failed in clinical stroke trials, and new therapies for both acute and chronic stroke are still needed. Translational failures in neuroprotection might have different reasons linked to differences between animal models and humans, i.e., comorbidities, age and sex, human genetic variations, the ratio of white matter to gray matter, etc. The time of stroke onset might also need to be taken into consideration. Circadian "circa diem" rhythms are daily cycles of physical and behavioral changes regulated by a highly phylogenetically conserved system. In the context of stroke, there may be a circadian pattern, e.g., strokes in patients occur mostly in the morning (8-10 AM, Zeitgeber ZT1-3) corresponding to ZT13-15 in mice. Stroke clinical trials of neuroprotection mostly recruit patients in the daytime, which corresponds to human's "awake" active phase. However, rodent experiments on cerebral ischemia are also performed during the daytime, which corresponds to their "sleep" and inactive phase, being that rodents are nocturnal animals. This difference creates a "circadian mismatch" between animal models and clinical trials of neuroprotection that might be one of many reasons for failure in translation to clinical trials.

Remote ischemic postconditioning is an endogenous neuroprotective mechanism where a short occlusion of a distant artery can protect the brain from a previously harmful ischemic insult in animal models, mostly rodents. We and others have shown that one of the mechanisms involved in neuroprotection might be linked to an increase in neuronal nitric oxide synthase (nNOS). Even though remote postconditioning represents a clinically translatable, feasible, and, most importantly, safe approach to treating cerebral ischemia, clinical trials have failed. Here, we asked whether opposite circadian cycles in nocturnal rodents versus diurnal humans may contribute to this failure in translation. Mice were housed in normal or reversed light cycle rooms for three weeks, and then they were blindly subjected to transient focal cerebral ischemia or remote conditioning. Twenty-four hrs post-ischemia triphenyl tetrazolium chloride staining revealed that remote postconditioning reduced infarct volumes in mice during the inactive (ZT3-9) but not active phase (ZT15-21). Western blot analysis showed a reduction in Per 2, one of the main genes involved in circadian regulation, in the remote postconditioning group compared to controls, during the inactive (ZT3-9) but not active phase (ZT15-21). On the other hand, nNOS increased after remote conditioning when stroke onset was at ZT3-9 but not at ZT15-21.

This proof-of-concept study indicates that in order to move forward, remote postconditioning mechanisms and targets should be re-assessed in rodent models within the appropriate circadian context. Understanding how circadian rhythm affects remote postconditioning would help define targets, find biomarkers, and potential therapy for stroke.

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Biomarkers for preconditioning white matter

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Most in vivo rodent models of ischemic preconditioning (IPC) consist of short episodes of hypoxia-ischemia that are not clinically applicable. Thus, a pharmacologically induced IPC would be an ideal therapy to apply to stroke patients. Furthermore, many IPC studies have been primarily focused on gray matter despite white matter (WM) injury contributing to clinical deficits. Increasing evidence supports the contribution of miRNAs to ischemic tolerance induced by preconditioning. We showed that CK2 inhibition with CX-4945 promotes recovery in WM by acting on Cdk5 and AKT/GSK3β pathways. Therefore, we investigated whether CX-4945 preconditions WM and whether preconditioning is mediated by alterations of miRNAs in WM and human serum samples from control and stroke patients.

Mouse optic nerves (MONs) were obtained from adult and aging male and female Thy1-CFP^{+} mice, and action potentials were evoked and recorded under baseline, ischemia, and recovery periods. CX-4945 (5μM) was applied for 30 minutes before ischemia. MONs were collected for miRNA profiling

using qPCR. CK2 inhibition using CX-4945 effectively preconditioned axon function in young and aging male and female MONs. Roscovitine (5μM) inhibition of Cdk5 equally preconditioned axon function in all age groups and sexes. Interestingly, AKT/GSK3β inhibition with ARQ-092 (500nM) selectively preconditioned axon function in young males but not young females. We identified miRs 375, 489, 200-a, 494 and 501-3p as significantly upregulated after ischemia in MONs and stroke patients' plasma, while CX-4945 attenuated miR-501-3p levels.

We are investigating how the changes in miRNA profiles underlie the effects of CX-4945 preconditioning of WM.

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Cytoprotection of nanoparticles-based formulations

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Despite recent progress in the treatment of several cardiovascular diseases (CVDs), new therapeutic approaches would be of great interest. Recently, nanoparticle-based drug delivery has gained significant popularity as an approach to improve the therapeutic potential of drugs. In fact, nanoparticles and nanocarriers- based therapies have emerged as essential tools for many fields of modern medicine, in order to optimize the targeted delivery of drugs in a controlled manner and to track the fate of the cells. Specifically, we tested different nanoparticles- based formulations containing xenon, olive oil or hydroxytyrosol (HT) for their capability to modulate inflammation and oxidative stress and to reduce cell mortality during hypoxia and reoxygenation (H/R) protocols. To test the effectiveness of these formulations, we used H9c2, a cardiomyoblast cell line derived from rat heart, and HMEC-1, a human microvascular endothelial cell line, exposed to normoxia (5% CO_2 and 21% O_2) or hypoxia (5% CO_2 and 95%N_2) in a hypoxic chamber. The cellular mortality was measured with MTT assay and the ROS production was detected with DCFDA kit assay. In addition, the level of several antiinflammatory markers was evaluated through different ELISA kit. Preliminary results showed a significant reduction of ROS production when cells were treated with HT or the other different formulations, highlighting the antioxidant role of HT and olive oil. Further studies are required to ascertain the protective potential of these compounds on cardiac I/R injury under in vivo conditions.

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Exploring the role of remote ischemic postconditioning in modulating gylmphatic system functions

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Acute ischemic stroke is a leading cause of morbidity and mortality worldwide, highlighting the urgent need for neuroprotection strategies to prevent irreversible brain damage.The "neuroprevention" aims to counteract the cascade of detrimental biochemical and molecular events leading to irreversible ischemic brain injury. Remote ischemic postconditioning (RIPC), a non-invasive therapeutic technique, has emerged as a potential endogenous neuroprotective mechanism, offering alternative pharmacological interventions for treating a broad range of acute brain pathologies. The glymphatic system (GS), a recently discovered macroscopic brain waste clearance system, has gained attention for its role in neurodegenerative diseases. The GS facilitates and supports the exchange of cerebrospinal fluid (CSF) and interstitial solutes throughout the brain, aided by aquaporin-4 (AQP4) water channels, mainly expressed in astrocyte end-feet. Glymphatic dysfunction has been observed in several neurological disorders, such as stroke, Alzheimer's disease, traumatic brain injuries, and type 2 diabetes, where brain clearance and impairment of CSF influx and ISF efflux emerge. Here, we asked whether remote ischemic postconditioning may play a neuroprotective role by improving glymphatic function after stroke injury.

Male mice were blindly subjected to 1) sham/ctr, 2) transient middle cerebral artery occlusion (tMCAo), or 3) femoral artery occlusion (FAO) in the hind limb to induce RIPC. Mice were sacrificed after 24 hours of reperfusion, and brain tissues were collected for biochemical analysis (e.g., TTC staining, western blot, immunohistochemistry). To evaluate glymphatic clearance, fluorescent tracers were injected into the cisterna magna, and in vivo 2-photon microscopy and ex-vivo analysis were performed. The data from this study support the hypothesis that FAO might provide a protective effect against brain damage caused by ischemia and could potentially be involved in the modulation of glymphatic system functions. The observed increase in AQP4 expression at the basal lamina around blood vessels in FAO-treated mice suggests that AQP4 polarization may contribute to the neuroprotective effects of FAO. AQP4, which is involved in water transport and astrocytic function, could ameliorate the brain clearance compromised after ischemic injury, resulting in a smaller injury. Overall, these results suggest that FAO offers significant neuroprotective benefits, reducing brain damage after an ischemic event. However, further research is needed to understand the detailed pathways involved and to translate these findings into potential clinical therapies for stroke patients.

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Tumor necrosis factor-stimulated gene-6 (TSG6) is implicated in neuroprotection exerted by cerebral ischemic preconditioning in mice subjected to transient middle cerebral artery occlusion

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Ischemic preconditioning (IPC) induced by a sub-lethal cerebral insult triggers brain tolerance against a subsequent severe injury through diverse mechanisms, including the modulation of the immune system. The hyaluronate (HA)-binding protein tumor necrosis factor (TNF)-α-stimulated gene 6 (TSG6) has recently been involved in the regulation of the neuroimmune response following ischemic stroke. Here, we evaluated whether the neuroprotective effects of IPC involve modulation of TSG6 in mice subjected to transient middle cerebral artery occlusion (MCAo).

Protein TSG6 expression was significantly elevated in the ischemic cortex of mice subjected to 1h MCAo followed by 6 to 48h of reperfusion, reaching a peak at 24h reperfusion (2.7-fold elevation vs SHAM-operated mice). The latter effect was potentiated by pre-exposure (i.e., 72h before) to IPC triggered by 15-min MCAo. By immunofluorescence analysis, we detected TSG6 expression mainly in astrocytes and myeloid cells populating the lesioned cerebral cortex, with a more intense signal in tissue from mice pre-exposed to IPC. By contrast, plasma TSG6 levels were significantly reduced after 24 h of reperfusion when compared to SHAM, which was

coincident with an upregulation of its corresponding miRNAs: miR-23a and miR-23b (1.6 and 1.4-fold elevation, respectively). Elevation of circulating levels of TSG6 by intravenous injection upon reperfusion of 30 μg of the recombinant mouse protein or by IPC resulted in significant neuroprotection, namely reduced brain infarct volume and neurological deficits.

In conclusion, our data demonstrate that neuroprotection exerted by IPC is associated with elevation of both central and peripheral TSG6 protein levels, further underscoring the beneficial effects of this endogenous immunomodulatory protein.

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Ischemic tolerance induced by time-restricted feeding is modulated by circadian rhythm

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Dietary control by either caloric restriction or time-restricted feeding (TRF) is known to promote better health and, importantly, resistance to metabolic and CNS diseases. We and others showed that TRF induces significant tolerance to stroke-induced brain damage in adult rodents by countering inflammation, oxidative stress, and neuronal death. Recent studies showed that the timing of an intervention is critical in deciding the efficacy of a stroke therapy. Hence, we investigated the influence of circadian timing of feeding/fasting in promoting ischemic tolerance. Cohorts of adult male and female C57BL/6 mice were subjected to TRF by fasting for 16 h/day aligned with either active phase (ATRF; fasting from ZT12 to ZT4) or inactive phase (ITRF; fasting from ZT0 to ZT16) compared against a control group fed ad libitum (AL). After 6 weeks of feeding regimens, focal ischemia was induced by intraluminal transient middle cerebral artery occlusion (MCAO) for one hour under isoflurane anesthesia. Sham-operated mice served as a control. Both ITRF/MCAO and ATRF/MCAO groups had better survival rates compared to the AL/MCAO group at 30 days of reperfusion. ATRF/MCAO, but not ITRF/MCAO cohorts, showed significantly better motor function recovery (rotarod and beam walk tests) than the AL/MCAO cohort between 3 and 14 days of reperfusion, compared with sex-matched AL cohorts. ATRF/MCAO cohorts also showed a significant increase in time spent in the platform quadrant on day 23 of reperfusion, indicative of better cognition compared to sex-matched AL/MCAO cohorts. ATRF, but not ITRF, reduced infarction (on day 10) and grey and white matter damage (on day 30) of reperfusion in both sexes compared with AL. ATRF also promoted the survival of dendritic spines, increased the expression of transcripts related to synaptic function, and PSD95-dependent structural plasticity after stroke compared with ITRF or AL cohorts. These findings indicate that the time of daily fasting is an important factor in inducing ischemic tolerance by TRF.

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Siah2-E3 ligase has a role in IPC-induced neuroprotection by regulating mitophagy and mitochondrial biogenesis

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Mitochondria are highly dynamic organelles able to adapt to cellular stress conditions; therefore, it is extremely important to guarantee the maintenance of a healthy mitochondrial network in response to cerebral ischemia-induced cell death. Cerebral ischemic preconditioning (IPC), is an adaptive mechanism of the brain that improves tissue tolerance to a subsequent lethal ischemic event. We previously demonstrated that the hypoxia-induced Seven in absentia Homolog 2 (Siah2) is an E3 ligase activated under ischemic conditions that is able to interact and ubiquitinate mitochondrial proteins involved in the regulation of mitochondrial function and morphology. In the present study, the role of Siah2 in the IPC-induced neuroprotection was investigated in cortical neurons exposed to 30 min of oxygen and glucose deprivation (OGD, sub-lethal insult) followed by 3hr-OGD plus 24hr- reoxygenation (lethal insult). Our results demonstrated that IPC induces Siah2 expression and LC3- II activation, a marker of mitophagy. This effect is counteracted by OGD/reoxygenation. Conversely, IPC reduced the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a marker of mitochondrial biogenesis, whereas its expression was increased after OGD/reoxygenation. Interestingly, IPC exposure was able to prevent neuronal death in the subsequent OGD/reoxygenation phase, thus suggesting that mitochondrial biogenesis activated in response to mitophagy leads to neuroprotection in preconditioned neurons. These findings were counteracted by Siah2 silencing. Collectively, our data indicate that the balance between mitophagy and mitochondrial biogenesis, due to the activation of the Siah2–E3 ligase, is involved in IPC-induced neuroprotection.

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Preconditioned Extracellular Vesicles derived from retinal astrocytes conferred neuroprotection in ischemic retinopathy

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Retinal ischemia is a clinical condition that occurs due to a lack of appropriate blood supply to the retina and is implicated in various ocular diseases, including glaucoma, hypertensive retinopathy and diabetic retinopathy. Ischemia/reperfusion injury leads to a cascade of events such as neuroinflammation, redox imbalance and abnormal vascular formation that culminates in retinal ganglion cell (RGC) death and vision loss. Extracellular vesicles, such as exosomes, are released during inflammation and may play a significant role in the pathophysiology of retinal ischemia. This study aims to investigate the effect of preconditioned extracellular vesicles obtained by astrocytes in in vitro model of retinal ischemia. To this aim, an in vitro preconditioning (PC) protocol was set up by exposing primary mouse retinal astrocyte cultures to 15 minutes of oxygen and glucose deprivation (OGD) followed by 24 hours of reoxygenation (RX). Extracellular vesicles were

then collected from the preconditioned medium, purified with differential centrifugation and characterized with Nanoparticles Tracking Analysis (NTA). Astrocytes that underwent preconditioning alone and the PC-astrocytes followed by 4 hours of OGD and 2 hours of RX exhibited decreased injury compared to those subjected only to the OGD protocol. Additionally, preconditioned astrocytes expressed lower levels of the proinflammatory markers C3 and inducible nitric oxide synthase. These experiments will enable us to explore the use of preconditioned extracellular vesicles as a potential pharmacological tool for treating retinal ischemia.

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Mitochondrial network in ischemic brain preconditioning: is the way for neuroprotection

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Ischemic preconditioning represents an important adaptation mechanism of CNS, which results in its increased tolerance to lethal cerebral ischemia. The molecular mechanisms responsible for the induction and maintenance of ischemic tolerance in the brain are complex and not yet completely clarified. Great attention has been devoted to unraveling the intracellular pathways activated by preconditioning and responsible for establishing the tolerant phenotype. Burgeoning evidence supports the hypothesis that mitochondria might act as master regulators of preconditioning-triggered endogenous neuroprotection due to their role in meeting the high metabolic demand of neurons by maintaining a constant energy supply through oxidative phosphorylation. In addition to energy production, mitochondria are essential for regulating several processes necessary for neuronal functions, including intracellular calcium homeostasis, production of reactive oxygen species, apoptotic signaling, and synaptic function. This functional adaptability is strictly related to the constant remodeling of the mitochondrial network in a series of processes, referred to as mitochondrial dynamics, involving organelle fusion and fission (division), the mitochondrial biogenesis and mitophagy that consist in the generation of new and the removal of damaged mitochondria. All these phenomena are also regulated by posttranslational modification, including phosphorylation, sumoylation, and ubiquitylation. In this scenario, evidence supporting the involvement of mitochondria within the preconditioning paradigm will be provided. Particular emphasis will be devoted to the role played by the SIAH2/NCX3/AKAP121 complex in the regulation of mitochondrial dynamics as a new molecular mechanism proposed for the establishment of an ischemic tolerant phenotype.

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Synaptic and metabolic targets for ischemic neuroprotection by conditioning paradigms

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Synaptic dysfunction has been linked to cognitive impairments after cerebral ischemia. The mechanisms underlying ischemia-induced synaptic dysfunction and cognitive impairments have not been fully elucidated. Although many mechanisms have been postulated, metabolic derangements have also been implicated since vascular and mitochondrial dysfunction also developed following cerebral ischemia. Our laboratory focuses on investigating the phenomenon of ischemic preconditioning and physical exercise, which result in increased tissue resilience. We have identified key mimetic molecules that mimic the synaptic and metabolic resilience observed following ischemic preconditioning. We have also shown that PE following cerebral ischemia promotes cognitive resilience by sparing key limbic nuclei networks. This presentation highlights our research group's findings on the signaling pathways that promote metabolic, synaptic, and cognitive improvements following cerebral ischemia. The results have important implications for the development of novel therapeutic strategies for ischemic brain injury.

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Plasmatic exosomes from remote ischemic post-conditioning as a strategy for transferring neuroprotection and functional recovery against hypoxic-ischemic injury in neonatal mice

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Remote ischemic conditioning (RIC) is a novel therapeutic intervention designed to protect the brain from ischemia-reperfusion injury, showing promise in clinical settings. RIC consists of brief, controlled episodes of occlusion followed by reperfusion applied on an artery distant from the organ exposed to the lethal ischemic event. The neuroprotection mechanisms activated through this process are multifaced, with microRNAs (miRNAs) emerging as critical mediators of the protection. miRNAs are small non-coding RNAs regulating gene expression and playing a pivotal role in supporting cellular resilience against ischemic injury. Originally, miRNAs were believed to operate only within the originating cells where they were produced, however, recent works have identified miRNAs in secreted exosomes and freely circulating in the plasma. Exosomes are intricate structures produced by different cell types, containing an array of cell surface receptors and encapsulating proteins, trophic factors, miRNAs, and other RNAs. Interestingly, it has been demonstrated that exosomes may be released in the circulation and that transferring their content to distant cells represents a tool for neuroprotective signals. The leading aim of the present study is to evaluate the effect of conditioned plasmatic exosomes on hypoxia-ischemic damage in neonatal mice. In particular, exosomes were isolated from the plasma of adult rats exposed to remote limb ischemic post-conditioning (RLIP). Purified exosomes were intraperitoneally administered in pups subjected to hypoxia-ischemia (HI) using the Rice-Vannucci model three hours after ischemia induction. The extent of neuroprotection will be assessed 24 hours after administration by Nissl staining. This innovative approach will highlight the potential of conditioned exosomes as a viable and translatable strategy for transferring neuroprotection against neonatal hypoxic-ischemic brain injury.

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The role of physical exercise in modulating neuroinflammation and promoting neurogenesis after ischemic stroke

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Neurorehabilitation has been emerging as one of the possible and reliable ways to deal with the devastating consequences of brain ischemia. Interestingly, physical exercise, applied after the ischemic event, revealed a beneficial effect on neurodegeneration, thus functioning as a post-conditioning stimulus. This protective effect may be related to the release of several humoral factors from skeletal muscle in response to physical exercise. Among them, a pivotal role has been proposed for irisin, a myokine generated by the cleavage of the membrane protein fibronectin type III domain-containing protein 5, FNDC-5, and able to cross BBB, which modulates several transductional pathways involved in stroke pathogenesis. The aim of the present study is to investigate the implication of irisin in the regulation of post-stroke neurogenesis and neuroinflammation in ischemic mice afforded physical exercise. To this aim, adult male mice will be subjected to 40' transient middle cerebral artery occlusion followed, 24h later, by 20' aerobic mild physical exercise applied every other day. The expression of irisin will be evaluated by western blot and PCR, while brain cell proliferation and differentiation will be analyzed by immunohistochemistry.

Collectively, the results of the present project will allow us to identify new players governing the crosstalk between muscle and brain and responsible for repairing processes after cerebral ischemia.

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Brain ischemic preconditioning through GATA6/TET3 epigenetic complex promotes DNA hydroxymethylation at NCX1 heart promoter in the brain

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NCX1 (sodium/calcium exchanger1) is a plasma membrane protein regulating intracellular calcium and sodium homeostasis in the brain. In particular, it has been demonstrated that NCX1, by sustaining pro-survival pathways, is an important player involved in the neuroprotective mechanisms of ischemic preconditioning. The transcription of the NCX1 gene is driven by three different promoters. It has been recently reported that the heart promoter (Ht) also drives the expression of NCX1 in the brain and that its downregulation is linked to stroke-dependent DNA methylation. However, the epigenetic mechanisms influencing NCX1 Heart expression in the brain during ischemic preconditioning have yet to be explored. In light of this information, we investigated the role of DNA hydroxymethylation in modulating NCX1 heart expression, and we found that ascorbic acid (vitamin C) increases NCX1 heart promoter activity, mRNA, and protein levels in SH-SY5Y cells. In addition, siTET3 (ten-eleven translocation enzyme3), but not siTET1 and siTET2, down-regulates NCX1 mRNA and protein expressions. Furthermore, TET3 physically interacts with the zinc-finger transcription factor GATA-binding factor 6 (GATA6), and TET3 or GATA6 silencing prevented their recruitment to the NCX1 Heart Promoter. Importantly, GATA6, TET3, and NCX1 protein expression increases in the temporoparietal cortex of rats subjected to ischemic preconditioning followed by tMCAO (transient middle cerebral artery occlusion) at 24h and 72h after reperfusion. In conclusion, upon preconditioning, the GATA6/TET3 complex epigenetically up-regulates NCX1 gene transcription.

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Exosomes from remote conditioning as a cargo of neuroprotective signals in brain ischemia

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Ischemic stroke is a leading cause of disability worldwide. Therefore, in the last few years, great attention was paid to non-pharmacological methods capable of evoking an endogenous resilient phenotype in individuals subjected to severe acute injury. Interestingly, our group and others recently demonstrated that a short occlusion of an artery is able to protect the brain from a previous harmful ischemic insult, even if the occluded artery is in a separate, distant district of the body; this phenomenon is known as remote postconditioning (RLIP). Among possible mediators of the cross-talk between the periphery and the brain, non-coding RNAs, such as miRNAs, have increasingly been recognized as key candidates. Over the last few years, we have demonstrated genetic reprogramming involving microRNAs after RLIP in the brain. However, evidence demonstrates that a variety of ncRNAs can be encapsulated and transported by exosomes, explaining their roles in intercellular communication. Thus, systemic exosome administration may be a strategy to deliver protective cell-released mediators to the CNS. More recently, we demonstrated that exosomes isolated from the plasma of animals subjected to RLIP and systemically administered to ischemic rats attenuated cerebral ischemia-reperfusion injury and improved neurological functions. Interestingly, two miRNAs, miR-702-3p and miR-423-5p, seem to be mainly involved in exosome protective action by modulating their targets NOD1 and NLRP3, two key triggers of neuroinflammation and neuronal death. Collectively, our results revealed exosomes as a possible promising tool for a successful therapeutic intervention during cerebral ischemia and as an attractive option to use them as a delivery vehicle of neuroprotection for stroke treatment.