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**Exploring Time-of-Day Influences on Neuroprotective
Strategies: New Insights from an Experimental
In Vivo Stroke Model**

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ABSTRACT

Annually, more than 15 million people suffer from a stroke accident, with 5 million dying and another 5 million remaining permanently disabled. Emerging evidence suggests that impairment of the glymphatic system — a brain-wide clearance network that relies on aquaporin-4 dynamics — may play a critical role in its development. Consistently, neuroprotective strategies aimed to counteract the cascade of event following an ischemic insult has shown remarkable neuroprotective potential. Among them, remote conditioning activates endogenous protective mechanisms involving both neural and humoral pathways that transmit signals from the limb to the brain.

Despite extensive experimental and clinical efforts, most pharmacological agents have failed to demonstrate efficacy in human stroke trials. Among the many contributing factors, the timing of stroke onset may critically influence therapeutic outcomes. Although both preclinical and clinical data show promise, the effectiveness of remote ischemic postconditioning in treating acute stroke in humans remains uncertain. Therefore, this work is divided in two sections aiming to provide new insight in stroke field.

Specifically, the study aims to:

- To unravel the mechanisms by which glymphatic dysfunction and remote ischemic conditioning interact in the pathogenesis of stroke, with the final goal of identifying novel therapeutic strategies to enhance fluid clearance and improve stroke recovery.
- To establish whether circadian differences represent a potential reason for translational difficulties and can modulate the protective effect of RIC depending on the time of day of its application.

The data obtained in this work, supports the remote conditioning strategy as a promising non-invasive intervention to elicit neuroprotection, by restoring AQP4 polarization at astrocytic end-feet and improving glymphatic clearance, thus counteracting glymphatic impairments post-stroke. The study further highlights the influence of circadian biology on stroke intervention, suggesting the potential use of remote conditioning for tailoring a specific subset of patients experiencing stroke during sleep phase.

The timing of intervention represents a critical factor of therapeutic outcome, emphasizing the importance of considering individual circadian biology when developing and optimizing neuroprotective strategies.

Key-words: Acute Ischemic Stroke; Remote Conditioning; Circadian Rhythms; Glymphatic System; Neuroprotection.

Abbreviations

2-VO or 4-VO Two- or four-vessel occlusion

7-NI 7-Nitroindazole

A β amyloid- β

AD Alzheimer disease

AKT Protein kinase B

AIS Acute ischemic stroke

AQP Aquaporin channel

ARNTL Aryl hydrocarbon receptor nuclear translocator-like protein 1

BBB Blood brain barrier

BMAL1 Brain and Muscle ARNT-Like protein 1

CBF Cerebral blood flow

CCA Common carotid artery

CLN Cervical lymph nodes

CLOCK Circadian locomotor output cycles kaput

CM Cisterna magna

CNS Central nervous system

CRY Cryptochrome protein

CT Computed tomography

ECA External carotid artery

ECs Endothelial cells

ESUS Embolic strokes of undetermined source

EVT Endovascular therapy

GFAP Glial fibrillary acidic protein

GR Glucocorticoid receptor

GS Glymphatic system

HIF-1 α Hypoxia-inducible factor 1-alpha

ICA Internal carotid artery

ICH Intracerebral haemorrhage

IL-1 β Interleukin-1 β

IL-6 Interleukin-6

ISF Interstitial fluids

IV-tPA Intravenous tissue plasminogen activator

IVT Intravenous thrombolysis

LDF Laser doppler flowmetry

MAPK Mitogen-Activated Protein Kinase

MCA Middle cerebral artery

miRNA MicroRNA

MMPs Matrix Metalloproteinases

MRI Magnetic resonance imaging

MTNR Melatonin receptors

mTOR Mammalian target of rapamycin

ncRNAs Non-coding RNA

NF- κ B Nuclear Factor kappa-light-chain-enhancer of activated B cells

NINDS National Institute of Neurological Disorders and Stroke

nNOS Neuronal nitric oxide synthase

NO Nitric Oxide

NOS Nitric oxide synthases

NPAS2 Neuronal PAS domain-containing protein 2

NVU Neurovascular unit

OGD/R Oxygen–glucose deprivation/reoxygenation

PER Period protein

PI3K Phosphatidyl inositol 3-kinase

PMCA Plasma membrane Ca²⁺-ATPase

pMCAO Permanent MCAO

PVS Perivascular space

REST RE-silencing transcription factor

RIC Remote ischemic conditioning

RISC RNA-induced silencing complex

RLIP Remote limb ischemic postconditioning

ROS Reactive oxygen species

SAH Subarachnoid haemorrhage

SCN Suprachiasmatic nucleus

siRNA Small interference RNA

STAIR Stroke Therapy Academic Industry Roundtable

TJs Tight junctions

tMCAO Transient middle cerebral artery occlusion

TNF α Tumor necrosis factor alpha

TTC 2,3,5-triphenyl tetrazolium chloride

ZT Zeitgeber time

1. INTRODUCTION

1.1. STROKE

Stroke represents a significant public health burden, and is recognized as the second leading cause of death and one of the main causes of long-term disability (Feigin et al., 2025). First introduced into medicine in 1689 by William Cole, the term “stroke” was formally defined by the World Health Organization in 1970 as “rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin” (Feigin et al., 2025). Stroke is a multifactorial disease characterized by a detrimental cascade of several cellular and molecular processes, triggered by an occlusion of a cerebral artery due to either an ischemic or haemorrhagic event (Laurent et al., 2025). More than 80 million patients have experienced a stroke episode, including all stroke subtypes (Capirossi et al., 2023). Eighty percent of them were differentiated in ischemic stroke (IS), while intracerebral haemorrhage (ICH) and subarachnoid haemorrhage (SAH) represented about 10% and 3%, respectively (Capirossi et al., 2023). Ischemic stroke subtypes include cardioembolism (approximately 22% of cases), large artery atherosclerosis (23%), small vessel occlusion (22%), other determined causes (3%), and undetermined etiology (26%) (Ornello et al., 2018).

Haemorrhagic strokes result from the rupture of a blood vessel within the brain, whereas ischemic strokes are caused by the obstruction of an artery supplying the brain (Unnithan et al., 2023). Both conditions cause local hypoxia that damages brain tissue. However, a small proportion of ischemic strokes remain of undetermined cause and are now defined as embolic strokes of undetermined source (ESUS) (Ntaios et al., 2024).

Based on the site of infarction, ischemia can be divided into global and focal ischemia. Global ischemia is often the result of a systemic reduction in blood supply to the entire brain, due to cardiac failure or prolonged systemic hypotension (Katan & Luft, 2018; Laurent et al., 2025), whereas focal ischemia occurs when a blood clot interrupts the flow in a specific cerebral artery (Campbell & Khatri, 2020). The clinical symptoms may vary depending on the affected brain region, and usually include motor impairment, aphasia, sensory loss, and visual disturbances (Campbell & Khatri, 2020).

1.1.1. Risk factors associated with stroke onset

Given the multifactorial nature of stroke, several factors have been identified and classified based on their potential modification or disease associated conditions into modifiable and non-modifiable (Boehme et al., 2017).

Non-modifiable risk factors include age, sex, ethnicity, geographic location, and heredity (Boehme et al., 2017). As reported in epidemiological studies, stroke incidence doubles after

the age of 55 (Ovbiagele et al., 2013). Men show a higher incidence of stroke than women at younger ages, but this trend reverses after the age of 75 (L. Li, Scott, et al., 2022), when women have a higher case fatality rate than men (Persky et al., 2010). Geographic and ethnic variations in stroke may be explained by differences in socioeconomic status, experiences of discrimination, lifestyle habits, and access to healthcare—although data remain limited (Cruz-Flores et al., 2011; Havranek et al., 2015; Williams et al., 2010).

Modifiable risk factors include diabetes (Boehme et al., 2017; Ojangba et al., 2023), hypertension (Wajngarten & Sampaio Silva, 2019), hypercholesterolemia (Hackam & Hegele, 2019), smoking (Pan et al., 2019), and a history of cardiac disorders such as atrial fibrillation (Gallanagh et al., 2011). Among them, hypertension represents the most prevalent risk factor, affecting approximately 64% of stroke patients (Wajngarten & Sampaio Silva, 2019).

Promoting a healthy lifestyle (Ojangba et al., 2023) and regular physical activity (Bushnell et al., 2024; Gordon et al., 2004) may prevent the majority of strokes. In particular, regular exercise has been shown to effectively reduce the risk of stroke by lowering both cardiovascular and cerebrovascular risk (Mahalakshmi et al., 2020). Physical exercise also promotes the expression of neuroprotective factors such as endothelial nitric oxide synthase (eNOS) (Green et al., 2004), brain-derived neurotrophic factor (BDNF) (H. Zhang et al., 2022), and insulin-like growth factor 1 (IGF-1) (Mahalakshmi et al., 2020).

Overall, identifying factors associated with clinical outcomes can help define effective treatment strategies that reduce case fatality and mortality, while improving the quality of life of patients with stroke.

1.1.2. Classification of stroke

1.1.2.1. Ischemic Stroke

Ischemic stroke is defined as “an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal infarction” (Sacco et al., 2013). The occlusion in ischemic stroke is usually caused by a blood clot in one of the arteries of the brain (Moskowitz et al., 2010). The tissue supplied by this artery forms the “the infarct core” (Laurent et al., 2025), and is surrounded by the “ischemic penumbra”, which maintains some blood supply through collateral arteries (S. Liu et al., 2010). With rapid reperfusion, this penumbral brain tissue can be salvaged and may recover normal function. Usually, when perfusion drops below 30%, brain cells die, making the timing of revascularization critically important (Kalogeris et al., 2012; Moskowitz et al., 2010). Seventy percent of all cases are characterised by obstruction of the middle cerebral artery (MCA) (Laurent et al., 2025). The common

symptoms in patients affected by MCA infarctions are contralateral hemiparesis, facial paralysis, and sensory loss affecting the face and upper extremities (Y. Gao et al., 2023).

1.1.2.2. Haemorrhagic Stroke

Although less common than ischemic stroke, haemorrhagic stroke has a significant public health impact, accounting for about 13% of all stroke cases (Unnithan et al., 2023). The haemorrhagic event is generated by a rupture of a weakened blood vessel, followed by the bleeding into the surrounding brain tissue (Magid-Bernstein et al., 2022). Vasculopathies and haemorrhagic conversion of an ischemic stroke or venous thrombosis can also lead to a haemorrhagic event (Paciaroni et al., 2018). The usual causes of spontaneous SAH (spontaneous subarachnoid haemorrhage) include ruptured aneurysms, arteriovenous malformations, vasculitis, and cerebral artery dissection (Ziu et al., 2023). Other contributing factors include coagulopathy, platelet dysfunction, and drug abuse (e.g., amphetamines or

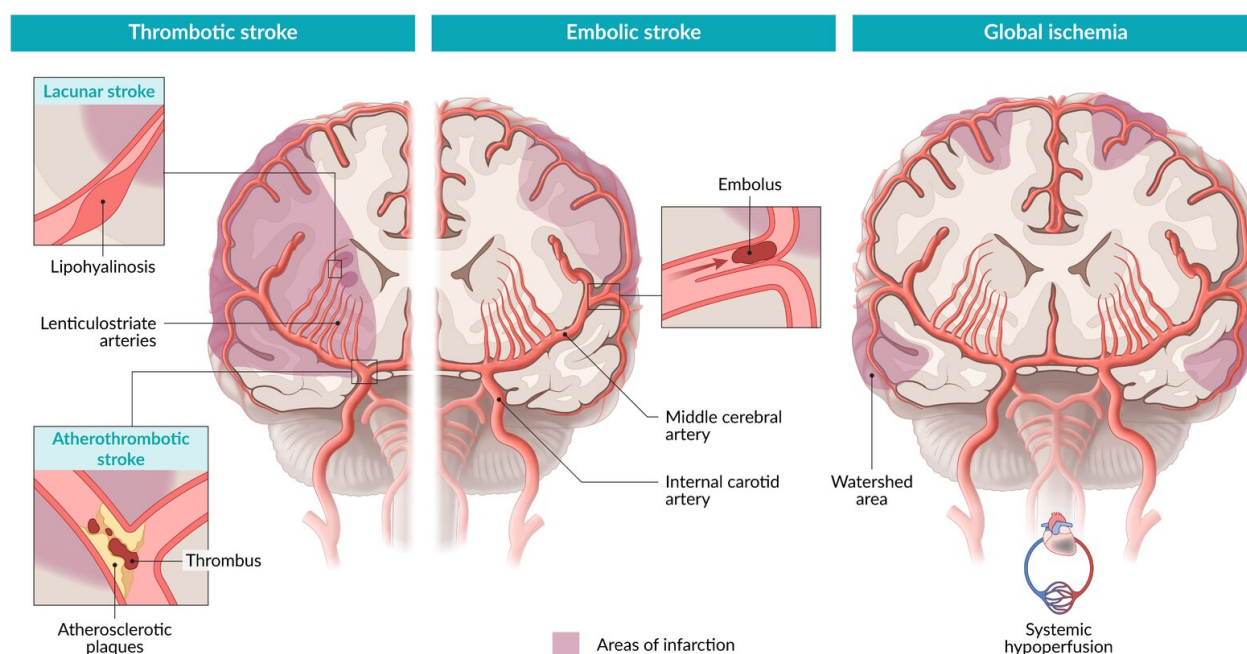


Figure 1.1 Schematic representation of different stroke subtype: focal (thrombotic and embolic stroke) and global ischemia

cocaine) (Ziu et al., 2023). In ICH (intracerebral haemorrhage), the “primary injury” results from the haemorrhage itself, which occurs immediately after the event and continues for several days (Magid-Bernstein et al., 2022). This is followed by a “secondary injury,” caused by downstream pathological processes activated in the presence of intraparenchymal blood (Magid-Bernstein et al., 2022). Neuroinflammation, oxidative stress, and iron- and blood-related toxicities contribute to the secondary injury, which unfolds over days to weeks (Yao et al., 2021).

1.1.3. Current management of ischemic stroke

The management of IS involves a multidisciplinary approach that, prior to the 1990s, was largely limited to symptomatic treatment, prevention and rehabilitation strategies (X. Li et al., 2024). A major breakthrough was achieved with the FDA's approval of intravenous tissue plasminogen activator (IV-tPA) in 1995, based on the study of the National Institute of Neurological Disorders and Stroke (NINDS) ("Tissue Plasminogen Activator for Acute Ischemic Stroke," 1995). This was followed in the 2000s by the introduction of endovascular therapy (EVT) (Berkhemer et al., 2015; Saver et al., 2015).

Currently, reperfusion therapy is considered the most effective treatment for IS. Its efficacy is based on the rapid restoration of the cerebral blood flow (CBF), achieved by either mechanical removal of an arterial thrombus or the administration of thrombolytic drugs (Bhaskar et al., 2018). Alteplase remains the only FDA- and EU-approved treatment for IS (Ringleb et al., 2008). Thrombolytic agents work by converting plasminogen to plasmin, a proteolytic enzyme responsible for the degradation of fibrin and fibrinogen, thereby promoting cerebral blood clot removal (Katz & Tadi, 2022). This treatment is generally recommended for administration within 4.5 hours of symptom onset (Wechsler, 2014) and can be given alone or in combination with EVT using mechanical thrombectomy in cases of large vessel occlusion, typically within 6 hours of symptom onset (P. Yang et al., 2020).

When combined with mechanical clot retrieval to restore arterial blood flow, IVT is indicated for most types of ischemic stroke (Mehta et al., 2017). Although the therapeutic time window is narrow, several trials have demonstrated that earlier IVT treatment is associated with better outcomes and a lower risk of secondary intracranial haemorrhage (Man et al., 2023). Unfortunately, only a small percentage of patients are eligible to receive IV-tPA (Messé et al., 2016). Multiple factors contribute to this limitation, including delayed hospital arrival (Lim et al., 2016), irreversible cell damage due to prolonged ischemia, and the high risk of haemorrhagic transformation in patients treated with rtPA (Thomas et al., 2021).

Alternative therapies in cases of revascularization or embolic complications include heparin, antithrombotic, and antiplatelet agents (Coull et al., 2002; Del Brutto et al., 2019; Mohr et al., 2001). The latter agents, such as argatroban (an antithrombotic) (Ahmed et al., 2025) and glenzocimab (an antiplatelet) (Mazighi et al., 2025), have shown potential in improving the effectiveness and durability of reperfusion. These therapies are commonly used in the management and secondary prevention of AIS. However, in the acute phase of IS, combining thrombolytic and antiplatelet therapy has not shown benefit and may increase the risk of bleeding complications without significantly reducing the risk of recurrent stroke as

shown in the MATCH and CHARISMA trials (Diener et al., 2004; Hilken et al., 2021; Norris & Barnett, 2006).

With the advances of new technologies, clinicians can better assess brain tissue perfusion status using computed tomography (CT) and magnetic resonance imaging (MRI) (Dani et al., 2011). These strategies facilitate the distinction between the irreversibly damaged infarct core and the potentially salvageable penumbra. Recent clinical trials have suggested that neuroimaging-based assessment, rather than determining symptom onset time, may effectively guide treatment in AIS (Abdalkader et al., 2023). Specifically, approximately 50% of wake-up or daytime strokes of unknown onset may be eligible for IVT when selected through MRI-based criteria, as recently reported in the WAKE-UP trial (Thomalla et al., 2018). Additionally, CT angiography and MR angiography can identify large vessel occlusions and clarify the etiology of AIS (Albers & Fisher, 2011). Considering these observations, researchers and clinicians have addressed for new therapeutic agents that could enhance brain tissue tolerance to ischemia (M. Zhang et al., 2024). These novel agents, defined as “neuroprotectants” aim to counteract the pathogenic mechanisms involved in AIS, targeting several components of the ischemic cascade (Iadecola & Anrather, 2011; Y. Yang et al., 2024). They are designed to be used in combination with approved treatments to further improve the outcomes in stroke patients (Babadjouni et al., 2017). Although neuroprotective therapy has shown promising results in clinical and preclinical trials, several questions remain as to why these positive findings have not yet been successfully translated into clinical practice (Haupt et al., 2023).

1.1.4. Molecular mechanisms of ischemic stroke

Over the past three decades, the notion of glutamate excitotoxicity (Neves et al., 2023), as a unique player in cell death following ischemia has been questioned (Annunziato et al., 2007). Growing studies have identified alternative pathways of neuronal death, linked to ionic homeostasis imbalance, with particular attention to some plasma-membrane proteins that control Ca^{2+} , Na^{+} , K^{+} , and H^{+} ion influx or efflux, through glutamate-independent excitotoxicity processes (Annunziato et al., 2007; Pignataro et al., 2014).

During an ischemic event, the sudden interruption of CBF triggers a cascade of events, collectively referred to as “ischemic cascade”, that leads to energy failure, and consequent ion and acid-base imbalances (C. Qin et al., 2022; Steliga et al., 2020). These alterations affect the “neurovascular unit” (NVU). Primarily, neurons fail to maintain their transmembrane ion gradients, thereby producing a dysfunctional neuronal signal (Steliga et al., 2020). Additionally, glial cell dysfunction, vascular abnormalities, and inflammatory responses

further exacerbate neuronal injury (Macrez et al., 2011; C. Qin et al., 2022; W. Zhang et al., 2023). As a consequence of the vascular obstruction, a massive amount of reactive oxygen species (ROS) is generated (Lai et al., 2014), and the associated oxidative stress amplifies neuronal damage, leading to functional impairments (Datta et al., 2020; Jia et al., 2021) and increased permeability of the BBB (Macrez et al., 2011). These mechanisms are usually accompanied by the infiltration of immune cells into the brain parenchyma—including leukocytes, monocytes, and macrophages—which can release neurotoxic and neurotrophic factors that influence the extent of injury (C. Gao et al., 2023).

Astrocytes become 'reactive' in response to ischemic insult (Z. Liu & Chopp, 2015). In the early stages, they undergo marked morphological and functional changes, collectively referred to as reactive gliosis (Choudhury & Ding, 2015). This process typically manifests 48–96 hours following the ischemic event and is accompanied by increased production of glial fibrillary acidic protein (GFAP), along with astrocyte hypertrophy and glial scar formation, which may impede neuronal regeneration (Koehler et al., 2006). Astrocytes also play critical roles in the regulation of CBF and vascular remodelling (Koehler et al., 2006). In parallel, a reduction in oligodendrocyte numbers is observed within the ischemic region, further impairing white matter integrity (C. Gao et al., 2023). Additionally, pericyte death has been documented, contributing to BBB disruption (Roth et al., 2025). The compromised BBB becomes increasingly permeable to peripheral inflammatory cells that further exacerbate neuroinflammation and BBB rupture (Gherzi-Egea et al., 2018).

In the ischemic cascade, calcium signalling plays a key role across all components of the NVU (Pignataro et al., 2014). To help neurons from excessive intracellular calcium, several Ca^{2+} -binding proteins facilitate the influx into organelles or the efflux across the plasma membrane. The extrusion process is mediated by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and the plasma membrane Ca^{2+} -ATPase (PMCA), which differ in affinity and transport capacity (Brini & Carafoli, 2011). NCX mediates recovery from transient calcium overload, and several studies have reported its neuroprotective role during ischemic conditions, in particular the isoform 1 of NCX (NCX-1) (Pignataro et al., 2020). During ischemic stroke, SLC8A1, the gene encoding NCX-1, is modulated by several complex transcriptional pathways (Guida et al., 2024). In particular, the activation of NF- κ B during anoxia enhances SLC8A1 expression in neurons, leading to increased Ca^{2+} accumulation within the endoplasmic reticulum (Sirabella et al., 2009).

One of the key transcription factors activated under ischemic and hypoxic conditions is the hypoxia-inducible factor 1-alpha (HIF-1 α) (Ziello et al., 2007), which is strongly associated

with oxidative stress and ischemic adaptation processes. Although its precise role remains under investigation, HIF-1 α activation appears to be closely linked to ROS production and redox imbalance, ultimately influencing cellular processes and metabolic pathways (Forrester et al., 2018). NCX-1 is also a transcriptional target of HIF-1 α , and inhibition of HIF-1 α reverses ischemic preconditioning–induced upregulation of SLC8A1 (Valsecchi et al., 2011). Conversely, the transcriptional repressor REST (RE1-silencing transcription factor) negatively regulates SLC8A1 under ischemic conditions, reducing NCX-1 expression and potentially disrupting calcium homeostasis in neurons (Formisano et al., 2013).

The oxidative damage induced by ROS contributes to apoptosis via multiple signalling cascades, involving the tumor suppressor protein p53 and the transcriptional activation of pro-apoptotic genes (Hong et al., 2010; H. Wang et al., 2023). These events are accompanied by overactivation of MAPKs and NF- κ B (Q. Guo et al., 2024; Howell & Bidwell, 2020), as well as downregulation of the PI3K/Akt survival pathway (Redza-Dutordoir & Averill-Bates, 2016). Ultimately, these processes compromise mitochondrial membrane integrity, induce chromatin condensation, and trigger endoplasmic reticulum stress (Fulda et al., 2010; H. Lee et al., 2011).

Overall, the pathogenesis of ischemic stroke is multifactorial, involving cellular excitotoxicity, ionic imbalance, mitochondrial dysfunction, neuroinflammation, and BBB disruption, all of which trigger multiple cell death pathways.

1.1.4.1. Role of nitric oxide in ischemic stroke

Nitric oxide (NO) is an inorganic gaseous signalling molecule capable of transmitting biological information throughout the body (Lundberg & Weitzberg, 2022). NO mainly acts as a potent vasodilator and angiogenic stimulator, contributing to the maintenance of thrombotic–thrombolytic homeostasis (X. Wang & He, 2024). Interestingly, NO has also been associated with the circadian variations that regulate blood pressure, and its plasma concentration shows circadian fluctuations (Zeng et al., 2024). Several cell types, including neurons, endothelial cells, and glial cells can produce NO.

Although several experimental studies have been conducted, the role of NO in the context of ischemic stroke remains under debate. During ischemia-reperfusion, NO release in the ischemic area triggers numerous molecular cascades, leading to BBB rupture and exacerbation of neuronal damage (X. M. Chen et al., 2013). NO is synthesised by three different isoforms of nitric oxide synthase (NOS): inducible (iNOS), neuronal (nNOS), and endothelial (eNOS) NOS (Iadecola et al., 2011). In ischemic stroke, depending on the spatial and temporal context, their activity can lead either to cell death and BBB disruption or to

neuroprotection (Pignataro, Esposito, et al., 2013; Samdani et al., 1997; Terpolilli et al., 2012)

At the early stage of ischemia, reduced blood supply leads to increased eNOS activity (X. M. Chen et al., 2012). Following energy depletion and cellular depolarization, calcium channels activation and increased intracellular Ca^{2+} induces NO production through nNOS stimulation. At the reperfusion stage, upregulated expression of iNOS results in excessive NO formation and consequent neurotoxicity (X. M. Chen et al., 2012). For several years, nNOS was considered a harmful factor in the pathogenesis of IS (Terpolilli et al., 2012), but its role has recently been reconsidered in the context of endogenous neuroprotective phenomenon such as brain conditioning (Gulati & Singh, 2014; Iadecola et al., 2011). Recently, (Pignataro, Esposito, et al., 2013) demonstrated that nNOS is responsible for the neuroprotective effects induced by remote conditioning. The protective role of NO in remote conditioning may involve NO-mediated vascular protection or NO-induced innate neuronal resistance to ischemia (Loukogeorgakis et al., 2005).

1.1.5. The role of the neurovascular unit in ischemic stroke

Coined in 2001 (Lo & Rosenberg, 2009), the term “neurovascular unit” identifies the complex environment within the brain parenchyma that includes neurons, glial cells, vascular cells (endothelial cells, pericytes, and smooth muscle cells), and the basal lamina matrix of the brain vasculature (Iadecola, 2017). This paradigm emerged as a novel concept to evaluate CNS pathologies, considering each component not as a single entity but as an integrated system that governs CNS physiology (Tiedt et al., 2022). *Figure 1.2* illustrates the complex cell-to-cell dynamics in the context of NVU that preserve brain homeostasis, and how dysfunction at this level can cause neurodegeneration.

The integrity of the NVU reflects BBB functions, neuronal and glial cell preservation, and immune and vascular response, thereby promoting effective “neurovascular coupling” (L. Wang et al., 2021). Neurovascular coupling involves the interplay of several factors, including ions, metabolites, and vasoactive molecules in close communication with neurotransmitters.

A stroke episode can cause dysfunction of the NVU, triggering a cascade of detrimental events that affect all its components (Lo & Rosenberg, 2009). For the sake of clarity, these processes can be briefly summarized as follows:

1. BBB rupture. BBB integrity mainly depends on the proper function of tight junctions (TJs) and endothelial cells (ECs), which are severely damaged following ischemic episodes (Nian et al., 2020). These dysfunctions, together with the release of matrix

metalloproteinases (MMPs) from glial cells (Chelluboina et al., 2015), lead to BBB breakdown and contribute to edema formation.

2. Cell survival. To preserve TJ and EC integrity, other BBB components, such as pericytes, can release neurotrophic factors in response to IS (Gaceb et al., 2017). Similarly, astrocytes can transfer mitochondria to vulnerable neurons to improve cell survival (Hayakawa et al., 2016).
3. Inflammatory response. In response to stroke, microglial and astroglial activation evolves over time, provoking a shift in glial phenotypes from pro-recovery to pro-damaging states (C. Gao et al., 2023; X. Y. Shen et al., 2021).
4. Neurovascular repair. Following the acute phase of IS, reactive glial cells promote the recovery of brain parenchyma by forming a glial scar that delineates the infarct core from the surrounding viable tissue. This attempt aims to minimize the extent of injury and inflammation. Similarly, in the later phase of ischemic stroke, pericytes regulate several angiogenic signals that promote neurovascular repair (Tiedt et al., 2022).

Collectively, the NVU exhibits a dual nature in neuro–glial interactions: detrimental effects during the acute phase of injury but beneficial during the recovery phase (L. Shi et al., 2018). Reconsidering the traditional “neuron-centric” view in favour of a neurovascular era offers novel opportunities for therapeutic intervention in stroke.

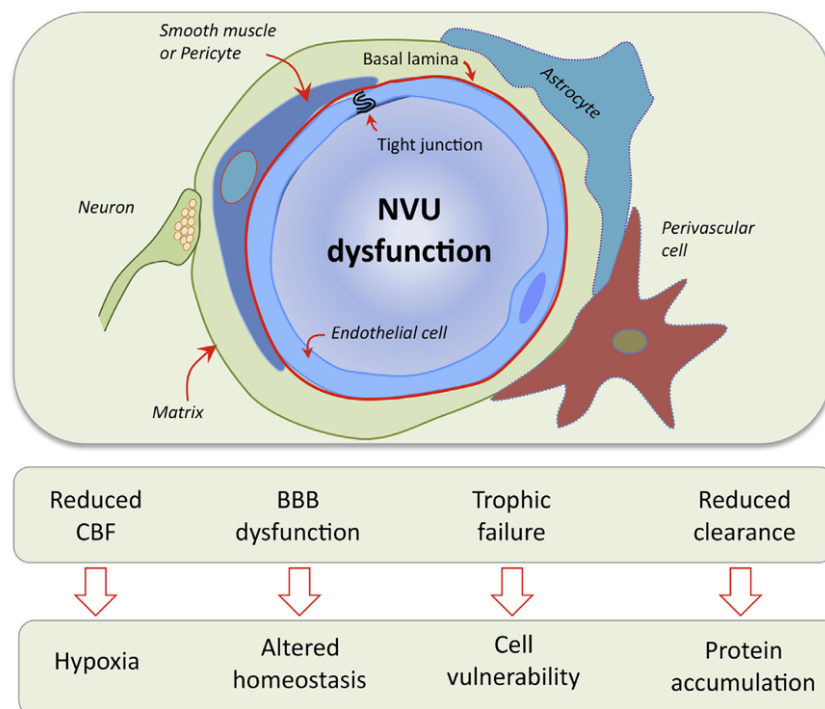


Figure 1.2 Overview of the potential pathogenetic mechanisms at the NVU (Iadecola, 2017)

1.2. NON-CODING RNAs: FOCUS ON THE ROLE OF MICRORNAs

Non-coding RNAs (ncRNAs) represent a family of RNA mainly able to regulate post-transcriptional gene expression (Wilkes et al., 2017). Non-coding RNAs can be classified as (a) short non-coding RNAs (Kaikkonen et al., 2011) - including microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNA), small nucleolar RNA (snoRNA), piwi-interacting RNAs (pi-RNAs) and other uncharacterized small RNA - and (b) long-non-coding RNAs (lncRNAs) (Statello et al., 2021). Among these, miRNAs are the most extensively studied and are widely involved in regulating numerous biological processes. Specifically, their expression changes reflect a variety of pathological processes.

MiRNAs are short noncoding RNAs, ~ 20–24 nucleotides in length, that regulate protein expression by targeting multiple mRNAs: around 30% of human protein-coding genes are regulated by miRNAs (Winter et al., 2009).

Briefly, primary miRNAs are cleaved by the endonuclease Drosha and transported from the nucleus to the cytoplasm via exportin-5. In the cytoplasm, they are processed into mature miRNAs through the activity of Dicer and TAR RNA-binding protein (J. Shen & Hung, 2015).

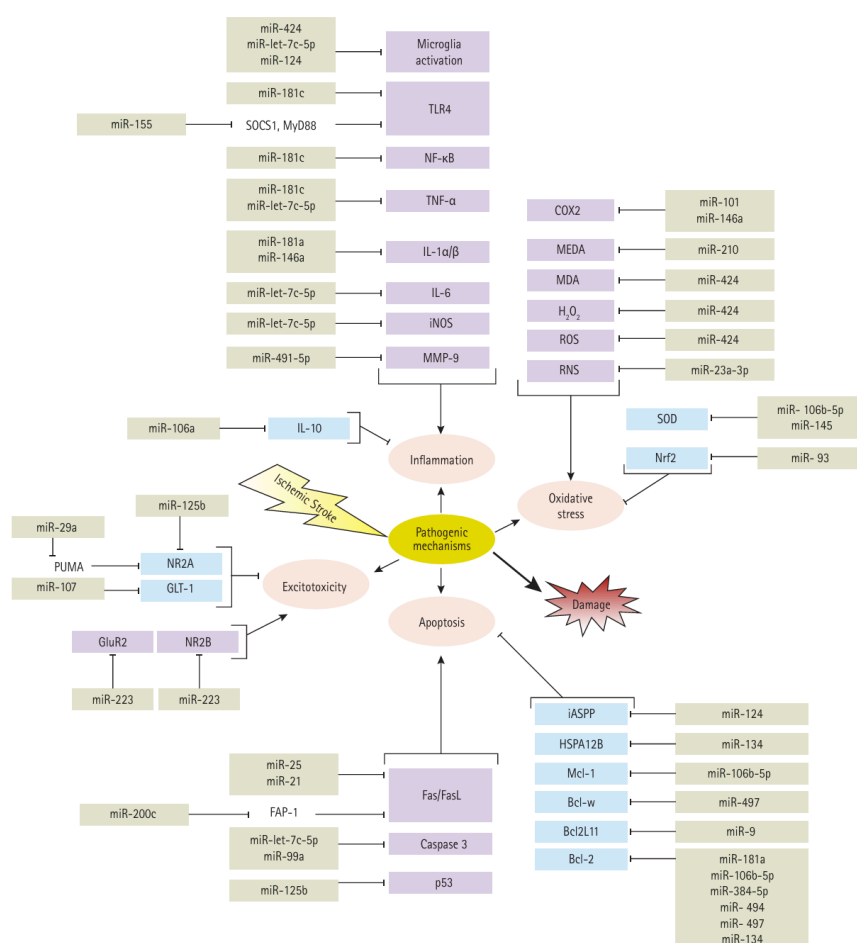


Figure 1.3 MicroRNAs involved in detrimental (purple boxes) and protective mechanisms (blue boxes) in the ischemic stroke (Khoshnam et al., 2017)

Then, these mature miRNAs then associate with the RISC complex (RNA-induced silencing complex) and bind to the 3' untranslated region (UTR) of target mRNAs. This binding can either inhibit the translation or promote the degradation of specific mRNAs, thereby reducing the expression of the corresponding proteins (Winter et al., 2009).

Initially, miRNAs were identified in tissues, but their detection in maternal plasma and blood highlighted their broader biological functions and suggested their potential as non-invasive molecular biomarkers (Ladomery et al., 2011).

Recent research has demonstrated that miRNAs play a pivotal role in the pathophysiology of ischemic stroke. Due to their ability to target multiple mRNAs, miRNAs regulate a wide range of cellular processes, including neuronal survival, differentiation, metabolism, and apoptosis, as illustrated in *Figure 1.3*.

A number of miRNAs, e.g. miR-21, miR-99a, have been implicated in the regulation of apoptotic death. In particular after IS, they suppress the expression of downstream factors (Fas ligand and caspase-3 pathway), thereby promoting neuronal survival (Chelluboina et al., 2025; Tao et al., 2015). In addition, other families of miRNAs regulate neuroinflammation after stroke, modulating the expression of downstream pro-inflammatory signalling (e.g., miR-181c) (Ma et al., 2016), as well as the microglial activation of pro-inflammatory mediators such as Arg-1 and IL-10 (e.g., miR-155) (W. Chen et al., 2021). Furthermore, miRNAs are implicated in edema formation and BBB dynamics. In particular, increased serum levels of miR-130a were observed in rodent models of ICH (M. D. Wang et al., 2016). Under these conditions, inhibition of miR-130a promotes an increase in caveolin-1 levels and a reduction of MMP-2/9 expression which correlates with improved BBB permeability, edema resolution and neurological outcomes (M. D. Wang et al., 2016; Y. Wang et al., 2018). Other studies have further proven the involvement of miRNAs in neurogenesis and angiogenesis after stroke, suggesting that these molecules may represent potential therapeutic targets for neuroprotection (Todorov et al., 2024; H. Zhang et al., 2019). While these experimental findings are promising, miRNA research remains relatively new, and the underlying mechanisms require further investigation.

A single miRNA can target numerous mRNAs, some of which may be beneficial, raising concerns about sudden side effects. Additional challenges include inefficient delivery and rapid degradation of miRNAs (Segal & Slack, 2020). However, despite these difficulties, miRNAs represent a promising therapeutic avenue with the potential to significantly impact ischemic stroke treatment.

1.2.1. MicroRNAs as Diagnostic and Prognostic Biomarkers in Ischemic Stroke

Circulating miRNAs can be measured in blood and plasma using RT-qPCR and microarray techniques. For example, specific miRNA profiles have been observed in patients diagnosed with IS (Sepmaniam et al., 2014). Similarly, in ICH patients the increased serum levels of miR-130a were correlated with greater brain edema and poor outcomes (M. D. Wang et al., 2016). Other authors measured miRNA levels (miR-30a, miR-126, and let-7b) in the plasma of stroke patients at different intervals after symptom onset (Long et al., 2013). Following blood collection, functional assessments were performed using the modified Rankin Scale (mRS), allowing the authors to correlate miRNA expression with clinical outcomes, and therefore suggesting that these miRNAs may serve as reliable tools for the diagnosis and outcome prediction of ischemic stroke. Similarly, serum exosomal miRNAs (miR-9 and miR-124) were found to be elevated in IS patients and positively correlated with NIHSS, infarct volume, and serum IL-6 concentrations (Ji et al., 2016). Their diagnostic value was also confirmed by ROC analysis (Ji et al., 2016). Another study evaluated plasma concentrations of miR-124-3p and miR-16, highlighting these as diagnostic tools capable of discriminating between SAH and IS (Leung et al., 2014).

Other biological fluids have also been used for miRNA profiling in stroke patients. Specifically, miRNAs associated with the regulation of downstream molecules like MMP-9, IL-1 β , IL-6, occludin, and E-selectin were measured in CSF of ischemic stroke patients. The data showed that 25 miRNAs were differentially expressed compared with controls (Pignataro, 2021).

Collectively, these studies offer new insight into the role of circulating microRNAs, which emerge as sensitive indicators of specific cellular pathophysiological alterations, useful to discriminate stroke subtypes and provide early diagnosis in individuals at elevated risk of stroke.

1.3. IN VIVO AND IN VITRO EXPERIMENTAL MODELS OF STROKE

1.3.1. *In vitro* models

Two principal strategies are employed to induce ischemia-like conditions in vitro: (i) deprivation of oxygen and glucose, and (ii) chemical or enzymatic inhibition of cellular metabolism (Holloway & Gavins, 2016). Among these, the oxygen–glucose deprivation/reoxygenation (OGD/R) paradigm is the most widely used. This approach deprives cultured cells (immortalized or primary) or organotypic brain slices of essential

metabolic substrates, thereby providing a platform to study stroke-related molecular mechanisms under controlled energy-deficient conditions.

To mimic ischemia-like conditions *in vitro*, the standard O₂/CO₂-equilibrated medium is replaced with N₂/CO₂-equilibrated medium and cells are incubated in a hypoxic chamber containing 95% N₂ and 5% CO₂ (Dubinsky et al., 1995). Typically, one-hour exposure to OGD can trigger neuronal death, although exposure times ranging from 30 minutes to 5 hours are commonly used (Holloway & Gavins, 2016).

Deprivation is terminated by removing the cells from the anaerobic chamber and incubating them in a normal O₂/CO₂ incubator (95% O₂ and 5% CO₂). The cells remain in normoxic conditions for a few hours of reoxygenation (RX), thereby mimicking the ischemia–reperfusion process observed *in vivo*.

Organotypic brain slices and primary cell cultures (neuronal or glial) can also be subjected to the OGD protocol. The advantage of using organotypic slices lies in their ability to provide a more physiologically relevant system that preserves neuronal–glial morphology and network connectivity (Croft et al., 2019).

Most *in vitro* models largely replicate conditions associated with global cerebral ischemia; however, several attempts have been made to better reproduce focal cerebral ischemia. For example, OGD medium can be applied in a specific area of a brain slice, while the surrounding tissue remains in standard medium. Interestingly, neurons adjacent to the insulted core gradually depolarize, mimicking the penumbral activity observed *in vivo* (Richard et al., 2010). The use of primary neuronal and glial cultures is suggested specifically to study cell-specific responses and dissect their roles in stroke pathophysiology (Holloway & Gavins, 2016).

Another important *in vitro* strategy is modelling the BBB, which plays a crucial role in stroke pathology, for example the use of mono- or co-culture transwell systems (Kaisar et al., 2016). This two-dimensional approach mimics the interactions among astrocytes, pericytes, microglia, and neurons through a semipermeable membrane, allowing researchers to investigate barrier properties in a controlled environment (Soliman et al., 2025).

Recent efforts have focused on recreating a three-dimensional matrix to better reconstruct the NVU and all BBB components. This approach includes the use of hydrogels and microfluidics. The hydrogel-based models of the BBB are composed of polymers that retain water, functionalized with cell-adhesion peptides to better replicate the extracellular matrix and provide a 3D environment that supports the growth of all the components of the NVU (N. R. Singh et al., 2023). In contrast, microfluidic BBB models (known as *organ-on-chip*)

integrate regulated fluid flow to simulate the mechanical shear stress produced by CBF in vivo (van der Helm et al., 2016).

1.3.2. *In vivo* models

Based on the extent of the injured area, animal models of stroke can be classified as global or focal ischemia, depending on whether blood flow is reduced throughout the entire brain or obstructed in a specific region (Kumar et al., 2016).

Global ischemia can be induced through several approaches. Some methods, such as decapitation (Abe et al., 1983), are now obsolete, while others, including cardiac arrest, can simulate clinical conditions but are characterized by high variability and systemic complications (Kofler et al., 2004). Two additional models of global ischemia include the two- or four-vessel occlusion (2-VO or 4-VO) (Durukan & Tatlisumak, 2007; Pulsinelli & Brierley, 1979). The 4-VO model consists of the occlusion of both common carotid arteries (CCAs) and the electrocauterization of the vertebral arteries, whereas in the 2-VO model, the two CCAs are occluded together with systemic hypotension. Both models reproduce transient ischemia; however, due to their high variability and hypotensive complications, their use remains limited.

The focal model of ischemia comprises approaches that either require or do not require craniectomy – such as embolic, intraluminal suture, and photothrombotic models (Kumar et al., 2016). To simulate the human thromboembolic stroke, in preclinical embolic models the MCA can be selectively occluded using (a) autologous blood clots, or (b) micro-emboli, as in the microsphere embolic stroke models, to induce multifocal permanent occlusions of pial and penetrating arteries (Durukan & Tatlisumak, 2007). The occlusion of the MCA (MCAO) can also be achieved through the intraluminal suture model (Longa et al., 1989). Specifically, a filament is advanced through the internal carotid artery (ICA) to the origin of the MCA to achieve vascular blockage. The suture can be left in place to mimic permanent ischemia or withdrawn to allow reperfusion (tMCAO; transient focal cerebral ischemia). Reperfusion intervals are typically 30–60 minutes in mice and 60–100 minutes in rats. Among preclinical stroke models, tMCAO is especially favoured due to its minimally invasive nature, absence of craniectomy, technical simplicity, and reproducible infarct volume within the MCA territory. Despite its widespread utility, the model has several limitations: (a) it does not replicate the primary etiology of human ischemic stroke, i.e., thromboembolism; (b) improper or incomplete filament insertion can result in partial MCA occlusion; (c) there is a risk of inadvertent SAH; (d) reperfusion occurs as an abrupt blood surge, contrasting with the gradual recanalization typical of human stroke; and (e) lesion size and location may exhibit

variability across species and strains (A. A. Singh et al., 2022). These factors contribute to experimental variability and may influence the interpretation of pathophysiological outcomes. Some procedural variability can be mitigated by monitoring cerebral blood flow and selecting appropriate filament dimensions tailored to species and strain (Durukan & Tatlisumak, 2007; Prager et al., 2009).

The photothrombosis model employs a photosensitive dye, such as Rose Bengal, administered systemically and activated by light through the intact skull (Watson et al., 1985). Photoactivation generates ROS, causing endothelial injury, platelet activation, and aggregation, which result in rapid ischemic cell death in the illuminated cortical regions supplied by end arteries.

The experimental approaches that require craniectomy involve the direct occlusion of the MCA employing i) electrocoagulation, ii) microaneurysm clips, or iii) ligatures. Infarct size depends on the occlusion site and duration; distal MCA occlusion produces primarily cortical lesions, whereas proximal MCA occlusion results in larger cortical and subcortical infarcts (Kumar et al., 2016).

Long-term neurological deficits and recovery are commonly assessed using a range of behavioural tests, each targeting distinct aspects of motor and sensorimotor function (Schallert, 2006). Simple assays, such as neurological deficit scoring, rotarod, and open field provide rapid evaluation of acute impairments, including limb weakness, gait disturbances, and reduced muscle strength (Balkaya et al., 2013; C. X. Li et al., 2025). Tests such as the adhesive label, cylinder, corner, and nest-building assays can differentiate sensory and motor deficits and are sensitive to long-term neurological changes, albeit with potential influence from learning or repeated testing (Bouet et al., 2009; X. Shi et al., 2021). Collectively, these paradigms offer complementary insights into both acute and chronic post-stroke impairments, recapitulating clinical manifestations such as limb weakness, sensory deficits, gait abnormalities, and fine motor dysfunction.

1.4. CONDITIONING MEDICINE: SELF-PROTECTION IN THE BRAIN

In the context of neurodegeneration, neuroprotection—or “neuroprevention”—may serve as a strategy to counteract the cascade of detrimental biochemical and molecular events that lead to irreversible ischemic brain injury (Haupt et al., 2023). Neuroprotective strategies aim not only to enhance the benefits of existing therapeutic interventions but also to offer alternative options for stroke patients who are ineligible for current treatments.

One potential neuroprotective mechanism involves the induction of “ischemic tolerance”, which can be achieved through a process known as “brain conditioning” (Vinciguerra et al.,

2018). This refers to the brain's ability to adapt and protect itself against injurious stimuli such as hypoxia or ischemia. A sublethal ischemic event—referred to as ischemic conditioning—can trigger endogenous responses that prepare the brain to withstand a subsequent, more severe ischemic insult, thereby establishing a state of tolerance (Pignataro et al., 2020).

Conditioning stimuli can be applied at different time points:

- Before ischemia (ischemic pre-conditioning).
- During ischemia (ischemic per-conditioning).
- After ischemia (ischemic post-conditioning).

These stimuli can be delivered either directly to the brain or to a remote organ, activating systemic protective pathways—a process known as remote ischemic conditioning (RIC) (Hess, Blauenfeldt, et al., 2015). Such adaptive biological processes can be harnessed to uncover mechanisms—still not fully understood—that may support brain protection and regeneration.

Ischemic pre-conditioning was first reported in 1986 in the heart, where cycles of occlusions and reperfusions of the coronary artery significantly reduced infarct size in a model of myocardial infarction (Murry et al., 1986). Similar protective effects have been observed in the brain (Valsecchi et al., 2011). However, the clinical use of preconditioning is limited to situations where ischemia is predictable, for example during complex surgical procedures like vascular neurosurgery, cardiovascular surgery, or brain tumour, to reduce the risk of ischemic complications (Chan et al., 2005; X. Q. Liu et al., 2009). In contrast, remote perconditioning and postconditioning offer more practical and feasible clinical applications, as reported in most clinical trials evaluating the potential efficacy of RIC in stroke (Beretta et al., 2025; Blauenfeldt et al., 2024; Hougaard et al., 2014).

1.4.1. Experimental model to reproduce ischemic conditioning *in vivo*

Barone et al. (1998) first introduced the concept of ischemic preconditioning in rats, applying tMCAO as a conditioning stimulus, prior to a permanent MCAO (pMCAO) (Barone et al., 1998). Specifically, these transient stimuli reduced infarct volume and improved neurological outcomes when applied 2 to 7 days before pMCAO (Barone et al., 1998). Similar paradigms employing one or three cycles of brief transient occlusion are sufficient to provide neuroprotection against experimental tMCAO in rats (Alkayed et al., 2002), and in mice (Hoyte et al., 2006).

Based on these findings, a three-day time window appears to represent the optimal interval for applying preconditioning stimuli before a severe ischemic insult in order to achieve maximal protection (Formisano et al., 2015; Pignataro, Cuomo, et al., 2013). This time-dependent effect suggests the activation of endogenous protective cascades, likely involving gene transcription, protein synthesis, and adaptive cellular signalling.

A similar strategy can also be applied after a harmful ischemic event through either a repetitive series or a single brief application of brief reperfusion/occlusion cycles to achieve ischemic postconditioning (Vinciguerra et al., 2018). Commonly used protocols include short cycles (5 minutes each) of reperfusion/occlusion following transient MCAO, or a single reperfusion/occlusion cycle (20/20 minutes in rats and 10/10 minutes in mice). In contrast, longer reperfusion intervals failed to confer protection (Vinciguerra et al., 2018).

Interestingly, both pre- and post-ischemic conditioning stimuli can also be applied remotely (Pignataro, 2021). For instance, transient occlusion of a distant organ, such as a limb via femoral artery occlusion, has been shown to exert a protective effect, a phenomenon defined as RIC (Licastro et al., 2025; H. Zhao et al., 2012). This strategy highlights the existence of systemic signalling pathways, likely involving neural, humoral, and immunological mediators, that can confer neuroprotection across organs.

1.4.2. Remote conditioning as a promising strategy in the treatment of stroke

RIC involves applying pressures above systolic blood pressure by repeatedly inflating and deflating a cuff around a limb (Epps et al., 2016). This procedure, in turn, activates endogenous protective mechanisms. RIC can remotely recruit neuroprotective pathways, with high therapeutic potential across a wide range of neurological conditions. Multiple mechanisms – including neuronal, humoral, or immune routes – have been proposed as possible paths through which the peripheral signal from the limb is transmitted to distant organs, such as the brain (Hess, Blauenfeldt, et al., 2015).

Preclinical studies in rodent models of stroke have shown the neuroprotective effects of RIC not only in reducing ischemic volume, but also throughout the entire brain, enhancing anti-apoptotic, antioxidant, and anti-inflammatory responses (Baranova et al., 2023). Similar effects have been reported in a murine model of vascular cognitive impairment, where remote conditioning improves CBF and reduces inflammation, neuronal death, and beta-amyloid (A β) accumulation (Hess, Khan, et al., 2015). Short episodes of RIC reduce neuronal death in the hippocampus when applied after experimental stroke (H. Qin et al.,

2025). Similarly, 20 minutes of RIC in rats, applied two days after global ischemia, significantly reduces the infarct area in the hippocampal region (L. Wang et al., 2024).

Overall, when the hippocampal area is rescued, improvements in neurological, functional, cognitive, and behavioural outcomes occur, partially explaining the efficacy of RIC, especially as a rehabilitation strategy. Experimental interventions – such as peripheral nerve or spinal cord transection, or the administration of nicotinic and opioid receptor antagonists, as well as pharmacological blockade of afferent fibers, autonomic ganglia, and parasympathetic efferent pathways – have been shown to abolish or attenuate the protective effects of RIC (Basalay et al., 2018). These findings support the hypothesis that RIC-mediated signalling is initiated via peripheral C-fiber sensory neurons, integrated within the CNS, and transmitted through efferent vagal pathways to elicit downstream organ protection (Baranova et al., 2023).

1.4.3. Remote ischemic conditioning: signal transmission to the brain

Remote conditioning neuroprotection can be divided into an early phase—starting right after the conditioning stimulus and lasting for several hours—and a late phase that appears 12–48 hours later (Q. Li et al., 2024).

Sub-lethal ischemic events can trigger the release of signalling molecules which activate CNS nuclei via afferent neural pathways. This activation, in turn, leads to the release of humoral factors into the bloodstream that target various organs. Factors responsible for the neuroprotection of the early phase include neurotransmitters, hormones, cytokines, and other RIC-related factors that promote the modulation of intracellular signal transduction pathways (W. Zhao et al., 2023). All these events trigger the regulation of key cellular processes involving receptor activity, ion transport, synaptic function, cytoskeletal structure, and mitochondrial homeostasis. During the late phase of neuroprotection, transcription factors modulate gene expression to promote the synthesis of protective proteins (Nikkola et al., 2015).

Several mediators have been proposed to transmit the protective signal from the limb to the brain during RIC (Pignataro, 2021; Vinciguerra et al., 2018). Increasing evidence supports the involvement of neural pathways in mediating RIC-induced neuroprotection. For instance, activation of the vagus nerve has been shown to stimulate the synthesis and release of NO and nitrites in visceral organs (Brack et al., 2009).

Experimental studies in rats by Chuancheng Ren et al. have demonstrated that postconditioning with RIC confers protection against stroke, both through immediate and

delayed application (up to 3 hours post-insult) (Ren et al., 2015). The use of cycloheximide, an inhibitor of protein synthesis, and capsaicin, the afferent nerve blocker, completely abolished RIC neuroprotective effects, suggesting that RIC mediates protection by modulating both protein synthesis and nerve signalling (W. Zhao et al., 2023).

Furthermore, Pignataro et al. showed that nNOS contributes to the neuroprotective effects of post-RIC intervention, mediated by the activation of pro-survival kinases such as p-ERK (Pignataro, Esposito, et al., 2013). Levels of nitrite are usually increased after RIC. Under hypoxic conditions, nitrite is converted to NO, a well-known vasodilator, by the activity of NOS (Lundberg & Weitzberg, 2005) which may modulate cerebral blood flow after RIC.

Humoral factors involved in RIC are likely delivered to target organs via endogenous extracellular vesicles or nanoparticles derived from endothelial cells or platelets (Gu et al., 2022). These vesicles carry bioactive molecules that contribute to neuroprotection (Cuomo et al., 2024; L. Zhang & Wrana, 2014). Through their cargo, they can downregulate pro-inflammatory pathways, promote angiogenesis, and inhibit apoptotic pathways. Some of them, known as exosomes, can be isolated from the plasma of RIC-treated rats and delivered to ischemic rats to promote neuroprotection (Cuomo et al., 2024).

Some humoral factors released during RIC may also act as neurotransmitters or neuromodulators. These molecules interact with receptors in the nervous system, contributing to the transmission of protective signals from peripheral tissues to the central nervous system (May et al., 2021).

RIC may also activate a cascade of intracellular signalling pathways – including PI3K/Akt and mTOR – that collectively enhance neuronal survival and confer resistance to ischemic injury (Breivik et al., 2011; Zare Mehrjerdi et al., 2013). RIC has also been shown to induce the cleavage of δ PKC, a PKC isoform typically associated with pro-apoptotic signalling and neuronal damage. Conversely, it enhances the phosphorylation of ϵ PKC, an isoform known to activate survival pathways (X. Gao et al., 2008).

Furthermore, RIC exerts anti-inflammatory effects by downregulating toll-like receptor 4 (TLR4), a key mediator of neuroinflammation (L. Cai et al., 2025). Suppressing TLR4 activation helps reduce the inflammatory response following ischemic injury, thereby mitigating secondary neuronal damage (J. Lv et al., 2021).

Another important neuroprotective mechanism induced by RIC is the activation of microautophagy (Wu et al., 2025). This selective form of autophagy facilitates the degradation and clearance of dysfunctional cellular components, particularly damaged mitochondria.

1.4.4. From preclinical to clinical conditioning medicine in neurological disease

Recently, several clinical studies have provided promising evidence on the benefit and efficacy of RIC in stroke (Hess, Blauenfeldt, et al., 2015).

A single-center randomized pilot trial was conducted to investigate the potential of RIC as an adjuvant therapy to alteplase in patients with IS (Hougaard et al., 2014). RIC was applied randomly to patients during transportation to the hospital. MRI was used to select patients eligible for rtPA treatment and clinical outcomes were evaluated after three months. Overall, the study showed that RIC was safe and well tolerated. Although RIC was not able to recover the ischemic penumbra or impact infarct size, a voxel-by-voxel logistic analysis — corrected for baseline severity of hypoperfusion — suggested that RIC promotes tissue survival after one month (Hougaard et al., 2014).

New promising data were obtained from the RESIST clinical trial (Blauenfeldt et al., 2020). Researchers evaluated whether combined RIC – preconditioning and postconditioning – could improve long-term functional outcomes in IS and SAH patients. After three months of follow-up, this combined strategy did not provide additional benefit; however, whether stroke etiology or adherence may influence the treatment response remains to be determined (Blauenfeldt et al., 2020). More recently, through a post hoc analysis of the RESIST trial, RIC was associated with improved functional outcomes at 90 days in IS patients due to small vessel disease (Blauenfeldt et al., 2024).

1.5. TRANSLATIONAL FAILURE FROM PRECLINICAL TO CLINICAL

Despite extensive efforts at both experimental and clinical levels, many agents failed to provide efficacy in human trials for stroke treatment (Dhir et al., 2020). Due to repeated negative outcomes and failures in clinical translation, a task force named the Stroke Therapy Academic Industry Roundtable (STAIR) was established in 1999. This group developed a set of guidelines, known as the STAIR criteria, aimed at improving the translation of preclinical findings into clinical success for stroke therapies (Saver et al., 2009).

The failure to translate neuroprotective strategies from bench to bedside may result from several factors, particularly the differences between animal models and human patients. These include comorbidities, age, sex, human genetic variability, and differences in brain composition, such as the ratio of white to gray matter. Collectively, factors that may contribute to poor translation include (Lyden et al., 2021):

1. Model selection and success criteria. Various *in vivo* models of stroke are commonly used – MCAO, global ischemia, photothrombosis, and thromboembolic stroke models.

Each has its limitations and may not fully replicate human stroke pathology (Kumar et al., 2016).

2. Outcome and functional assessment. In animal studies, neuroprotective effects are typically assessed by measuring infarct size and performing functional tests such as the Morris Water Maze (MWM) and Novel Object Recognition (NOR) (Lissner et al., 2021). In contrast, clinical evaluations rely on standardized scoring systems, including the NIH Stroke Scale and the Barthel Index (Harrison et al., 2013).
3. Therapeutic time window. In many preclinical studies, therapeutic agents are administered immediately before or after stroke induction (Narayan et al., 2021). However, in clinical settings, stroke diagnosis and treatment initiation often take significantly longer, limiting the applicability of preclinical timeframes (Jauch et al., 2013).
4. Age and comorbidities. Preclinical research generally involves young, healthy animals, whereas stroke predominantly affects older adults who often present with multiple comorbid conditions (Candelario-Jalil & Paul, 2020).
5. Sex. Preclinical stroke studies frequently use male animals to avoid variability caused by hormonal fluctuations during the estrous cycle, which can complicate data interpretation. This practice, however, reduces the generalizability of findings to both sexes (Barus et al., 2023).
6. Dosage and administration regimen. In animal studies, drugs are usually given in low doses and for short durations to minimize toxicity. In contrast, human treatments vary widely, ranging from single intravenous doses to long-term oral therapies lasting several months.

In addition to these considerations, the timing of stroke onset may also play a critical role in the effectiveness of therapeutic interventions (Esposito et al., 2020). The opposite circadian cycles in nocturnal rodents versus diurnal human patients may be a cause for failure in translation. On one side, most clinical stroke trials enrol patients during the daytime, corresponding to the human active (awake) phase. On the other side, preclinical studies on rodent models of cerebral ischemia are typically conducted during the human daytime as well, when rodents, being nocturnal animals, are sleeping. This circadian mismatch between animal models and human clinical trials may contribute to the challenges in translating stroke therapies (Licastro et al., 2025).

1.6. CIRCADIAN BIOLOGY

Circadian ("circa diem") rhythms represent an evolutionarily conserved system that produces daily cycles of physical and behavioural changes (Kramer et al., 2022). As reported in *Figure 1.4*, these rhythms control a wide range of cellular and physiological functions, including gene expression, metabolism, and stress responses (Fagiani et al., 2022). The central pacemaker, represented by the suprachiasmatic nucleus (SCN), governs the circadian rhythms, synchronizing peripheral oscillators in various tissues (Gillette & Mitchell, 2002). The core molecular mechanism driving circadian rhythmicity is a gene-regulatory network composed of transcription–translation feedback loops, primarily controlled by CLOCK:BMAL1-driven transcription of the Period and Cryptochrome genes (Takahashi, 2016). These genes encode repressors that inhibit CLOCK:BMAL1 activity, creating an oscillatory gene expression cycle with a period of approximately 24 hours (Langmesser et al., 2008). In addition to the hypothalamic master clock regulation, the synchronization of peripheral clocks is mediated by both humoral and non-humoral pathways (Kalsbeek et al., 2006): (a) autonomic innervation of peripheral tissues (Nader et al., 2010), (b) endocrine system (Begemann et al., 2025), and (c) body temperature regulation (Refinetti, 2020). In particular, the SCN controls the circadian secretion of

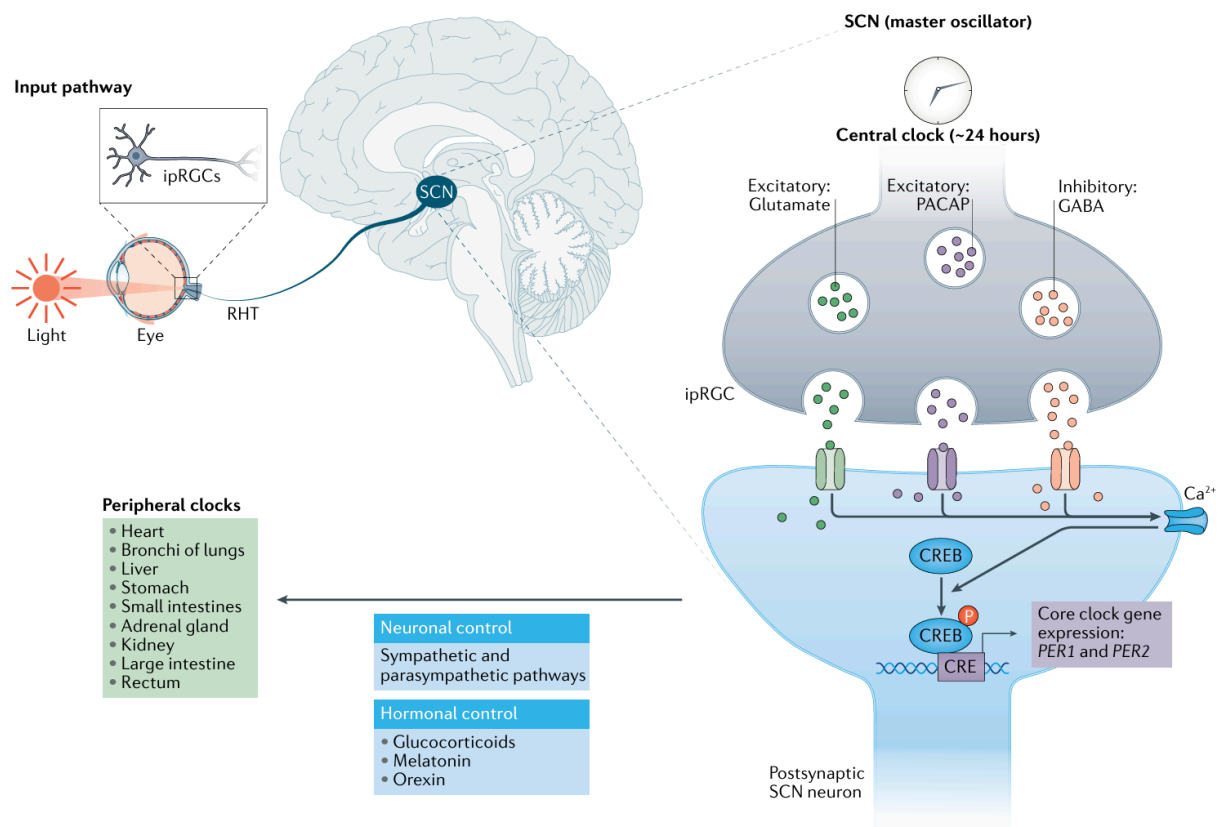


Figure 1.4. Hierarchical organization of the mammalian clock system (Ruan et al., 2021)

melatonin from the pineal gland (Arendt and Aulinas, 2022), as well as glucocorticoids and catecholamines secreted from the adrenal cortex (Ikeda et al., 2013), via the HPA axis (hypothalamic–pituitary–adrenal axis) (Nader et al., 2010).

The reciprocal crosstalk between the circadian molecular clock and key intracellular pathways highlights fundamental aspects of mammalian physiology. Moreover, circadian rhythms can be disrupted by environmental factors and social behaviours (Fishbein et al., 2021). Thus, misalignment in circadian timing may contribute to pathophysiological processes such as cancer (Y. Lee, 2021), inflammatory diseases (Inokawa et al., 2020; Leproult et al., 2014), and neurological conditions (Lo et al., 2021).

1.6.1. The circadian autoregulatory feedback loop

The circadian clock machinery is governed by transcription-translation feedback loops (TTFLs) that set the body's rhythmicity (Takahashi, 2016). The transcription factors CLOCK (circadian locomotor output cycles kaput) and its paralog NPAS2 (neuronal PAS domain-containing protein 2) (Murgo et al., 2023; L. U. Peng et al., 2021) are key transcription factors that form heterodimers with the aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL; also known as BMAL1) (H. Xu et al., 2015). The resulting CLOCK:BMAL1 complex translocates from the cytoplasm to the nucleus, where it binds to the Enhancer Box (E-box) sequences in clock-regulated genes (Y. Liu & Sancar, 2025). In particular, it regulates the expression of period (PER1-3; period circadian protein homologues 1, 2, and 3) and cryptochrome proteins (CRY1-2) (Fagiani et al., 2022). During the late evening, PER and CRY proteins form cytoplasmic complexes that migrate to the nucleus to inhibit CLOCK:BMAL1 transcriptional activity (Cao et al., 2021). The expression levels of PER and CRY are also regulated via polyubiquitination and degradation by specific E3 ligases (Srikanta & Cermakian, 2021). The decrease in PER and CRY protein levels reduces, in turn, the suppressive activity on the CLOCK:BMAL1 complex, allowing a new oscillatory cycle to begin (Pilorz et al., 2018). Other components of the circadian clock include the nuclear receptors REV-ERBs (also known as REV-ERB α /NR1D1 and REV-ERB β /NR1D2), which work as repressors of BMAL1 expression (Delezie et al., 2016), and the retinoic acid receptor-related orphan receptors (RORs; ROR α , ROR β , and ROR γ) (Ikeda et al., 2019), which positively regulate BMAL1 gene promoter via the RORE sites (Guillaumond et al., 2005).

Additional outputs and inputs to the transcriptional clock are represented by membrane depolarization, intracellular calcium flux (Onodera et al., 2023), and the activation of cAMP

signalling (Brenna et al., 2024; Koronowski & Sassone-Corsi, 2021). All these signals are integrated at the molecular clock level to sustain the ~ 24-hour circadian cycle.

1.6.2. Role of melatonin in sleep-wake cycle

Day-night circadian rhythm is regulated by melatonin synthesis in the pineal gland in the human brain (Pandi-Perumal et al., 2006). Other organs synthesize and secrete melatonin, including the bone marrow (Y. Zhou et al., 2020), retina (Felder-Schmittbuhl et al., 2024), thymus, lens, intestine (Bubenik, 2002), and epithelium of the respiratory tract (Ahmad et al., 2023; Arendt & Aulinas, 2022). Within these tissues, it exerts antioxidant functions that help counteract oxidative damage (Hardeland, 2012; Megha et al., 2024).

Melatonin reaches a peak in the bloodstream about 2–3 hours after the onset of darkness, whereas its levels remain low or undetectable during the daytime (Rzepka-migut & Paprocka, 2020). Melatonin synthesis is suppressed during the daytime due to activation of the SCN by retinal light input (Ruan et al., 2021). Conversely, when light stimuli are low, the SCN triggers a neural output signal that induces melatonin synthesis and release (J. Zhang et al., 2024). The melatonin receptors (MTNR1A, also known as the MT1 receptor and MTNR1B, also known as the MT2 receptor) mediate the functions of melatonin as G-protein-coupled receptors (Pandi-Perumal et al., 2008).

Sleep-related effects are mainly mediated by MT1 receptors (J. Liu et al., 2015), which are predominantly expressed in the SCN and act to reduce neuronal firing in this region, promoting sleep onset and maintenance (Poirel et al., 2002). Through MT2 receptors, melatonin also exerts a phase-shifting function on the circadian rhythm, affecting the timing of the biological clock via pathways not directly dependent on modulation of circadian gene expression (Dubocovich & Markowska, 2005; Nosjean et al., 2001).

Overall, melatonin plays a crucial role not only in sleep regulation but also in synchronizing various physiological processes with environmental light-dark cycles.

1.6.3. Circadian rhythms and their role in physiological processes

Circadian biology functions not only as the generator of daily rhythmicity but also as a fundamental regulator of physiological processes across nearly all tissues and organs (Thakur & Kishore, 2025). In particular, it exerts its influence through multiple mechanisms: the regulation of cell proliferation (Masri et al., 2013), metabolism (Masri & Sassone-Corsi, 2013), DNA repair and damage responses (Sancar et al., 2010), angiogenesis (Jidigam et al., 2022), and immune function (Scheiermann et al., 2018).

Evidence indicates that the molecular machinery of the circadian rhythms shares structural and functional similarities with the cell cycle regulatory machinery. In fact, several cell cycle regulatory factors display circadian expression patterns, and many genes governing cell cycle transitions contain E-box regulatory motifs within their promoters, which are transcriptionally activated by the CLOCK:BMAL1 heterodimer (Hunt & Sassone-Corsi, 2007). For instance, circadian clock components modulate the G1/S transition via transcriptional regulation of *Cdkn1a*, encoding the cyclin/Cdk inhibitor p21 (Farshadi et al., 2019). Perturbation of circadian timing mechanisms results in aberrant p21 expression and dysregulated cellular proliferation (Y. Lee et al., 2019). Furthermore, the circadian system participates in the DNA damage response through direct interactions with cell cycle checkpoint proteins, thereby safeguarding genomic integrity (Antoch & Kondratov, 2010). This function appears to involve critical mediators such as p53 and PER2.

Circadian regulation also extends to angiogenesis, principally through BMAL1-mediated interactions with E-box sequences in the promoters of *VEGF* and *HIF1 α* (Jidigam et al., 2022). Moreover, immune cell populations, including T lymphocytes, granulocytes, and macrophages, undergo daily oscillations in abundance and functional activity, with many of these rhythms being dependent on BMAL1 (Hand et al., 2020).

In addition to direct transcriptional regulation of immune mediators by clock genes, inflammatory processes are modulated via the glucocorticoid receptor (GR) (Strickland et al., 2022). Endogenous glucocorticoids exert circadian control over inflammatory cytokine expression by binding GR, which suppresses pro-inflammatory transcription through glucocorticoid response elements (GREs) and by modulating transcription factors such as NF- κ B (Shimba & Ikuta, 2020). GREs have also been identified in the promoters of *PER* genes, and glucocorticoid administration (e.g., dexamethasone) has been shown to selectively induce PER1 expression at concentrations corresponding to the nocturnal nadir of circulating cortisol (Fagiani et al., 2022).

1.6.4. Circadian disruption in neurological disorders

Circadian misalignment, often driven by social behaviours (e.g., exposure to artificial light, shift work, jet travel, irregular sleep and eating schedules) and environmental factors, has been linked to several pathologies (Inokawa et al., 2020; Leproult et al., 2014; Potter et al., 2016). Sleep disorders and jet lag are primarily associated with phase dissociation (Auger & Morgenthaler, 2009), whereas cancer, metabolic diseases, and neurodegenerative disorders appear to be influenced by disrupted circadian rhythms (Ruan et al., 2021). Mechanistically, circadian misalignment can lead to altered hormonal secretion patterns

(Potter et al., 2016), impaired glucose metabolism, endothelial dysfunction, and a pro-inflammatory state (Y. Shen et al., 2023). Observational studies have shown that these dysfunctions are frequently accompanied by a reduction in high-density lipoprotein cholesterol, elevations in triglycerides, flattening or phase shifts in cortisol rhythms, increased levels of C-reactive protein, and persistent elevations in blood pressure (Y. Shen et al., 2023) — all of which are recognised risk factors for cerebrovascular events (Boehme et al., 2017).

In the context of stroke, clinical data suggest that both the frequency and severity of stroke vary depending on the time of day. In a meta-analysis, the authors reported that the recurrence of ischemic stroke was higher between 6:00 a.m. and 12:00 p.m. compared with other times of the day (X. Wang et al., 2022). This morning effect may be influenced by circadian regulation of cardiovascular and hemodynamic variables, such as increased platelet aggregability, sympathetic activity, elevated blood pressure, and increased cortisol and catecholamine levels during the early active phase (Morris et al., 2012). However, the variation in the timing of first detection may, besides reflecting a genuine circadian influence on the pathophysiology of stroke, account for a confounding effect of wake-up strokes, in which the observed time of symptoms may not correspond to the actual time of stroke onset (Peter-Derex & Derex, 2019). Wake-up strokes account for 8% to 28% of all ischemic strokes, contributing to the clustering of first symptom observations in the mid-morning (Mackey et al., 2011). Even after adjusting for wake-up strokes, large-scale observational studies have demonstrated a substantial diurnal variation in the biological onset of ischemic stroke (Lo et al., 2021). A recent multicentre observational clinical study in Korea investigated whether the diurnal variation of stroke incidence influences outcomes within the first 72 hours after onset (Ryu et al., 2022). The study found that night-onset strokes were associated with greater neurological severity, increased infarct growth, higher rates of stroke recurrence, a greater risk of symptomatic haemorrhagic transformation, and poorer 3-month functional outcomes compared with day-onset strokes (Ryu et al., 2022). These differences may be related to the neurovascular environment during the rest phase, including reduced CBF and time-of-day-dependent regulation of neuroprotective or pro-apoptotic gene expression (Licastro et al., 2025; Moskowitz et al., 2010). Thus, the timing of stroke onset, in relation to different Zeitgeber times (ZT), should be considered in epidemiological studies and clinical trials evaluating neuroprotective agents for acute ischemic stroke (Licastro et al., 2025). Preclinical work supports this concept: infarct size and worse behavioural

outcomes have been observed, where rodents exhibited larger infarct volumes at noon—corresponding to their sleep phase—compared with other time points (Kamat et al., 2021). Additional impacts on stroke rehabilitation are related to disturbances in sleep (Pérez-Carbonell & Bashir, 2020). Disruptions in sleep patterns — such as obstructive sleep apnea (OSA), insomnia, and abnormal sleep durations (either too short or too long) — are recognized risk factors for stroke, primarily through the mechanism of circadian rhythm disruption (Hale et al., 2023; Rissardo et al., 2025). Patients experiencing stroke during sleep exhibit greater levels of disability and an increased mortality risk within the first month post-stroke compared to those who experience stroke during the awake phase (H. Cai et al., 2021). In preclinical studies, clock genes regulate sleep via post-translational modifications of core clock proteins (Thakur & Kishore, 2025). Under sleep deprivation, there is an alteration of the binding of Bmal1, Clock, and Npas2 to specific clock genes that contributes to poorer outcomes and potentially leads to more severe stroke prognoses (Mongrain et al., 2011).

1.6.5. Mechanisms of circadian disruption in stroke

Circadian biology influences stroke outcomes through several mechanisms. Key physiological functions that affect stroke prognosis—including metabolism, blood pressure, heart rate, coagulation, and immune responses—are governed by circadian clocks and exhibit daily fluctuations over a 24-hour cycle (Mergenthaler et al., 2024). *Figure 1.5* summarises the mechanisms and pathophysiological events linking circadian biology and stroke. First, under ischemic episodes, HIF-1 α is generated as a response to the hypoxic environment (Adamovich et al., 2017). The expression level of HIF-1 α regulates the clock proteins PER1 and CRY; notably, deletion of HIF-1 α results in reduced expression of these clock components (Dimova et al., 2019). Second, changes in glucose metabolism and mitochondrial function caused by hypoxia are modulated by the circadian clock (Puig et al., 2018). Other cellular processes strictly related to circadian rhythmicity include apoptosis, autophagy, mitochondrial activity and humoral processes. Apoptosis, among several processes, is triggered by TNF α and IL-6, which are secreted in a circadian manner by macrophages (Abreu et al., 2018). Mice lacking CRY proteins exhibit increased production of TNF α (Hergenhan et al., 2020). Interestingly, levels of TNF α and other inflammatory cytokines rise following stroke, contributing to neuroinflammation. Similarly, other factors that trigger apoptosis via the intrinsic pathway act as critical mediators of cell damage and neuronal death in the ischemic penumbra. Among these factors, the expression of the BCL-2 family exhibits a diurnal pattern in the rat brain (Montes-Rodríguez et al., 2009).

Specifically, the anti-apoptotic protein BCL-2 shows peak expression in the prefrontal cortex, while the pro-apoptotic protein BAX (BCL2-associated X, apoptosis regulator) is at its lowest expression in the striatum during the awake phase. This pattern suggests that this area may be more resilient to apoptosis during the awake phase compared to the sleep period, and BMAL1 may counteract apoptosis, at least in part, by regulating BCL-2 family proteins (Mergenthaler et al., 2024; Montes-Rodríguez et al., 2009).

Glucose serves as the brain's primary energy source, making the brain particularly susceptible to acute injury during stroke (Sifat et al., 2022). Metabolism itself is under circadian control (Masri & Sassone-Corsi, 2013). Additionally, mitochondrial dynamics, including fission and fusion, are regulated by circadian clock genes, and most mitochondrial proteins are differentially regulated according to their specific metabolic functions (J. Kim & Sun, 2024).

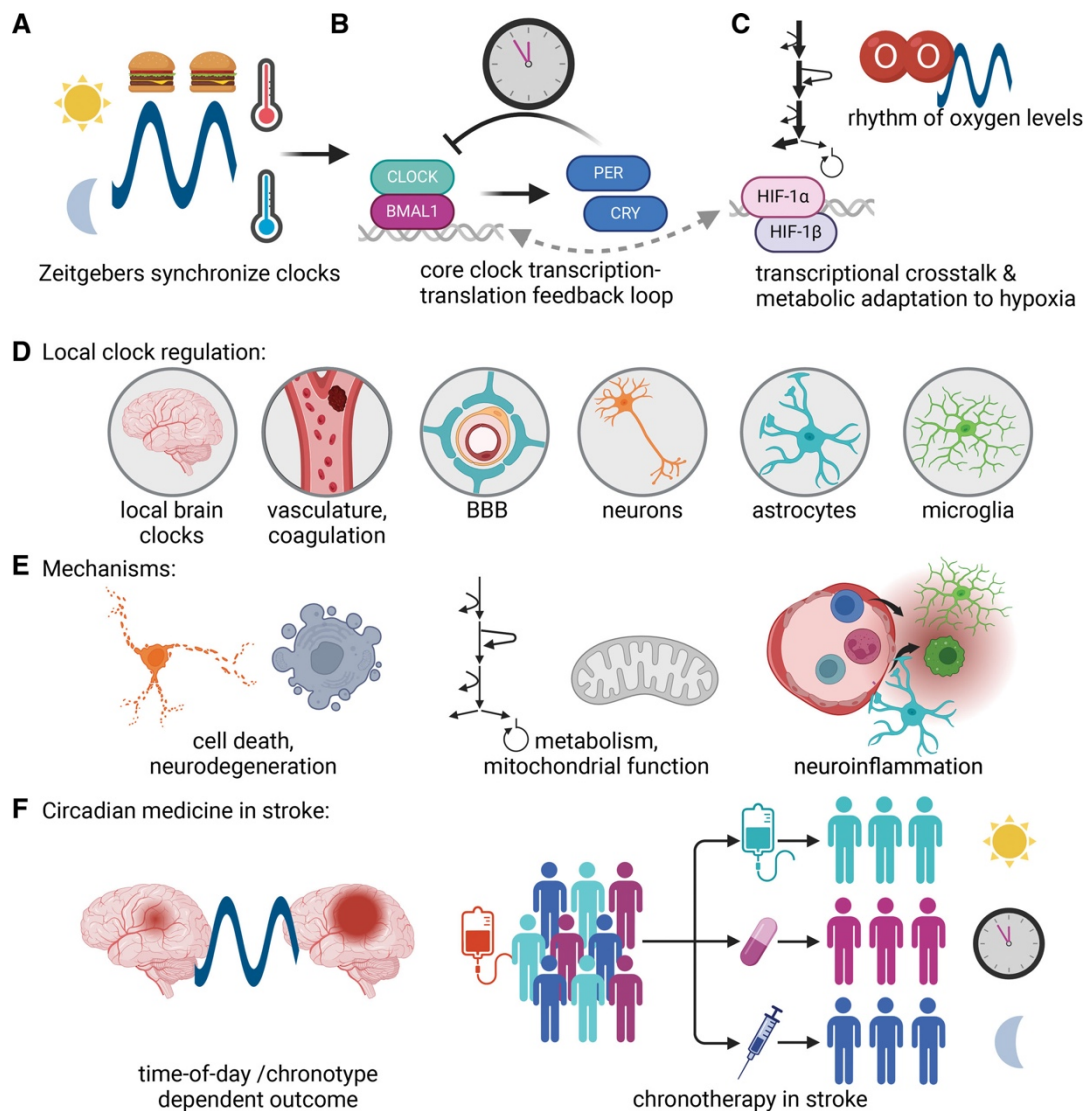


Figure 1.5. Overview of the mechanisms linking circadian rhythms and stroke (Mergenthaler et al., 2024)

The autophagy machinery plays a crucial role not only in cellular bioenergetics but also in maintaining protein quality control (Y. Xie et al., 2015). Autophagic flux has been found to be upregulated during the awake period and is associated with smaller infarct sizes in murine models of stroke (Buckley et al., 2014). A key regulator of autophagy, mTOR (Y. C. Kim & Guan, 2015), is under circadian modulation, influenced by the entrainment of the SCN to light and other signals — such as energy homeostasis tightly regulated by circadian rhythms (Ramanathan et al., 2018).

Recent research has demonstrated that the immune response to stroke is dependent on the time of stroke onset (Kamat et al., 2022). Specifically, after tMCAO, T-cell counts in both the bloodstream and brain infiltration were higher during the animals' sleep phase compared to their awake phase (Esposito et al., 2022). Furthermore, T-cell levels in the bloodstream correlated with infarct volume only during the sleep phase, with no such association observed during wakefulness (Esposito et al., 2022). This highlights the critical influence of circadian timing on post-stroke immune responses and outcomes (Hergenhan et al., 2020).

1.6.6. Circadian biomarkers in stroke

Circadian rhythms can be assessed using circulating physiological biomarkers (Reid, 2019). The diurnal variation of melatonin production is commonly used as a marker of endogenous circadian timing (Lewy et al., 1999). Melatonin itself can also modulate circadian phase (phase-shifting) and promote sleep by reducing the firing rate of SCN neurons, thereby creating a sleep-permissive state (Dubocovich, 2007). Melatonin levels can be evaluated in biological fluids, such as plasma and saliva, while its major metabolite, 6-sulphatoxymelatonin (aMT6s), is measured in urine. Patients with AIS have been reported

	Circadian variation
Clinical features	
Risk	Shift work, jet lag, weekday-weekend activity transition increase stroke risk factors
Onset	Morning surge for ischemic stroke, TIA, and hemorrhagic stroke
	Morning surge for LAA, CE, SV, and CRY ischemic stroke
Presenting severity	Lesser deficit severity for ICH with daytime onset
Physiology	Heart rate, blood pressure, temperature higher in daytime
	Prothrombotic factors higher in daytime
	Inflammatory responses increased in daytime
Outcomes	Increased mortality for ICH and SAH with morning onset
	More frequent ICH hematoma expansion with daytime onset
	More good outcomes for thrombolytic-treated ischemic stroke with daytime presentation
Chronopharmacology	Antihypertensives more effective with evening administration
	Low-dose aspirin more effective with evening administration
Biomarkers	
Melatonin	Night-time levels lower in acute stroke
Cortisol	Reduced circadian rhythmicity after stroke
PAI-1	Early morning peak

Table 1.1. Circadian Variation in Clinical and Biomarker Features of Human Stroke (Lo et al., 2021)

to exhibit lower nocturnal circulating and urinary melatonin levels compared with healthy controls (Q. Sun et al., 2024). Similarly, patients with post-stroke insomnia demonstrate reduced circulating melatonin levels, which can be restored with light therapy (W. H. Kim et al., 2022).

Another important circadian marker is cortisol, which regulates approximately 60% of the synchronisation of the peripheral circadian transcriptome (Fagiani et al., 2022). In stroke, cortisol rhythmicity is often disrupted, supporting the hypothesis that circadian disturbances are common in this population (Andreadi et al., 2025; Walker et al., 2020). However, further research is needed to determine whether melatonin or cortisol can serve as reliable markers for identifying patients at risk of circadian disruption, as well as for monitoring circadian changes post-stroke. Validated circulating biomarkers could also help assess the impact of lesion size, lesion location, and time of onset on circadian rhythm integrity.

Such biomarkers may also guide the optimisation of drug regimens. Certain factors, such as PAI-1, exhibit circadian variation, and their levels vary according to the time of stroke onset (Lo et al., 2021). These fluctuations in haemostatic factors could influence responsiveness to thrombolytic therapy. In addition, circadian biomarkers may provide insights into stroke progression and outcomes — for example, by correlating penumbra and core lesion volumes with circulating markers of neuroaxonal injury to determine whether strokes occurring at different times of day follow distinct trajectories of tissue damage (Kamat et al., 2022; Reidler et al., 2023). Finally, circadian biomarkers may help identify persistent circadian misalignment in the post-stroke period.

1.6.7. Targeting circadian rhythms in clinical applications

Clinical study on evaluation of time-of-day effects in IVT provide initial evidence that improved outcomes are correlated with daytime treatment (Burbano et al., 2023). An evaluation of data from the SITS registry, recruiting a cohort of approximately twenty-thousand individuals, indicated that therapeutic interventions during daytime hours (08:00–19:59) were associated with a slightly higher likelihood of favourable clinical outcomes, as well as a potential decrease in the occurrence of symptomatic SAH (Körv et al., 2014; Toni et al., 2012). Similarly, diurnal administration of IVT with alteplase correlates with higher recanalization rates and improved outcomes at 90 days post-treatment (Vilas et al., 2012). However, a small cohort study of AIS patients treated with IVT found no significant influence of treatment timing on clinical outcomes (Stan et al., 2023).

Similarly, investigations on circadian effects of EVT on clinical outcomes have yielded mixed results (Sreekrishnan et al., 2024). Several studies suggest better outcomes when EVT is

administered during morning hours, while one study reported improved outcomes for patients admitted between 18:00 and 08:00 (Hajdu et al., 2021). Additional research, grouping patients into four 6-hour time blocks in accordance with recent consensus recommendations, found a significant association between treatment timing and 90-day clinical outcomes, with patients treated between 05:00 and 10:59 exhibiting superior recovery compared to other time intervals (Truksinas et al., 2022). As reported in the SELECT Late study, the beneficial effects of EVT seem to be more pronounced in patients with stroke onset at specific times of the day (Sarraj et al., 2023).

The timing of symptom onset in relation to *zeitgeber* signals may not only serve as a prognostic indicator but could also modulate the efficacy of neuroprotective interventions, reperfusion strategies, and rehabilitation protocols (Allada & Bass, 2021). For instance, therapies targeting excitotoxicity, inflammation, or oxidative stress may demonstrate time-dependent differences in effectiveness, reflecting the molecular and physiological states governed by the circadian system (Ruan et al., 2021). The development of chronopharmacology and chronotherapeutics may contribute to time-tailored therapeutic approaches that optimise outcomes in patients with stroke.

1.7. THE GLYMPHATIC SYSTEM

Firstly described *in vivo* in 2012 by Iliff et al. (J. J. Iliff et al., 2012), the glymphatic system consists of a glial cell-dependent lymphatic- system, responsible for the transport of CSF (*Figure 1.6*). This system is crucial for tissue homeostasis and promotes the efficient elimination of waste products, such as protein aggregates or toxic metabolites (Hablitz & Nedergaard, 2021). Before this finding, the clearance processes were largely attributed to intracellular and extracellular degradation mechanisms, as well as solute exchange across the BBB. Following this report, more evidence has emerged showing how this clearance system contributes to the distribution of non-waste molecules such as lipids, glucose, nutrients and neurotransmitters within the brain (Jessen et al., 2015). This system is mainly active during sleep, when it supports the removal of toxic compounds (L. Xie et al., 2013). In contrast to peripheral organs — where lymphatic vessels drain proteins and interstitial fluids (Louveau et al., 2016) — the CNS lacks a conventional lymphatic system (Hladky & Barrand, 2018). Thus, the discovery of the glymphatic system has been crucial in advancing

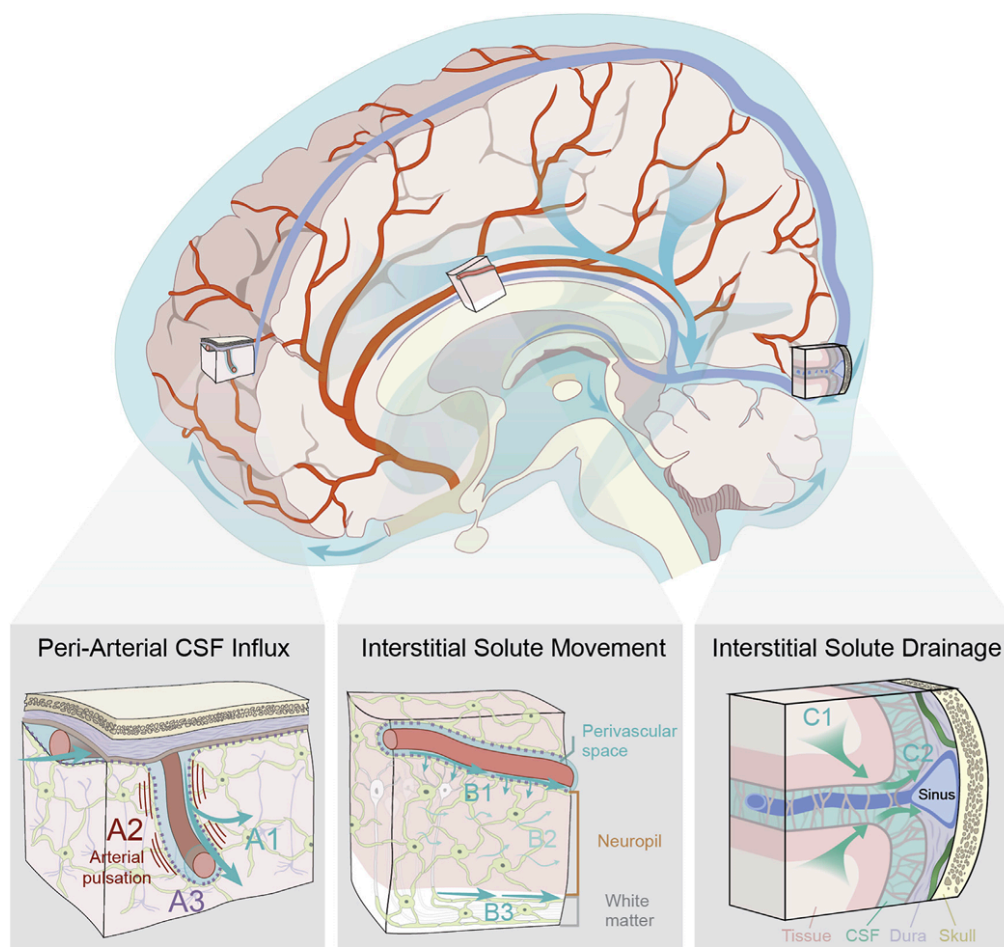


Figure 1.6. The organization of the glymphatic system (Bohr et al., 2022)

our understanding of how fluids and solutes circulate in the brain (Licastro et al., 2024). Dysfunctions of this system may contribute to the pathophysiology of neurological disorders, including stroke, emphasizing its potential as a promising target for therapies that could help restore brain fluid dynamics.

1.7.1. The physiology of the glymphatic system

The CSF is a colourless fluid that surrounds and protects the CNS, moving through a network of cavities: ventricles, subarachnoid space in the brain and the central canal of the spinal cord (Telano & Baker, 2022). The CSF is mainly produced by the choroid plexus epithelium, from which it flows through the four ventricles into the subarachnoid space. CSF production and absorption are tightly regulated, maintaining a total volume of 150-270 mL within the CNS (Bothwell et al., 2019). In humans, CSF production is under circadian regulation, with higher production at night (Nilsson et al., 1992, 1994).

From the subarachnoid compartments, CSF is transported into perivascular spaces (PVS) through areas called Virchow-Robin spaces, which surround the penetrating arteries of the brain parenchyma (Mestre et al., 2017; Wardlaw et al., 2020). The PVS have a pivotal role in supplying energy substrate and regulating blood flow. Given these functions, the PVS have a critical impact on neurological disorders associated with blood flow impairment, including stroke. Indeed, it has been extensively shown that during stroke, the neuroinflammatory reaction and edema formation often originate in the PVS (Gherzi-Egea et al., 2018).

The aquaporin-4 (AQP4) – a water channel mainly expressed in astrocyte end-feet, as well as in ependymal epithelial cells and choroid plexus epithelial cells (Day et al., 2014) – is a major contributor to CSF flow within the interstitium where it communicates with the interstitial fluid (ISF), aiding the clearance of toxic compounds through the PVS (Salman et al., 2021). Then, efflux fluids are drained into lymphatic vessels of the meninges and subsequently into the bloodstream through cervical lymphatic vessels (Hladky & Barrand, 2018).

Several findings have established the role of AQP4 in the GS, as it represents the main clearance pathway of interstitial fluid solutes from the brain (J. Iliff & Simon, 2019; Plog & Nedergaard, 2017). Conversely, CNS disorders, including epilepsy, edema, stroke are associated with altered AQP4 activity and expression. In particular, the polarization of AQP4 at the perivascular astrocytic end-feet contributes to glymphatic fluid movement and A β clearance in rodents (Mestre et al., 2018; Simon et al., 2022). As a result, AQP4 plays a crucial role in promoting glymphatic function and waste product clearance. Consequently,

AQP4 knockout mice displayed an impaired glymphatic transport and clearance of A β compared to wild-type littermates (Mestre et al., 2018; Simon et al., 2022). Moreover, inhibition or downregulation of AQP4 activity – often observed during stroke or cerebral edema – results in a reduction in edema size and formation. However, in vasogenic edema, the loss of AQP4 function exacerbates the injury, due to the reduced capacity to withdraw excessive water from the brain parenchyma (Manley et al., 2009; Vella et al., 2015). Targeting glymphatic function and AQP4 polarization may represent a promising therapeutic strategy to mitigate tissue injury and improve neurological outcomes (Licastro et al., 2024).

1.7.2. Regulation of the glymphatic system

Multiple mechanisms, including arterial pulsation arising from the cardiac systolic pressure (Mestre et al., 2018), respiration, CSF pressure gradients, and sleep (Chong et al., 2022; L. Xie et al., 2013), modulate the CSF dynamics, facilitating the clearance function. Age is also a factor influencing the glymphatic system, whose activity is reduced with aging (L. Li, Ding, et al., 2022; Nedergaard & Goldman, 2020).

In preclinical studies, Iliff et al. demonstrated that the administration of dobutamine, an adrenergic agonist that enhances cardiac contractility, increases the arterial pulsatility, thus promoting greater CSF penetration into the brain parenchyma (J. J. Iliff et al., 2013). Similar findings were corroborated by other studies, in which mice that underwent ICA ligation showed a reduced CSF influx (Venkat et al., 2017), suggesting that cardiac failure may impair glymphatic fluid exchange by reducing vascular tone. More recent evidence highlights the relationship of cardiac and respiratory cycles with CSF pulsatility, respectively involved in periarterial CSF movements and centripetal perivenous fluid movements (Kiviniemi et al., 2016). As mentioned earlier, the GS works predominantly during sleep. In rodents, the rest phase is characterized by enhanced CSF influx and improved A β removal (L. Xie et al., 2013). This function seems to be related to an increase in the interstitial space volume, likely because of reduced locus coeruleus–dependent noradrenergic activity. Similar findings were obtained in humans, wherein sleep improves glymphatic clearance efficiency compared to awake states (S. Lee et al., 2021) and sleep deprivation (Eide et al., 2021). Based on these findings, sleep promotes a shift of the brain into a functional state that improves the glymphatic clearance of waste products of neural activity accumulating during the awake phase (Boespflug & Iliff, 2018).

The extent to which glymphatic fluid movement is under circadian control remains debated, particularly in light of the circadian regulation of cardiovascular function and sleep (Thosar et al., 2018). Recent evidence has revealed that glymphatic function exhibits daily

rhythmicity, peaking around mid-day in mice, when they are in an inactive/sleep state (Hablitz et al., 2020). Furthermore, circadian rhythms regulate day/night variations in glymphatic dynamics, including solute clearance and drainage to the lymph nodes.

These oscillations may be dependent on two main mechanisms: (1) the suppression of the periarterial influx when the brain is active, and (2) the reduction of AQP4 polarization, which decreases CSF/ISF dynamics (Hablitz & Nedergaard, 2021; L. Xie et al., 2013). Given that the deletion of AQP4 abolishes diurnal variation of CSF movement and lymphatic drainage, the circadian regulation of GS may be subject to the circadian regulation of AQP4.

In the SCN, astrocytes may be responsible for setting behavioural cyclicity (Brancaccio et al., 2017; Duhart et al., 2013), and may also contribute to the circadian control of bulk fluid movement through CSF–interstitial fluid exchange (Hablitz et al., 2020).

Although there is a growing body of data, it is still unclear how sleep exerts its restorative effects and how its disruption impairs brain function.

1.7.3. Glymphatic dysfunction in neurological disorders

Given the multiple critical roles of CNS, dysfunction of the glymphatic pathway has been associated with neurologic diseases, including AD, Parkinson's disease, traumatic brain injury, epilepsy or diseases with vascular defects such as type 2 diabetes, all of which are characterized by reduced cerebral clearance and impaired CSF movements (Braun & Iliff, 2020; T. Lv et al., 2021; Rasmussen et al., 2018).

Aging brains have been shown to exhibit reduced penetrating arterial pulsatility, resulting in diminished glymphatic CSF influx and accumulation of interstitial solutes, including A β (Kress et al., 2014). Some hallmarks of glymphatic impairment include reduced vascular compliance and AQP4 mislocalisation, which lead to a reduction in CSF influx (Rasmussen et al., 2018). In both rodent models and human, the presence of soluble and insoluble A β plaques has been related to AQP4 mislocalisation and CSF influx reduction (W. Peng et al., 2016; Z. Xu et al., 2015). In patients with AD, a reduced CSF clearance rate is inversely correlated with A β levels (De Leon et al., 2017). Post-mortem analyses of AD brains revealed AQP4 mislocalization and an increase in total AQP4 expression compared with individuals without the disease (Zeppenfeld et al., 2017).

In stroke, the excessive accumulation of fluids causes edema, BBB disruption, neuroinflammation which contributes to glymphatic system impairment. These alterations in glymphatic efficiency may contribute significantly to brain edema complications (X. Zhou et al., 2021). During CNS injury, AQP4 redistributes from the perivascular astrocytic end-feet to other regions, suggesting a role for the GS in fluid outflow during the recovery stage after

brain edema (J. Iliff & Simon, 2019). Although initially edema or neuroinflammation processes can initially have harmful effects, these responses are also essential for subsequent cell debris removal (Willis et al., 2013). As a result, the function of the glymphatic system in the pathophysiology of brain edema can be more complex.

During ischemic stroke or other CNS injuries (Golanov et al., 2018; Goulay et al., 2017), alterations in AQP4 expression and distribution, as well as reduced CSF influx, have been reported (Gaberel et al., 2014). In particular, the reduction of arterial pulsation caused by vessel occlusion may explain the impairment of GS. Following 24 hours of reperfusion from MCA occlusion, the glymphatic perfusion returned to normal values (Gaberel et al., 2014). These observations likely reflect the consequences of glial hyperplasia, the loss of AQP4 polarization, and in turn reduced expression of the total AQP4.

1.7.4. Molecular targets of the glymphatic system in the treatment of stroke

Future directions targeting the mechanisms underlying glymphatic system's role in the brain can be fundamental to promote the recovery and improve patient outcomes after stroke. Among potential therapeutic targets, AQP4 has emerged as a key candidate due to its pivotal role in CSF dynamics (Manley et al., 2009). Several AQP candidates have been developed (Beitz et al., 2015; Soveral & Casini, 2017); however, they failed in clinical translation due to pharmacokinetic issues and adverse side effects.

Recent findings have identified miRNAs (O'Brien et al., 2018) as endogenous modulators of AQP expression and polarization (Gomes et al., 2018). Among them, several findings have highlighted the role of miR-320a, which suppresses AQP1 and AQP4 gene expression both *in vitro* and *in vivo* in a cerebral ischemia rat model, whereas anti-miR-320a increases AQP1 and AQP4 expression with consequent reduction of infarct volume (Sepmaniam et al., 2010). Additionally, several research groups have demonstrated that AQP4 down-regulation mediated by miR-145 (L. Zheng et al., 2017), miR-130b (Y. Zheng et al., 2017) and miR-29b (Y. Wang et al., 2015) is neuroprotective against IS. Recent findings have further indicated that AQP4 silencing, concomitant with upregulation of miR-224 and miR-19a, may reduce astrocytic connectivity and alter parenchymal fluid movement (Jullienne et al., 2018). However, how modulation of AQP4 protein expression affects AQP4 localization at astrocytic end-feet, and ultimately glymphatic flow, remains unclear and warrants further investigation. The answer may guide the identification of promising therapeutic targets in cerebral ischemic stroke.

1.7.5. Methods to study the glymphatic system

Imaging techniques have been extensively used to observe and describe the glymphatic system. These technologies allow researchers to dissect and study the CSF dynamics: CSF influx via PVS, CSF-ISF exchange, and CSF clearance via the lymphatic pathway (Wardlaw et al., 2020). The visualization of these dynamics can be assessed either *in vitro* or *ex vivo*, as illustrated in *Figure 1.7*.

1.7.5.1. *In vivo* imaging

The first *in vivo* visualization of the glymphatic system was performed using two-photon microscopy (J. J. Iliff et al., 2012). Fluorescent tracers are traditionally administered into the cisterna magna (CM), the brain parenchyma, or the periphery, allowing visualization *in vivo* or *ex vivo*. Tracer distribution depends on molecular size:

- Small-molecular-weight tracers (>1-10 kDa) usually distribute around the penetrating periarterial spaces into the cortical ISF after injection. They allow the visualisation of both influx and clearance.
- Medium-molecular-weight tracers (>40 kDa) usually do not diffuse into the ISF, remaining trapped around the injection site and astrocytic end-feet.
- Very large tracers are useful for labelling the perivascular spaces.

Moreover, depending on the animal's sleep state, they provide information about tracer transport and distribution (Jessen et al., 2015).

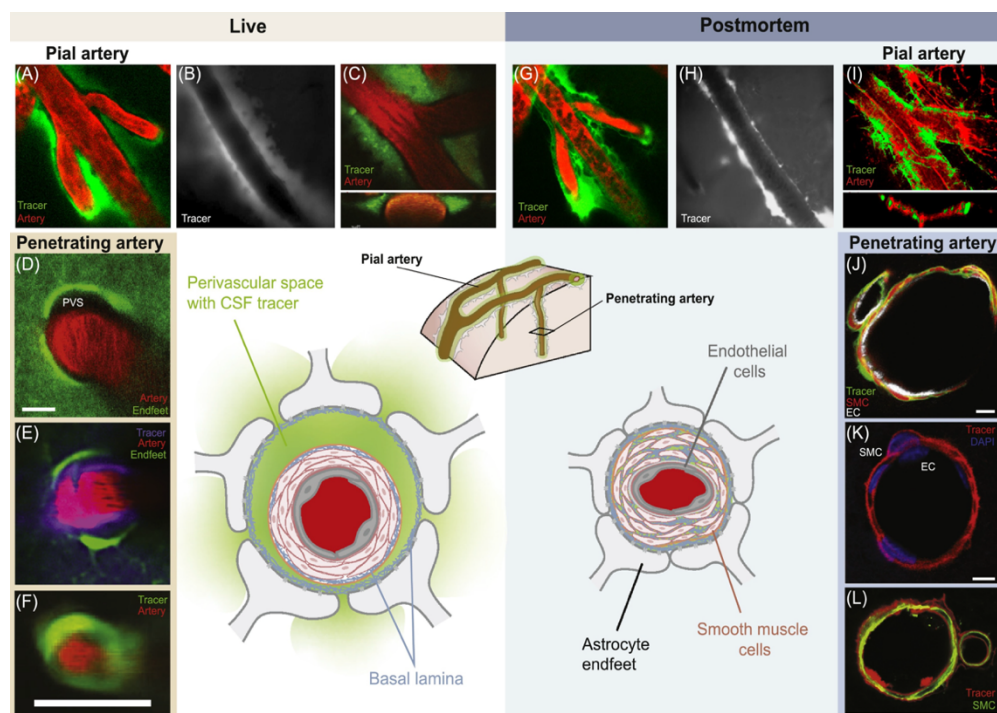


Figure 1.7 CSF tracers in the perivascular spaces (Mestre et al., 2020)

The two-photon microscopy offers high resolution, but its tissue penetration is limited (up to ~200 μm depending on contrast) and often requires the creation of a cranial window. Moreover, only a small field on the cortical surface can be visualized.

In contrast, epifluorescence microscopy may be used to visualize the entire dorsal cortex without affecting the integrity of the skull. This approach is useful in awake mice to track the perivascular CSF tracer influx. However, its main limitation remains the lack of depth resolution, making it difficult to differentiate tracers in the subarachnoid space from those within cortical tissue (Mestre et al., 2020).

More advanced approaches include the use of MRI, which employ gadolinium-based contrast agents (GBCAs), to assess glymphatic function by measuring signal changes in the tracer concentration or in brain water content (e.g., phase-contrast MRI, blood-oxygen-level-dependent MRI) (Iyad et al., 2023). This technique can be used in human to study glymphatic dynamics. Through this imaging technique, (Ray et al., 2024) have confirmed that BBB leakage in AD patients correlates with cognitive decline, reflecting the role of glymphatic dysfunction in AD pathology.

Other techniques to investigate water movement, as a reflection of glymphatic activity, are the diffusion tensor imaging along the perivascular space (DTI-ALPS) (Boyd et al., 2024), computed tomography (CT) and positron emission tomography/single-photon emission computed tomography (PET/SPECT) scans (Boyd et al., 2024; Lilius et al., 2022). These methods are useful tools to measure not only fluid diffusion around the PVS, but also CSF tracer influx and clearance in vivo.

1.7.5.2. *Ex vivo* analysis

After tracer infusion into the CSF or brain parenchyma, brain slices can be analysed to assess tracer distribution, particularly providing information on efflux and clearance (Bork et al., 2024). These slices can be processed with immunofluorescence protocols combined with neuronal or glial markers to determine whether CSF tracers localize within the PVS or the NVU. Imaging is typically performed using confocal laser scanning microscopy. *Ex vivo* techniques are also useful for investigating other key components of the glymphatic system, such as dural lymphatics, which play a crucial role in immune surveillance (Ayyappan et al., 2025). An alternative to traditional *ex vivo* imaging is light-sheet microscopy. Although more costly, this technique enables whole-brain imaging within approximately 25 minutes by illuminating samples with a thin sheet of light, exciting fluorophores across multiple slices and reconstructing a 3D image of tracer distribution (Hubert et al., 2025).

2. AIM OF THE STUDY

Annually, more than 15 million people suffer from brain injury, with 5 million dying and another 5 million remaining permanently disabled (Campbell & Khatri, 2020). Emerging evidence suggests that impairment of the glymphatic system — a brain-wide clearance network that relies on aquaporin-4 (AQP4) dynamics — may play a critical role in the development of brain injury (Licastro et al., 2024). While pharmacological modulation of AQP channels has faced limitations due to poor selectivity and toxicity, recent findings suggest that miRNAs may act as endogenous regulators of AQP4 expression and localization, providing an innovative therapeutic approach. Understanding the molecular mechanisms that regulate glymphatic function after stroke could provide novel strategies to enhance fluid clearance, reduce edema, and improve neurological recovery.

The ischemic tolerance achieved by remote ischemic postconditioning has shown remarkable neuroprotective potential (Hess, Blauenfeldt, et al., 2015). RIC activates endogenous protective mechanisms involving both neural and humoral pathways that transmit signals from the limb to the brain. Experimental studies demonstrate the RIC-induced neuroprotective effects on infarct volume, cerebral blood flow, and cognitive and functional recovery (Cuomo et al., 2024; Pignataro, 2021; Torres-Querol et al., 2021). Importantly, RIC may also influence circulating miRNA profiles, raising the possibility that it could directly or indirectly modulate glymphatic function and AQP4-related mechanisms.

Therefore, the primary aim of this study is to unravel the mechanisms by which glymphatic dysfunction and remote ischemic conditioning interact in the pathogenesis of stroke, with the final goal of identifying novel therapeutic strategies to enhance fluid clearance and improve stroke recovery. Specifically,

1. Characterize alterations in glymphatic clearance following ischemic stroke.
2. Elucidate the molecular and cellular pathways underlying glymphatic dysfunction in the post-stroke brain.
3. Evaluate whether RIC can restore or enhance glymphatic function and improve neurological outcomes after stroke.
4. Investigate the role of microRNAs as endogenous modulators of AQP4 expression and their potential use as biomarkers.

The expected outcomes primarily aim to: (a) clarify the mechanisms of the glymphatic pathway and support the development of treatments that modulate this system as complementary therapies for post-stroke recovery; (b) investigate how RIC influences glymphatic function and AQP4-related mechanisms, offering insights into alternative therapeutic strategies for stroke patients.

Taking this premise into account, the second part of the study aims to provide insights into translation from preclinical to clinical studies. Although both preclinical and clinical data show promise, the effectiveness of remote ischemic postconditioning in treating acute stroke in humans remains uncertain (Esposito et al., 2020). While some clinical studies have demonstrated beneficial effects, others have failed to show significant outcomes. Such translational failures in neuroprotection are likely due to fundamental differences between animal models and humans, including comorbidities, age, sex, genetic variability, and other physiological factors. Importantly, the timing of stroke onset may critically influence the effectiveness of therapeutic interventions. Furthermore, the opposite circadian cycles in nocturnal rodents compared with diurnal human patients may also contribute to translational challenges (Lo et al., 2021).

Based on this premise, the secondary aim of the study is to establish whether these circadian differences represent a potential reason for translational difficulties. In particular,

- Evaluate whether experimental stroke exhibits circadian variations.
- Determine whether the protective effect of RIC depends on the time of day of its application.
- Elucidate the molecular pathways underlying the potential diurnal variations in RIC-mediated neuroprotection.
- Investigate the contribution of circadian-regulated systemic factors in modulating the neuroprotective efficacy of RIC.

Ultimately, by integrating these premises, this thesis aims to bridge the translational gap between experimental models and clinical practice, paving the way for more effective, personalized therapies for stroke recovery. Understanding how circadian rhythm affects remote postconditioning could help define therapeutic targets, discover biomarkers and develop potentially effective therapy in stroke physiopathology.

3. MATERIALS AND METHODS

3.1. Cell cultures

C2C12 cells were obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin–streptomycin, and 2 mM L-glutamine in a humidified incubator at 37 °C with 5% CO₂.

For primary astrocyte cultures, cerebral cortices were isolated from postnatal day 1–3 mouse pups. After decapitation, the brains were rapidly removed under sterile conditions. Meninges and visible blood vessels were carefully removed to prevent contamination by meningeal fibroblasts and endothelial cells. Cortical tissues were then mechanically dissociated using fine forceps and enzymatically digested with 0.25% trypsin and DNase/MgSO₄ at 37 °C for 30 min. The single cell suspension was centrifuged at 1000 rpm for 5 min and suspended in DMEM supplemented with 100U/mL penicillin, 100 µg/mL streptomycin and 10% FBS. The cells were plated in 25 cm² T-flasks, then cultured in a humidified incubator at 37°C with 5% CO₂. The day after plating, astrocytic primary cells were washed with PBS, and the medium was replaced every two days. When cells grew to confluence (10-14 days), the flasks were shaken at 37°C to remove loosely attached microglia and oligodendrocyte precursor cells. The remaining attached enriched astrocytes were used for AQP4 silencing experiments. In particular, after 15-20 DIV, cells were plated in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 2 mM L-glutamine on glass coverslips pre-coated with Poly-D-Lysine and subjected to transient transfection.

3.2. Cell transfection

For C2C12 cells, AQP4 knockdown was achieved by transient transfection with three preselected siRNAs targeting mouse AQP4 mRNA (siAQP4#1, ID: 160736; siAQP4#3, ID: s62529; siAQP4#4, ID: s62530; Thermo Scientific). Transfections were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 2 mM L-glutamine. After 24 hours, the culture medium was replaced with Opti-MEM. The siRNA–Lipofectamine 2000 complexes were prepared at final concentrations of 50, 25, or 10 nM in Opti-MEM. Cells were incubated with the transfection mixture or the negative control siRNA (reported as CTR in the figures) for five hours at 37 °C in a CO₂ incubator, after which the medium was replaced with fresh complete medium. Twenty-four hours post-transfection, cells were collected for Western Blot analysis.

Similarly, primary astrocyte cultures were seeded onto Poly-D-Lysine–coated glass coverslips in fresh complete medium. Twenty-four hours after seeding, cells were incubated

with siRNA–Lipofectamine 2000 complexes (siAQP4#3, final concentration 10 nM, or siCTR (CTR)) for 3 hours, followed by replacement with fresh complete medium for an additional 24 hours. The cells were then processed for immunocytochemistry analysis.

3.3. Animals

All experiments were conducted in accordance with protocols approved by the Animal Care Committee of “Federico II”, University of Naples, Italy.

Male C57BL/6J mice (weighing 23 to 28 g, sourced from Charles River Laboratories) were randomly housed for three weeks prior to surgery and for 24 hours or 3 days post-surgery in two separate rooms. The standard light cycle (LD) room has 12 hours of light from 7 a.m. to 7 p.m. and 12 hours of darkness from 7 p.m. to 7 a.m., while the reversed light cycle (DL), had the opposite schedule with 12 hours of darkness from 7 a.m. to 7 p.m. and 12 hours of light from 7 p.m. to 7 a.m. Mice from the LD room were used for the study of remote conditioning and glymphatic system; for the study of the circadian rhythms, animals from both rooms were randomly divided into two groups: one group received remote limb postconditioning, while the other group received standard poststroke care.

During any surgical or invasive procedure, animals were anesthetized using 2% sevoflurane, 60% N₂O, and 38% O₂, and the rectal temperature was maintained at 37±0.5°C with a heat-controlled mat (Harvard Apparatus).

3.4. Experimental model of transient focal cerebral ischemia

Transient focal ischemia was induced by inserting a 6-0 surgical monofilament nylon suture (Doccol) through the internal carotid artery (ICA) into the middle cerebral artery (MCA) for 60 minutes (Ginsberg & Busto, 1989; Longa et al., 1984).

Briefly, under an operating stereomicroscope (Nikon SMZ800, Nikon Instruments, Florence, Italy), after identification and exposure of the right common carotid artery (CCA), the external carotid artery (ECA) near the bifurcation was cut and electrocauterized to create a stump. The 6-0 filament was inserted through the stump and gently advanced from the ECA into the ICA until it blocked the origin of the MCA. After 60 minutes, the filament was removed to allow reperfusion. Adequate ischemia was confirmed by continuous Laser Doppler Flowmetry (LDF) (Perimed, North Royalton, OH, USA). Animals that did not show a significant reduction in LDF values to less than 30% of baseline during MCAO were excluded. Rectal temperature was monitored and maintained at 37 ± 0.5 °C using a thermostatically controlled heating pad during surgery and a heating chamber for 10 minutes after surgery.

3.5. Remote limb ischemic postconditioning model

Remote conditioning was performed through a single cycle (10 minutes) of transient occlusion of the femoral artery (FAO) applied 10 minutes after the transient occlusion of the MCA, as previously described (Pignataro et al., 2013, 2009). Specifically, the femoral artery was isolated and a micro-serrafine clip was placed to transiently block blood flow for 10 minutes. After one cycle of FAO, mice were placed back to their cages.

3.6. Evaluation of the ischemic volume

The infarct area was evaluated by 2,3,5-triphenyl tetrazolium chloride (TTC) staining. Specifically, the brains were removed at 24 or 72 hours after stroke and cut through a brain matrix into 1 mm coronal slices and incubated with 2% TTC. The ischemic volume of 8 sections was calculated using the “indirect” morphometric method with ImageJ software.

3.7. Evaluation of neurological behaviour

Functional outcome after stroke was assessed by neurological severity scores, foot-fault test, and adhesive tape removal test, as previously reported (Licastro et al., 2025).

Modified Neurological Severity Score

Total motor scores assess spontaneous activity (1, does not rise up; 2 does not approach to all cage sides; 3, normal exploration), motor (1, minimal movement of 1 side; 2, abnormal walking and mild weakness; 3, normal symmetry), climbing (1, hang for seconds and falls; 2, hangs but cannot displace mesh; 3, hangs and displace mesh), balance and coordination (1, holds but falls; 2, holds but unable to walk; 3, holds but able to walk). Total sensory scores evaluate body proprioception (1, no reaction body; 2, slowed reaction; 3, normal), vibrissae (1, no reaction; 2, slowed reaction; 3, normal), forepaw tactile response (1, no reaction; 2, slowed reaction; 3, normal), and hind-paw tactile response (1, no reaction; 2, slowed reaction; 3, normal). Total modified Neurological Severity Score (mNSS) was obtained by summing total motor and sensory scores.

Foot-Fault Test

Mice were placed on elevated square grids, free to move their forelimbs and hindlimbs along the wires of the grid. During 2 minutes of recording, each paw that fell or slipped while moving across the grid was scored as a foot fault. The total number of steps and total number of foot faults for each paw were counted (Hayakawa et al., 2016).

Adhesive Tape Removal Test

Adhesive tape strips were placed on each forepaw of mice. During 300 seconds of recording, the time to contact, defined as the moment when the mouse reacts to the

presence of the tape, and the time to remove were measured. Data represent deficits revealed by the removal time of the adhesive tape on the contralateral side (left side) (Bouet et al., 2009).

3.8. Drugs and chemicals

7-Nitroindazole (25 mg/kg, intraperitoneal) was dissolved in dimethyl sulfoxide solution. The drug solution was freshly prepared before use and administered 30 minutes after cerebral ischemia induction (Pignataro et al., 2013).

SiRNA control (CTR) or siRNA against AQP4 (siAQP4#3, 10 μ L, 5 μ M) was reconstituted in 0.9% NaCl solution and administered 30 minutes before tMCAO and 3 hours post-reperfusion at the following coordinates from bregma: AP -0.6 mm, LL -1.6 mm, depth -3.2 mm. At 24 hours post-reperfusion, mice were euthanized, and TTC staining was performed on brain slices. The ipsilateral striata were collected for Western Blot analysis.

3.9. Nitric oxide detection with the Griess method

Nitric oxide production was evaluated in blood serum by the measurement of nitrite using the Griess method (Nitrite Assay Kit MAK367 Sigma-Aldrich) (Giustarini et al., 2004). The sera were reacted with the same volume of Griess reagents: Griess Reagent I (0.1% N-1-naphthylenediamine dihydrochloride) and Griess Reagent II (1% sulphanilamide in 1% phosphoric acid) for 10 minutes, and absorbance at 540 nm was measured by a spectrophotometer. Background correction was achieved using a mix between Griess I and Griess II as the blank. Concentration of nitrite in serum samples was extrapolated from a nitrite standard curve.

3.10. Plasma sample collection

Blood from mice was collected 24 hours post stroke in an EDTA-tube. After centrifugation at 3000 rpm for 8 minutes at 4°C, the supernatant was removed and the whole blood was used for RNA extraction, as described in the following paragraph.

Whole blood was collected from ischemic patients hospitalized in the stroke unit of “Azienda Ospedaliera Universitaria Federico II, Napoli”. The study protocol for the use of human plasma samples was approved by the Ethics Committee of the same hospital. Plasma was collected after centrifugation at 3000 rpm for 8 minutes at 4°C, and immediately stored at -80°C. The resulting pellet was used for RNA extraction.

An observational pilot study involving patients affected by obstructive sleep apnea syndrome (OSAS) was carried out at Department of Neuroscience, ENT Unit of AOU Federico II.

Inclusion criteria were about sleep apnea episodes or continue snoring during night with an increase of daily fatigue. Blood samples of the participants (one healthy control and seven patients) were collected by venous blood drawn. Plasma was collected after centrifugation at 3000 rpm for 8 minutes at 4°C, and immediately stored at -80°C. The resulting pellet was used for RNA extraction.

The sample size was determined on the basis of feasibility and the availability of eligible participants during the recruitment period: the sleep apnea cohort used to investigate glymphatic dysfunction–associated microRNAs, and the circadian-stroke cohort employed to assess time-of-day–dependent variations in circulating miRNA profiles.

3.11. Whole Blood RNA Extraction

RNA was extracted from whole blood samples using the GENEJET Whole blood RNA Purification Kit (K0761 - Thermo Scientific™) according to the manufacturer's protocol. Whole blood (500µL) was centrifuged at 3000 rpm for 8 minutes at 4°C. After removing the supernatant, the whole blood was mixed with 600 µL of Lysis Buffer supplemented with β-mercaptoethanol to enhance RNA stability. The lysate was vortexed vigorously and incubated for 5 minutes at room temperature. After incubation, 600 µL of 70% ethanol was added to the lysate, and the solution was mixed thoroughly. The mixture was then transferred to a purification column and centrifuged at 12,000 x g for 1 minute to allow RNA binding. The column was washed with Wash Buffer I and Wash Buffer II followed by centrifugation at 12,000 x g for 1 minute after each wash step. Residual ethanol was removed by an additional spin at full speed. The RNA was eluted with 20 µL of Nuclease-Free Water.

3.12. MicroRNA Reverse Transcription

For miRNA quantification, reverse transcription was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Invitrogen). In brief, 10 ng of total RNA (2µL) was

miRNA Reverse Transcription Mix	Volume (µL)
RNase inhibitor	0,1
100mM dNTPs	0,2
Reverse Transcription Buffer	1
MultiScribe™ Reverse Transcriptase	0,6
Nuclease-free water	4,6
TaqMan™ MicroRNA-specific stem-loop primer	1,5
RNA (10ng)	2
Total	10

Table 3.12 *miRNA Reverse Transcription Mix*

reverse transcribed in a 10 μ L reaction volume, as reported in the *Table 3.12*. The reaction was carried out under the following conditions: 16°C for 30 minutes, followed by 42°C for 30 minutes, and 85°C for 5 minutes to inactivate the reverse transcriptase. The cDNA was then stored at -20°C until further use.

3.13. Real-Time PCR for miRNA Quantification

Real-time PCR for miRNA quantification was performed using the TaqMan™ miRNA Assay (Applied Biosystems). Each reaction was carried out in a 10 μ L volume, as reported in *Table 3.13*. The reactions were run in triplicate for each miRNA of interest. Thermal cycling conditions were as follows: initial denaturation at 95°C for 20 seconds followed by 40 cycles of 95°C for 1 second (denaturation) and 60°C for 20 seconds (annealing/extension).

The relative expression levels of the miRNAs (hsa-miR130a ID 000454; hsa-miR218 ID 000521, hsa-miR146a ID 000468, hsa-let7-a ID 000377, hsa-miR23a ID 000399, hsa-miR16 ID 000391) were normalized to the endogenous control, U6 snRNA (ID 001973) calculated using the comparative Ct ($\Delta\Delta C_t$) method (also known as the $2^{-\Delta\Delta C_t}$ method).

Real time PCR Mix	Volume (μ L)
TaqMan™ Fast Advanced Master Mix,	5
TaqMan™ miRNA Assay 20x	1
Nuclease-free water	3
cDNA	1
Total	10

Table 3.13 Real-Time PCR Mix

3.14. Protein extraction and Western Blot analysis

Whole cell extracts from isolated brain tissue or cultured cells were prepared using the Lysis Buffer (9803; Cell Signaling Technology) according to the manufacturer's protocol.

Brain tissue samples were homogenized using a semi-automatic pestle, and total protein concentration was determined by the Bradford dye-binding assay (Bio-Rad Laboratories). Proteins were separated on 4–12% Tris–glycine SDS-PAGE gels and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for one hour at room temperature in Tris-buffered saline (TBS-T) containing 3% bovine serum albumin (BSA). Primary antibodies were then incubated overnight at 4 °C against PER2 (PA5-100107; Thermo Fisher Scientific), nNOS (76067; Abcam), AQP4 (59678; Cell Signaling Technology), GFAP (130300; Thermo Fisher Scientific), and β -actin (A5441; Sigma-Aldrich). After washes with TBS-T, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (anti-mouse NA931 or anti-rabbit NA934; GE

Healthcare). Protein bands were visualized by chemiluminescence, and band intensity was quantified using ImageJ software (National Institutes of Health, USA).

For cultured cells, cells were detached using trypsin–EDTA and centrifuged at $700 \times g$ for 5 minutes at 4°C . Whole-cell extracts were prepared after 30 minutes of incubation in lysis buffer, followed by centrifugation at 14,000 rpm for 30 minutes at 4°C . A total of 20 μg of protein per sample was separated by SDS-PAGE and transferred onto PVDF membranes. The expression of AQP4 and β -actin was analysed as described above.

3.15. Immunofluorescence analysis

3.15.1. Immunohistochemistry

For histological analysis, animals were deeply anesthetized with sevoflurane and transcardially perfused with 0.9% saline solution. Brains were rapidly removed and fixed overnight at 4°C in 4% paraformaldehyde (PFA). After fixation, tissues were cryoprotected in 30% sucrose dissolved in PBS and subsequently embedded in Optimal Cutting Temperature (OCT) compound. Coronal brain sections (20 μm thick) were obtained using a cryostat and mounted onto Superfrost microscope slides. Sections were post-fixed in 4% PFA and permeabilized with PBS containing 0.3% Triton X-100. Samples were then blocked for one hour at room temperature in 3% BSA prepared in PBS with 0.1% Triton X-100, followed by overnight incubation at 4°C with primary antibodies against AQP4 (59678; Cell Signaling Technology), GFAP (130300; Thermo Fisher Scientific), syntrophin (ab11425; Abcam), and collagen-IV (1340-01, Southern Biotech). After washing with PBS containing 0.05% Triton X-100, sections were incubated for two hours at room temperature with fluorescence-conjugated secondary antibodies (anti-rabbit FITC, anti-mouse, or anti-rat Alexa Fluor 594, Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and coverslips were applied using a mounting medium.

Fluorescent images were acquired using an inverted Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss, Germany) equipped with a 40 $\times$ oil-immersion objective. Images were captured at a resolution of 2048×2048 pixels, and identical laser power and exposure settings were maintained across all experimental groups.

3.15.2. Immunocytochemistry

Astrocytic primary cells were fixed in 4% PFA for 15 minutes. Following fixation, they were permeabilized with 0.3% Triton X-100 and subsequently blocked with 3% BSA. Cells were then incubated with the appropriate primary antibodies, followed by fluorescently conjugated secondary antibodies (anti-rabbit FITC and anti-rat Alexa Fluor 594; Invitrogen). Nuclei were

counterstained with DAPI. Fluorescent images were acquired using a Zeiss LSM 700 confocal microscope equipped with a 63× oil-immersion objective.

3.16. *Ex vivo* fluorescence imaging

All fluorescent CSF tracers were constituted in PBS at a final concentration of 0.5%. The dyes used were the following: Texas Red-Dextran 3kDa, Alexa Fluor 680-Dextran 70kDa and FITC-Dextran 2000kDa. Anesthetized mice were fixed in a stereotaxic frame, and a Hamilton syringe was inserted into the CM. A total volume of 10 μ L of CSF tracers was administered at a rate of 1 μ L/min over 10 minutes, as previously reported (J. J. Iliff et al., 2012). After saline perfusion, brains were fresh frozen and embedded in OCT. Coronal sections (60 μ m thick) were mounted with Prolong Anti-Fade Gold with DAPI (Invitrogen), and imaged *ex vivo* by conventional fluorescence microscopy. Whole-slice montages were acquired with the NanoZoomer S60 Slide scanner system (Hamamatsu Photonics, Japan). Exposure levels were determined based on non-injected control slices. Tracer quantification was analysed in ImageJ software, as described in detail previously (J. J. Iliff et al., 2013).

3.17. *In vivo* imaging

A craniotomy (around 3 mm in diameter) was made over the cortex 1 mm lateral and 0.5 mm posterior to bregma. The skull was thinned but left intact with the dura, which was covered with artificial CSF, and sealed with a glass coverslip (Shin et al., 2023). Mice were allowed four weeks to recover fully from surgery before imaging.

To visualize the cerebral vasculature, 0.2 mL of , Alexa Fluor 680-Dextran 70kDa (0.5 % in saline) was injected intra-arterially via retro-orbital injection immediately before imaging. *In vivo* imaging was performed using a two-photon microscope integrated with a custom-built multimodal imaging system (Shin et al., 2023). A pulsed laser (InSight DeepSee; Spectra-Physics, tuning range: 680 nm to 1300 nm) was used as an excitation light source. A 20x water immersion objective lens (XLUMPLFLN20XW; Olympus), mounted on a motorized Z-axis stage (M-112.1EG; Physik Instrumente), was used to image the cortex. The cerebral vasculature was first imaged with 512 x 512 pixel frames from the surface to a depth of 500 μ m with multiple z-steps. After CM injection, tracer movement was conducted with dual channels (AlexaFluor680 and Texas Red). The cortex was repeatedly scanned from the surface to a depth of 300 μ m with 51 z-steps at 2 μ m intervals throughout the imaging session.

3.18. Metabolomic profiling

Global metabolomic profiling was performed on skeletal muscle tissue to evaluate circadian variations in metabolic activity under physiological and ischemic conditions (Benton et al., 2014). Muscle tissues were fresh-frozen in liquid nitrogen and stored at -80°C for further analyses. Metabolites were extracted using a biphasic extraction protocol based on a modified Folch method, as previously reported (Di Minno et al., 2021). Briefly, frozen tissue was homogenized in pre-chilled methanol/water (8:2, v/v) containing stable isotope-labeled internal standards. Chloroform was then added, and after vigorous mixing, the solution was centrifuged at 4°C to separate the phases. The upper aqueous phase, containing the polar metabolites and the lower organic phase, containing the lipid fraction were carefully collected, dried under vacuum, and reconstituted in appropriate solvents prior to analysis. Metabolite identification and principal component analysis (PCA) were performed to identify temporal differences in the metabolic profile. Enriched pathways were determined through KEGG-based pathway analysis, as previously reported (Miro et al., 2025). To further explore circadian modulation of the arginine–citrulline–nitric oxide (NO) pathway, serum nitrite levels were measured in samples collected during the inactive (ZT3) and active (ZT15) phases. In parallel, targeted metabolomic analysis of arginine and citrulline was performed in muscle extracts collected under physiological conditions and following tMCAO at 6 h and 24 h post-reperfusion. The arginine-to-citrulline ratio was used as an indirect index of NOS activity.

3.19. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software Inc., La Jolla, CA, USA). The sample size was predetermined using an online calculator (<https://www.danielsoper.com/statcalc/calculator.aspx?id=47>). The calculation was based on Cohen's d value, with standard deviation and mean estimated from historical and preliminary data. For *in vivo* studies, a total of 175 male mice were used, 14 mice died during tMCAO procedure, and 32 mice were excluded based on LDF values to avoid SAH complications. A total of 54 mice were used for the first aim of this thesis; 75 mice were used for the second part of the study.

All experimental procedures and analyses were conducted in a blinded and randomized manner. Results are expressed as mean $\pm$ SEM. Comparisons between two groups were performed using an unpaired two-tailed t -test. For experiments involving more than two groups, one-way or two-way ANOVA was applied as appropriate, followed by Tukey–Kramer or Bonferroni post hoc tests for multiple comparisons. A p -value of < 0.05 was considered statistically significant.

4. RESULTS

4.1. Remote limb postconditioning as a strategy for endogenous neuroprotection

To determine whether remote limb postconditioning can confer neuroprotection, male mice were randomly subjected to either transient focal ischemia (tMCAO) or remote limb ischemic postconditioning (RLIP). At 24 hours after the ischemic injury, brains were harvested and stained with TTC to evaluate infarct size. As illustrated in *Figure 4.1a*, mice treated with RLIP exhibited a significant reduction in ischemic volume compared with the tMCAO-only group. The neuroprotective effect was particularly evident in sections 1–4 (*Figure 4.1b*), areas directly supplied by the middle cerebral artery (MCA), including the striatum and prefrontal cortex. These findings suggest that RLIP can represent a potential strategy for achieving neuroprotection.

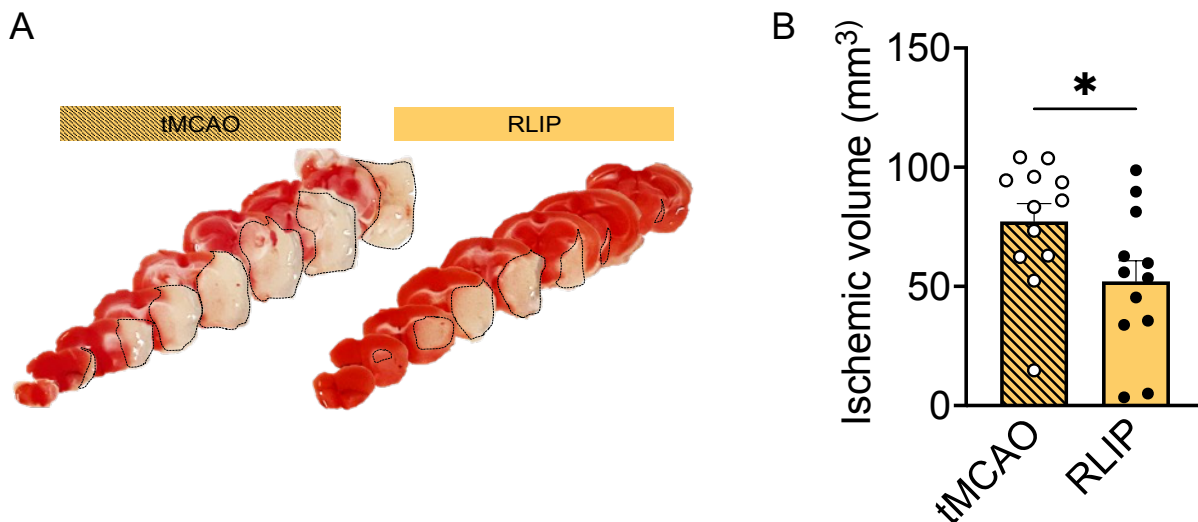


Figure 4.1a Neuroprotection elicited by remote limb ischemic postconditioning

(A) Eight brain coronal sections – 1mm thick - were stained with TTC. The white region identifies the ischemic area. The right panel (B) shows the quantification of the infarct area. RLIP significantly reduces the ischemic volume compared to tMCAO.

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0,05$, tMCAO $n = 12$, RLIP $n = 12$.

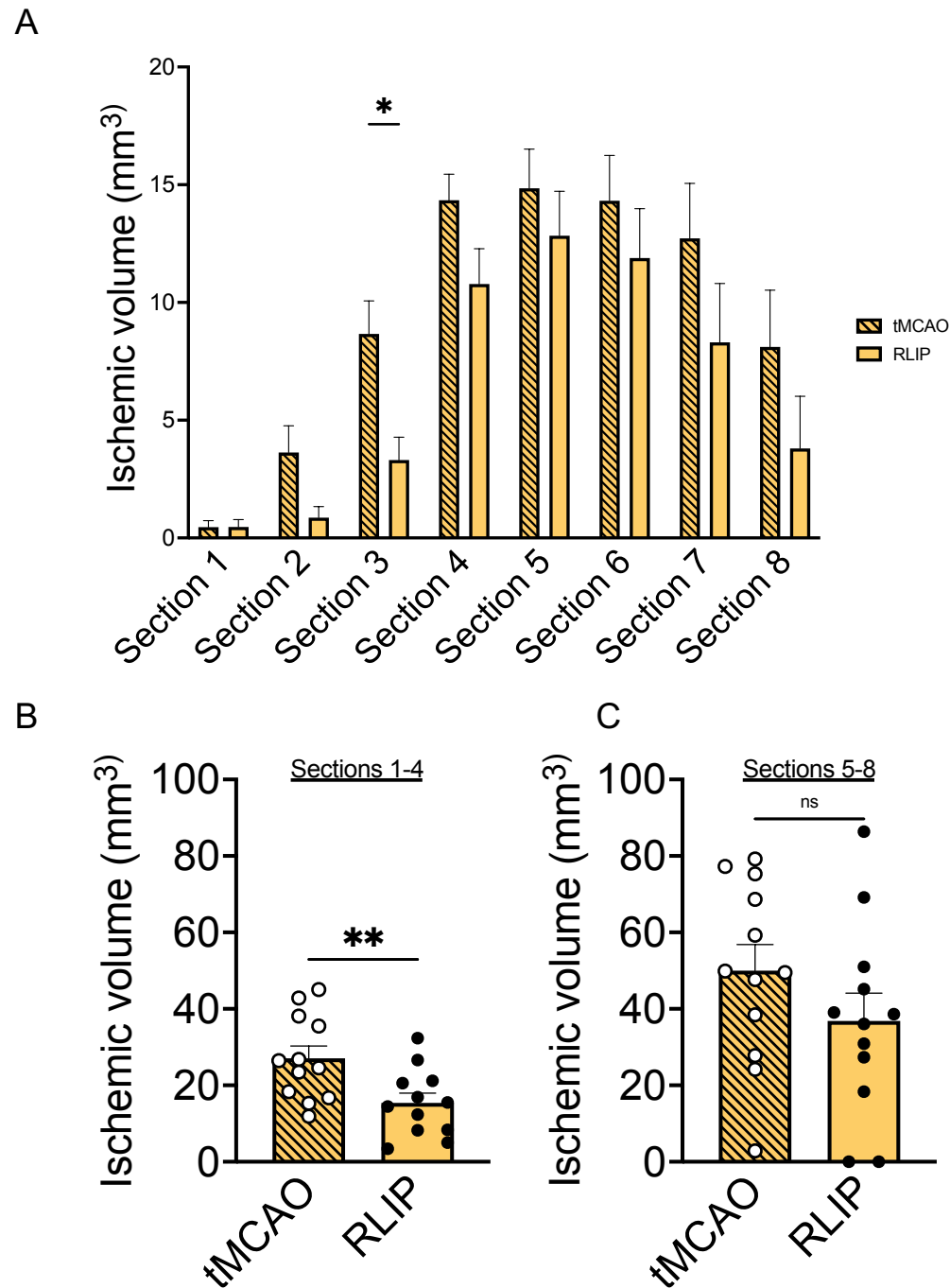


Figure 4.1b Remote limb ischemic postconditioning exerts neuroprotection in the prefrontal area.

Eight brain coronal sections were stained with TTC. The graphs show the quantification of the infarct area of each section (**A**), or the quantification of the anterior area— sections 1-4 —(**B**) and posterior area— sections 5-8 (**C**). RLIP significantly the ischemic volume in the brain sections 1-4 compared to tMCAO (**B**). No effects in the sections 5-8 (**C**). Results are expressed as mean $\pm$ SEM, One-way ANOVA and post hoc Tukey test for multiple comparison, * $p < 0.05$, tMCAO $n = 12$, RLIP $n = 12$.

4.2. Potential mechanisms involved in the neuroprotection elicited by remote limb postconditioning

At 24 hours post-stroke, brains were collected, and the ipsilateral striatum was dissected to investigate the potential mechanisms underlying RLIP-mediated neuroprotection. The expression of aquaporin-4 (AQP4), a well-established marker of glymphatic function, and glial fibrillary acidic protein (GFAP), a marker of astrocytic activation, was evaluated. Western blot analysis showed a significant increase in AQP4 protein expression in the striatum of RLIP-treated mice compared with the tMCAO group, that is accompanied by a slight increase in GFAP levels (*Figure 4.2*). These findings suggest a potential involvement of glymphatic function in mediating the beneficial effects of RLIP during post-stroke recovery. Moreover, the increase in GFAP expression and AQP4 upregulation, primarily localized to astrocytic end-feet, may suggest an early mobilization of astrocytes in the infarcted area, potentially contributing to the protective effects observed with RLIP.

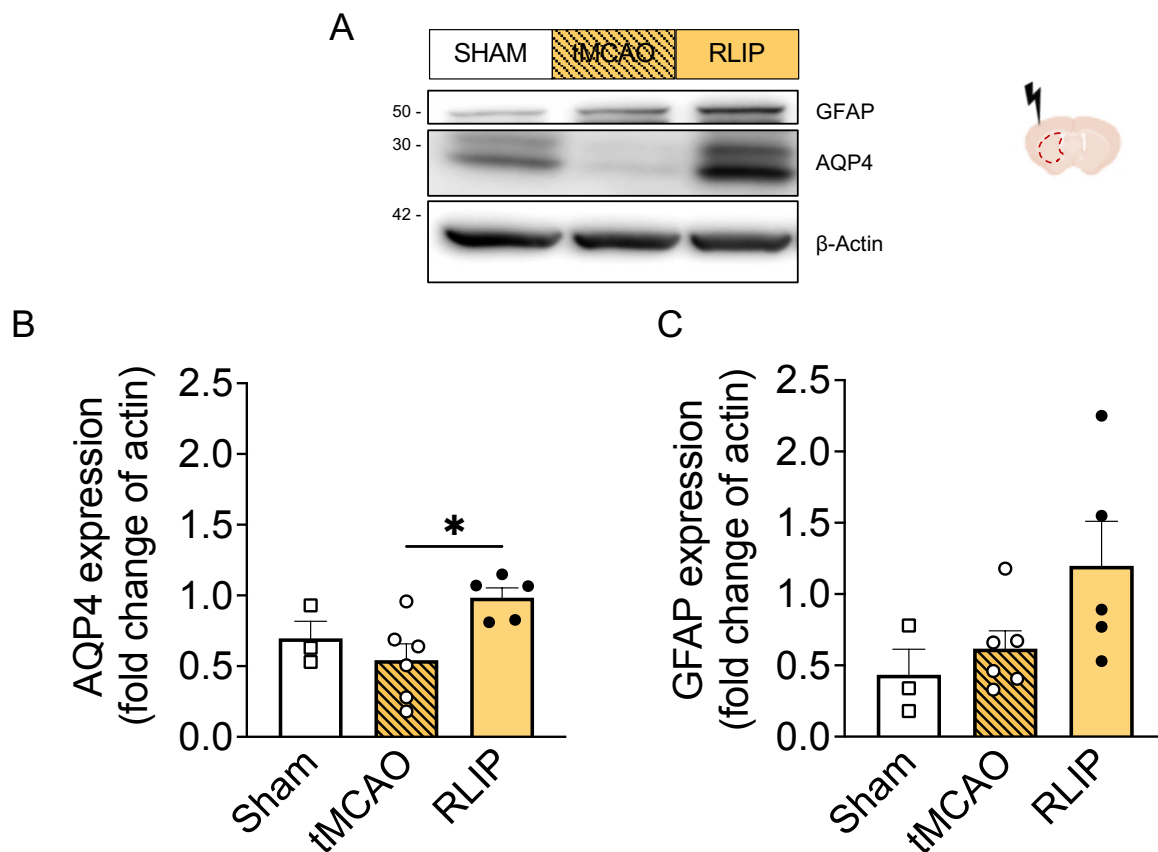


Figure 4.2 Mechanisms potentially involved in the neuroprotection elicited by remote limb ischemic postconditioning

Western blot analysis was performed on the striata of tMCAO and RLIP (**A**) at 24h post reperfusion. AQP4 expression significantly increases when RLIP was applied (**B**), accompanied by an increase of GFAP levels (**C**). Results are expressed as mean $\pm$ SEM. One-way ANOVA and post hoc Tukey test for multiple comparison, * $p < 0.05$ vs tMCAO, SHAM $n = 3$, tMCAO $n = 6$, RLIP $n = 6$.

4.3. Remote limb postconditioning modulates AQP4 polarization

To further strengthen the role of AQP4 in mediating RLIP-induced neuroprotection, immunofluorescence analyses were performed to evaluate its localization and polarization within the peri-infarct region, as illustrated in the *Figure 4.3*.

Brain sections from both tMCAO and RLIP groups were stained for AQP4 with vascular and astrocytic markers, including Collagen-IV, GFAP, and the membrane-associated scaffolding protein syntrophin. The results showed that RLIP-treated mice had a significant increase in

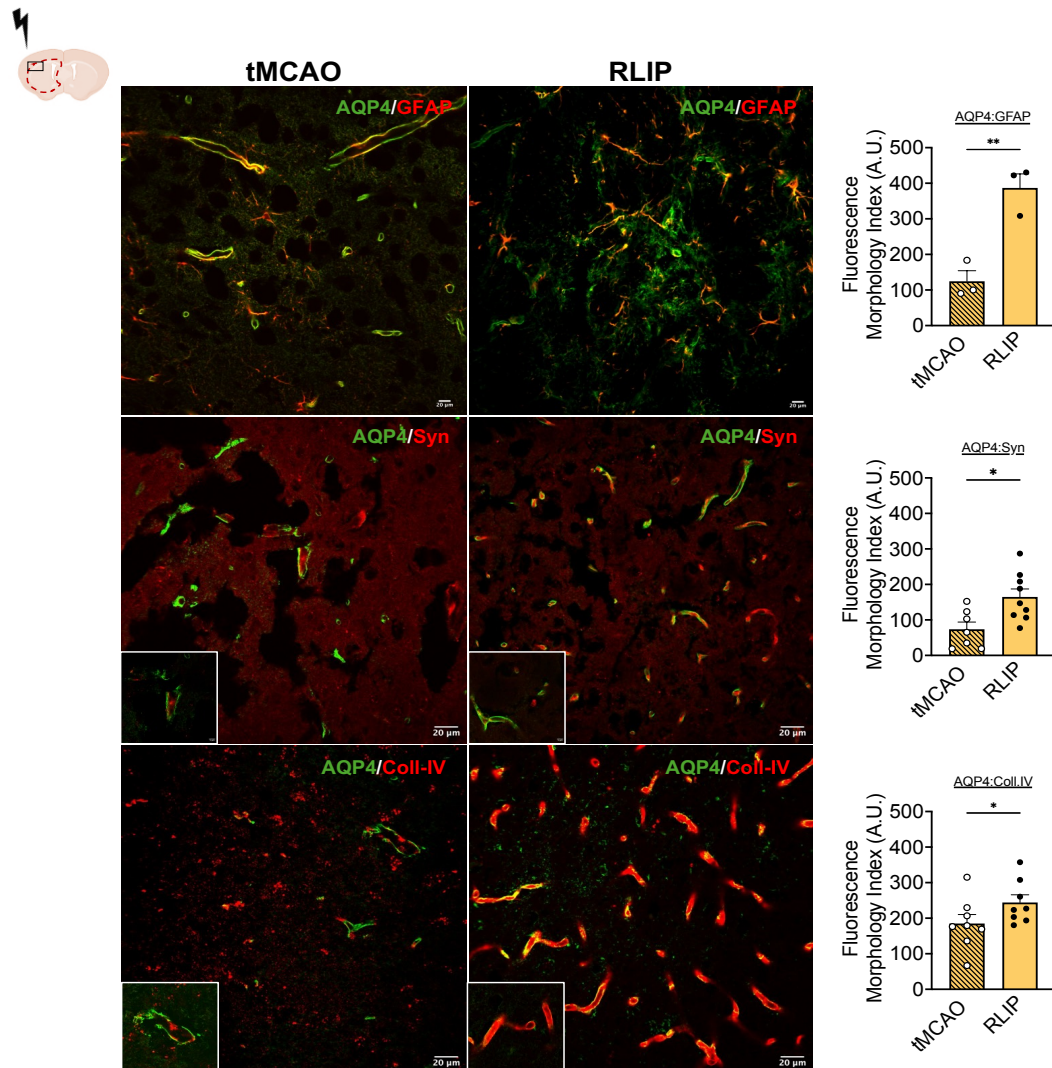


Figure 4.3 Remote limb ischemic postconditioning increases AQP4 polarization

Representative immunohistochemistry analysis of the co-staining between AQP4 and GFAP (**upper** panel), and Syntrophin (Syn) (**middle** panel) and AQP4 with Collagen IV (Coll-IV) (**bottom** panel). Remote limb ischemic postconditioning increase the number of GFAP+ astrocytes expressing AQP4 compared to stroke. Specifically, remote limb ischemic postconditioning increases the polarization of AQP4 at the vascular level as demonstrated by the increase co-localization of AQP4 with Syn and Coll-IV.

Three different ROIs of periinfarct area were acquired and fluorescence morphology index was evaluated. Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed, * $p < 0.05$, tMCAO $n = 3$ to 7, RLIP $n = 3$ to 8. Scale bar = 20 μ m

GFAP+ astrocytes expressing AQP4, accompanied by enhanced co-localization of AQP4 with Collagen IV and syntrophin (Figure 4.3).

Collectively, these findings indicate that RLIP counteracts AQP4 mislocalisation following ischemic injury, thereby restoring its vascular polarity. This effect is partly due to syntrophin-dependent anchoring of AQP4 at astrocytic end-feet (Figure 4.3). The increase in Collagen IV expression further supports an improvement in blood–brain barrier integrity, structures typically compromised after stroke, suggesting a potential role of RLIP in preserving these functions.

4.4. *In vitro* selection of the siRNAs for AQP4

Three commercially available siRNAs for AQP4 were tested *in vitro* to evaluate the ability to selectively block AQP4 expression and the best among them was selected for the following

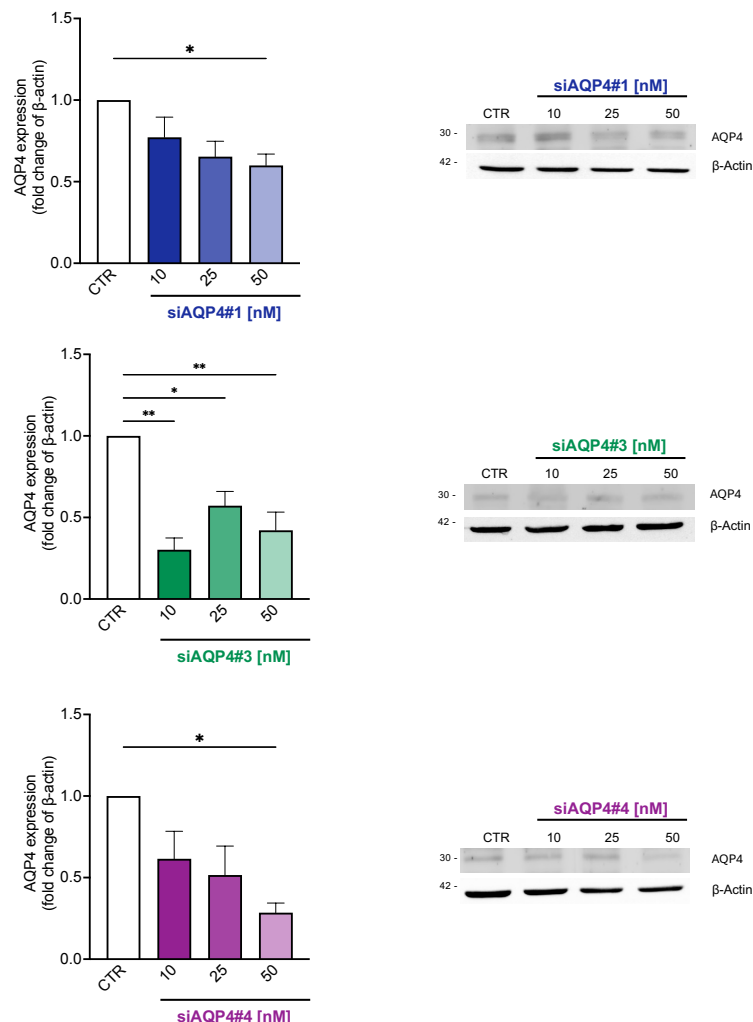


Figure 4.4a Silencing of AQP4 in C2C12 cells

Representative western blot analysis shows a concentration-dependent reduction of AQP4 expression following siRNA treatment. siAQP4#3 shows the best capacity to selectively reduce AQP4 expression at the lower concentration. Results are expressed as mean $\pm$ SEM, of $n=3$. One-way Anova and post Tukey t-test for multiple comparison. * $p<0.05$, ** $p<0.01$ vs CTR

in vivo study. As mentioned in the methods section, three concentrations were tested for the three siRNAs (Figure 4.4a). Among them, the siAQP4#3 exhibited the best capacity to reduce AQP4 expression, as shown by Western Blots. The immunocytochemistry analysis revealed that the reduction in AQP4 expression is also associated with a mislocalisation of the protein in the astrocytic end-feet (Figure 4.4b). Based on these results, siAQP4#3 was chosen as a potential candidate for the *in vivo* evaluation of AQP4 role in RLIP-mediated neuroprotection.

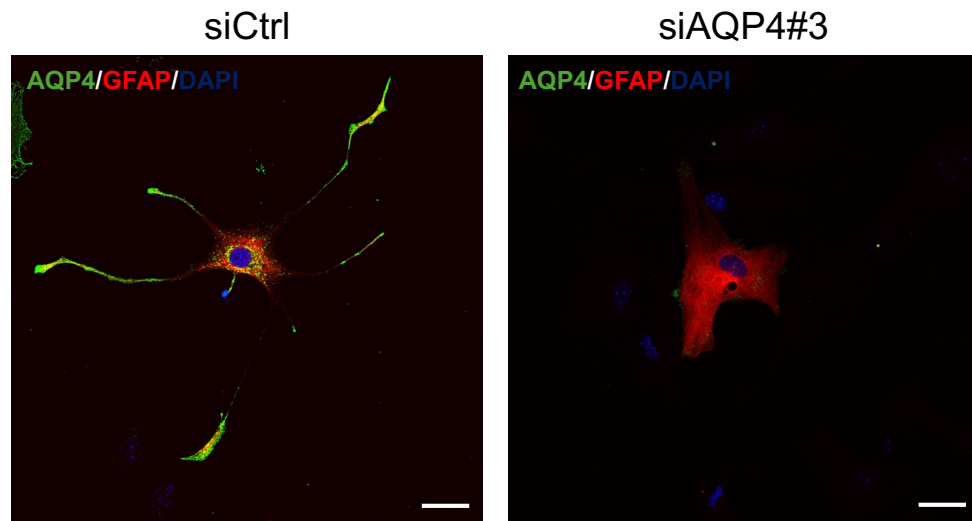


Figure 4.4b Silencing of AQP4 in astrocytic primary cell

Representative immunocytochemistry on astrocytic primary cell analysis reveals a reduction in AQP4 expression and mislocalisation after silencing with siAQP4#3, 10nM).

Scale bar=20 μ m

4.5. Remote limb postconditioning neuroprotection requires AQP4 activity

To evaluate the role of AQP4 in the neuroprotection elicited by RLIP, siRNA control or siRNA against AQP4 (siAQP4#3, 10 μ L, 5 μ M) was i.c.v. administered 30 minutes before tMCAO and 3 hours post-reperfusion. At 24 hours post-reperfusion, mice were euthanized, and TTC staining was performed. The reduction of AQP4 expression, confirmed by Western blot (Figure 4.5a), abolished the neuroprotective effect of RLIP. As shown in the left panel (Figure 4.5b), no difference was observed in the infarct area between RLIP or tMCAO groups.

These findings suggest that AQP4 plays a critical role in the reduction of ischemic volume induced by RLIP.

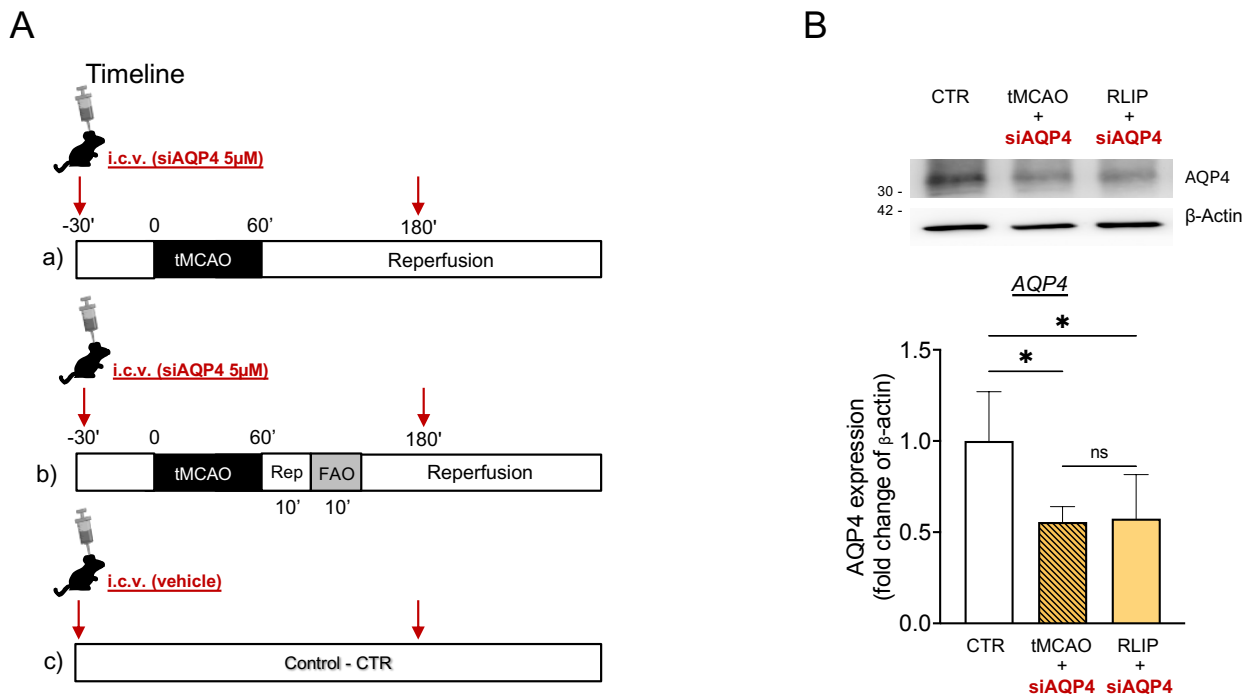


Figure 4.5a AQP4 expression is suppressed by the siAQP4#3

Experimental design (**A**): mice subjected to tMCAO or RLIP were treated with siAQP4#3 (5 μ M; 10 μ L) 30 minutes before tMCAO induction, and 3h after reperfusion then Western blot analysis was performed 24 hours later. Control mice (CTR) were injected with siCTR (vehicle, 10 μ L). Under AQP4 inhibition, protein levels were drastically reduced (**B**).

Results are expressed as mean $\pm$ SEM. One-way ANOVA and post hoc Tukey test for multiple comparison, * $p < 0.05$ vs tMCAO, SHAM $n = 3$, tMCAO $n = 6$, RLIP $n = 6$.



Figure 4.5b AQP4 is necessary for the neuroprotection elicited by remote limb ischemic postconditioning

Representative TTC staining (**A**). Under AQP4 inhibition, the neuroprotective effects of RLIP are abolished. As shown in the right panel (**B**), no difference in the ischemic volume was observed between tMCAO and RLIP-treated mice.

Results are expressed as mean $\pm$ SEM. Tukey t-test two tailed, tMCAO n=6, RLIP n=7.

4.6. *In vitro* methods to evaluate the glymphatic system

To evaluate the flow and the clearance dynamics within the compartments of the glymphatic system, three fluorescent dyes with different molecular weights were injected into the cisterna magna (CM). Brains, cervical lymph nodes (CLN), and meninges were collected at three time points after CM injection (0, 30, and 60 minutes). CLN were immediately analysed under fluorescence microscopy. As illustrated in *Figure 4.6a*, dye clearance was both size- and time-dependent.

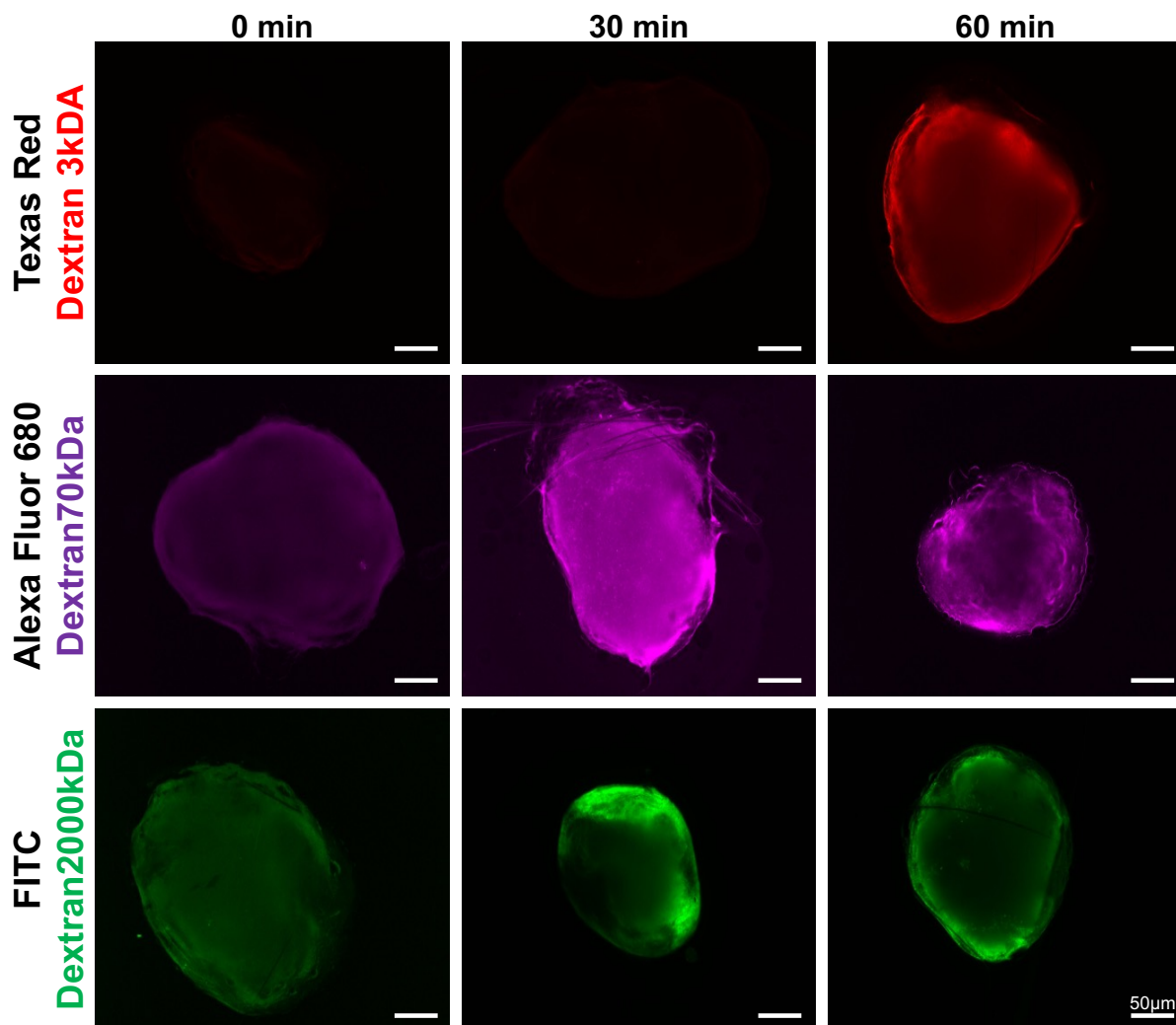


Figure 4.6a Evaluation of the glymphatic flow in the cervical lymph nodes

Representative immunofluorescence of cervical lymph nodes (CLN). Under stereotaxic instrument, mice were injected into the cisterna magna (CM) with 3 different dyes. Dyes were allowed to circulate at three different time points: 0 – 30 and 60 minutes after completing the injection. CLN were collected and visualized immediately under fluorescence microscopy. The clearance of the dyes appears to be size- and time- dependent.

Scale bar= 50 µm.

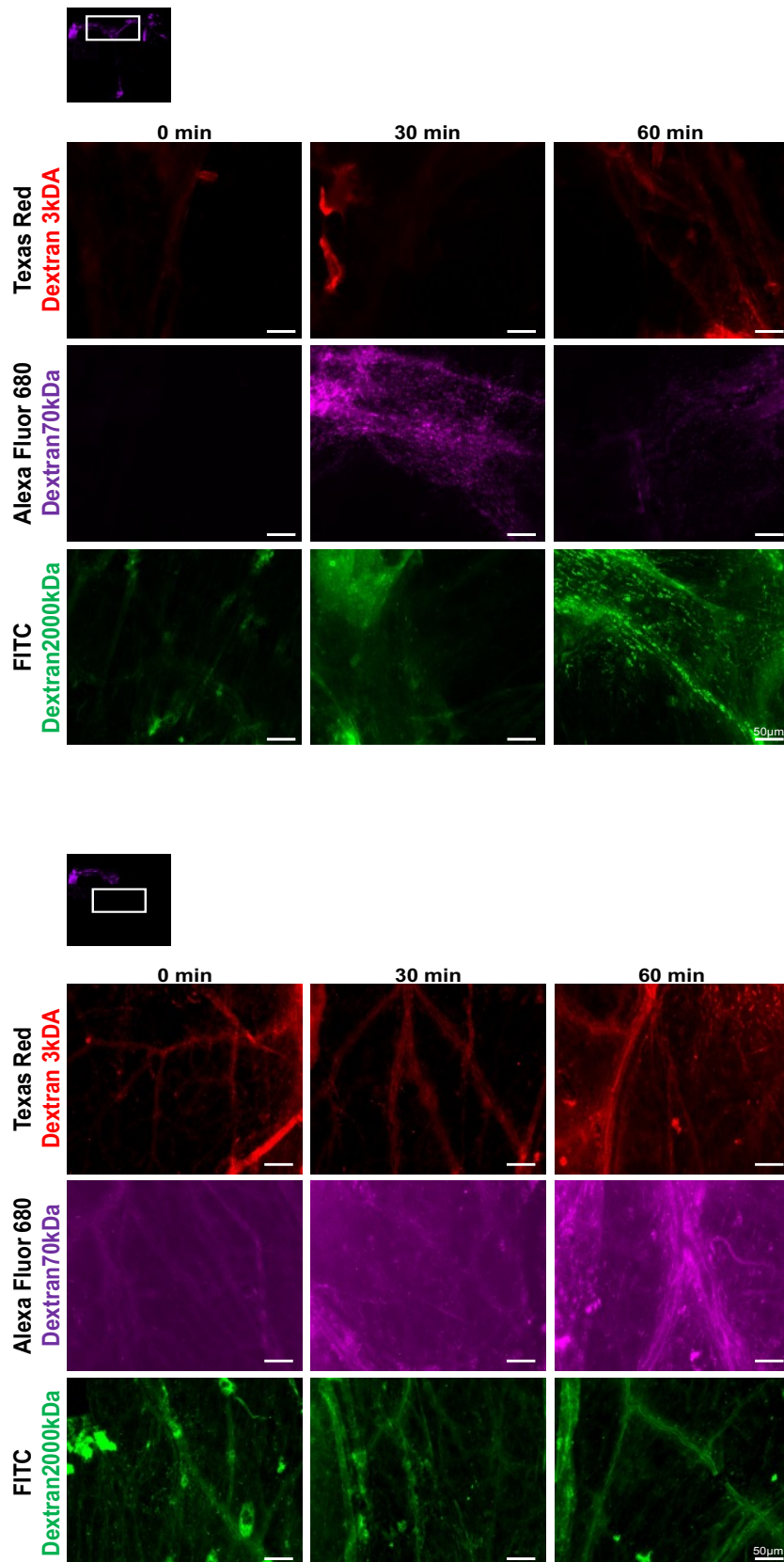


Figure 4.6b Evaluation of the glymphatic flow in the meninges

Representative immunofluorescence of meningeal lymphatic vessels. Under stereotaxic instrument, mice were injected into the cisterna magna (CM) with 3 different dyes. Dyes were allowed to circulate at three different time points: 0 – 30 and 60 minutes after completing the injection. Meninges were collected and whole-mounted in glass cover slip. The clearance of the dyes appears to be size- and time- dependent. Scale bar= 50 µm.

Similar results were observed in the analysis of brain sections and meninges (*Figure 4.6b* and *Figure 4.6c*). Specifically, 60 μm coronal brain sections and whole-mount meninges were examined to assess dye distribution and clearance. Collectively, dye circulation within the analysed compartments depended on molecular size and time after injection. High-molecular-weight dyes (70–2000 kDa) were unable to penetrate the vascular compartments and remained outside the vessels in both the brain and meninges. In contrast, the 3kDa Dextran penetrated the brain parenchyma and was gradually cleared through the CLN within 60 minutes. The 70kDa Dextran exhibited an intermediate distribution pattern, with limited penetration into the parenchyma.

Based on these findings, the 70kDa Dextran was selected for in vivo visualization of vasculature following intravenous (i.v.) injection, whereas the 3kDa Dextran was used to visualize glymphatic flow following CM injection.

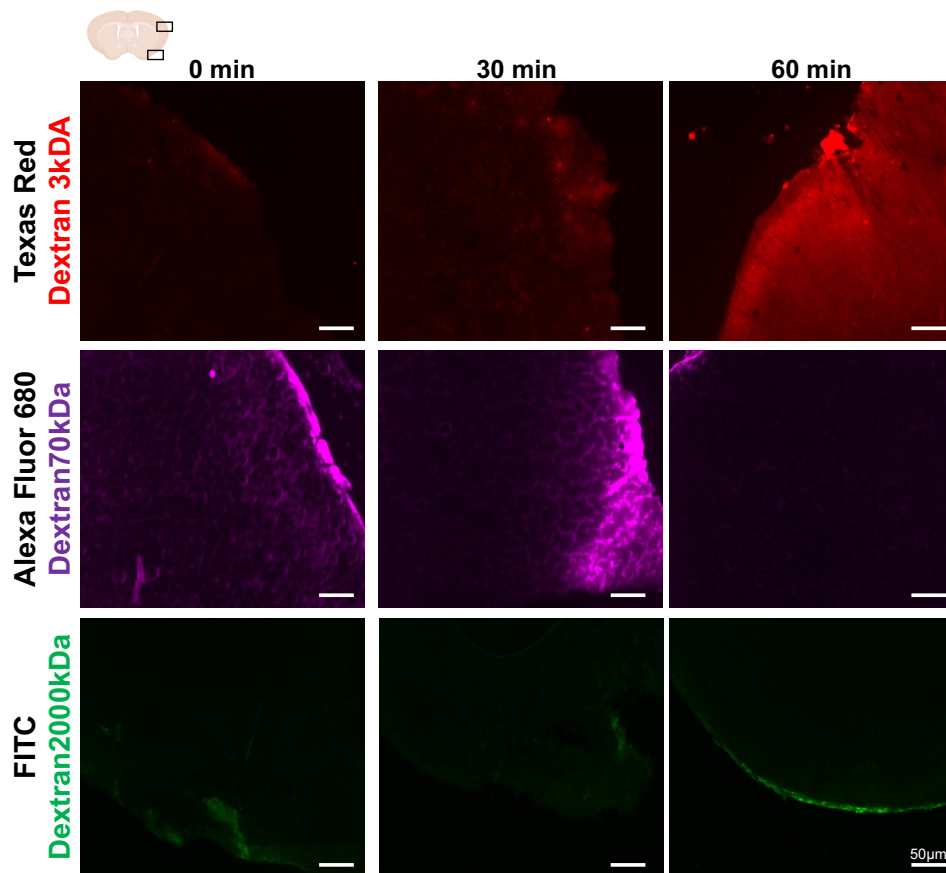


Figure 4.6c Evaluation of the glymphatic flow in the brain

Representative immunofluorescence of brain section. Under stereotaxic instrument, mice were injected into the cisterna magna (CM) with 3 different dyes. Dyes were allowed to circulate at three different time points: 0 – 30 and 60 minutes after completing the injection. Brains were collected and sectioned in 60 μm coronal section. Brain sections were visualized under fluorescence microscopy. The clearance of the dyes appears to be size- and time- dependent.

Scale bar= 50 μm .

4.7. *In vivo* methods to evaluate the glymphatic system

CSF can be visualized *in vivo* through two-photon microscopy. Two different dyes were used: Alexa Fluor 680-Dextran70kDa was injected retro-orbitally (RO) to visualize the vasculature, and the Texas Red-Dextran 3kDa was injected into the CM to visualize the CSF.

A cranial window (around 3 mm in diameter) was prepared by gently thinning the skull with a high-speed drill (*Figure 4.7a*). Then, a circular glass coverslip was placed over the area. The two-photon microscope objectives (4x to select the window, 20x for the *in vivo* acquisition session) were placed over the cranial window. This technique allows the visualization of the pial vessels above the skull to a depth of approximately 500 μm , depending on the contrast after RO injection.

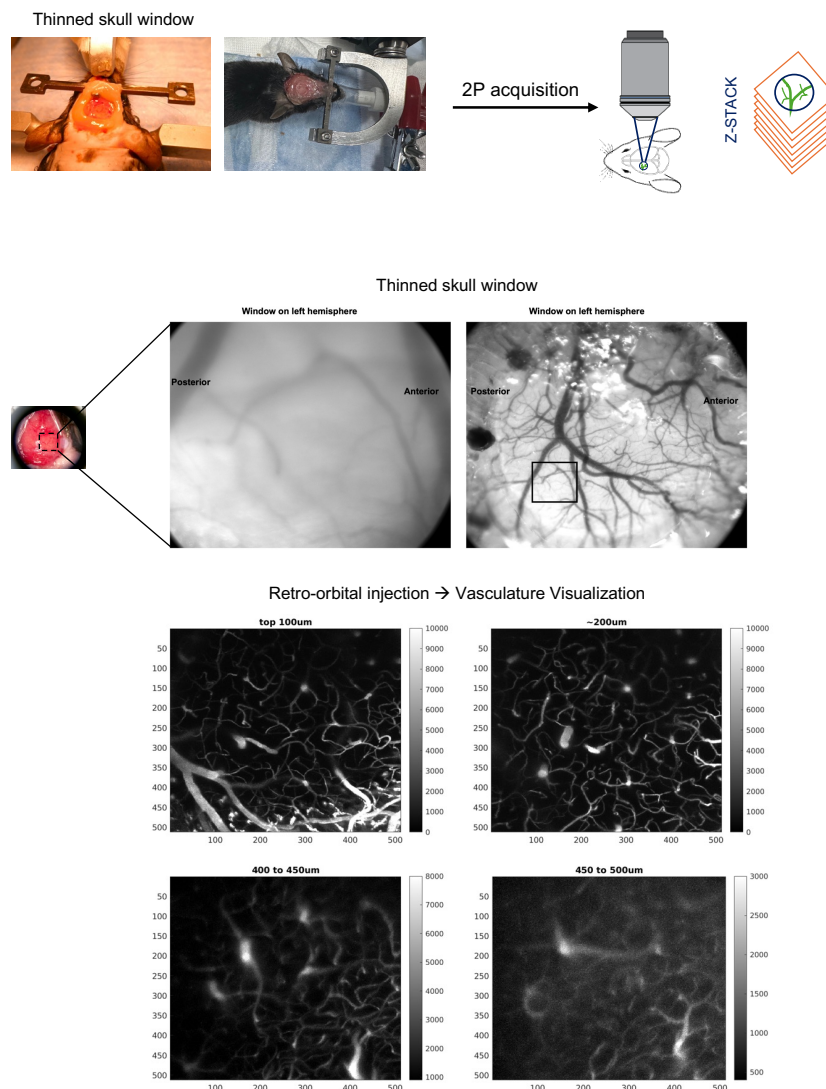


Figure 4.7a *In vivo* two photon imaging of CSF dynamics

A cranial window (around 3 mm in diameter) was prepared by gently thinning the skull with a high-speed drill (upper panel). Then, a circular glass coverslip was placed over the area.

The two-photon microscope objectives (4x to select the window, 20x for the *in vivo* acquisition session) were placed over the cranial window. Mice were injected retro-orbitally with Alexa Fluor 680-Dextran 70kDa and 1h of two-photon imaging was performed to acquire multiple z-stack. Image stacks were acquired at 2 μm step.

The CM injection allows the visualization of the CSF, as highlighted in the volume viewer of the *Figure 4.7b*, to a depth of approximately 250 μm above the skull.

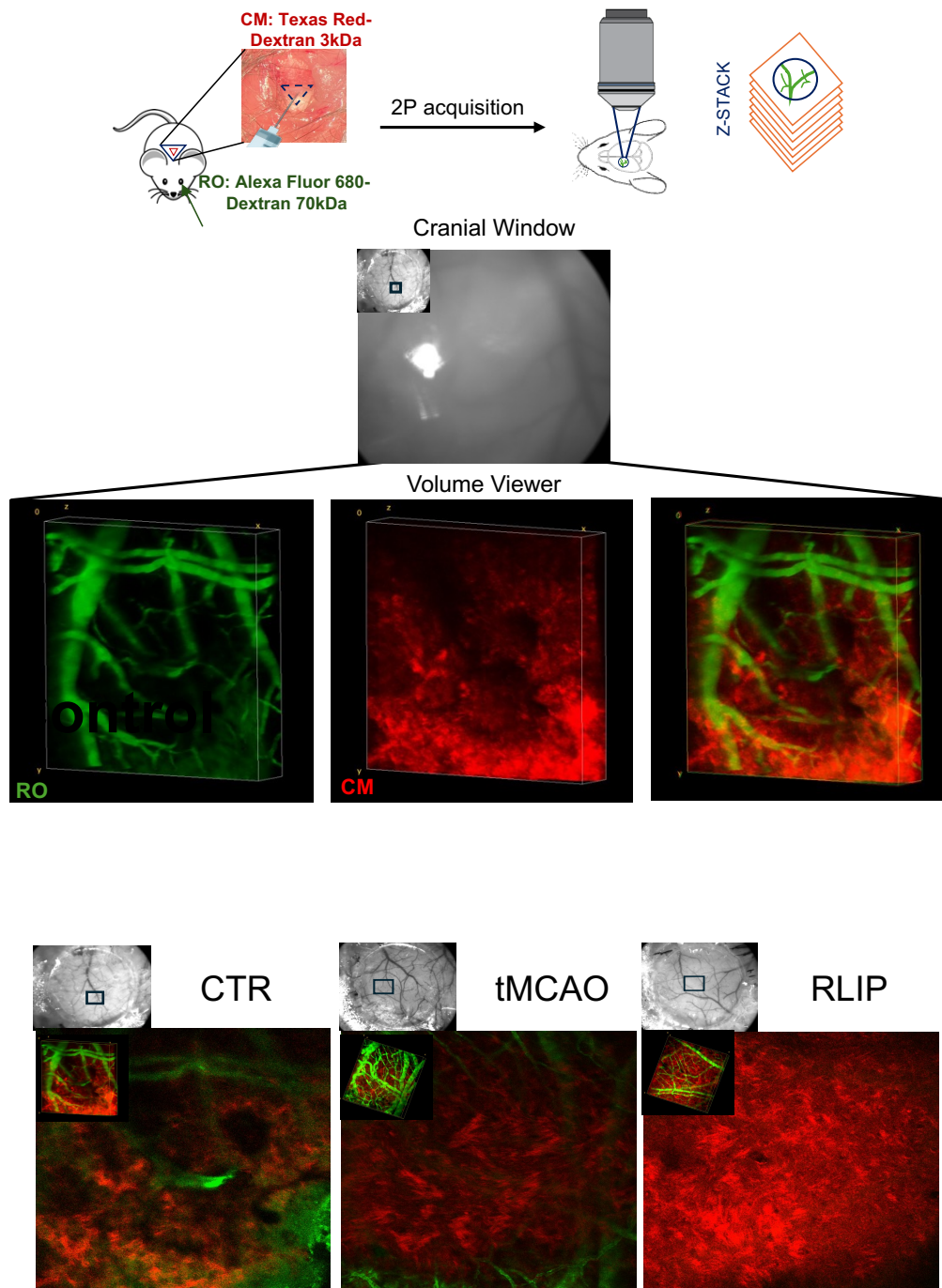


Figure 4.7b *In vivo* two photon imaging of CSF dynamics

Cranial window was made through thinned skull to avoid perturbation of CSF dynamics. Mice were injected retro-orbitally with Alexa Fluor 680-Dextran 70kDa and 1h of two-photon imaging was performed to acquire multiple z-stack. Then, mice were subjected to CM injection with Texas Red-Dextran 3kDa to visualize the CSF movements. In the lower panel, representative acquisition of control (CTR), tMCAO and RLIP mice.

Image stacks were acquired at 2 μm step. The 3D volume viewer was made with Image J and includes 51 z-stack of 2 μm /step.

4.8. Remote limb postconditioning reduced impairments in the glymphatic flow after stroke injury

Mice subjected either to tMCAO or RLIP, were injected into CM with Texas Red-Dextran 3kDa 24 hours post-stroke. After 30 minutes, CLN, brains and meninges were harvested and collected to visualize *ex vivo* the clearance of the dye, as illustrated in the experimental design of the *Figure 4.8*. CLN and meninges were immediately analysed under fluorescence microscopy. Brains were cut in 60 μ m section and visualized with laser scanning microscopy that acquired the whole section to assess dye accumulation.

RLIP significantly reduces the accumulation of the dyes in the brain parenchyma, as shown by the lower intensity in the whole brain section (*Figure 4.8*).

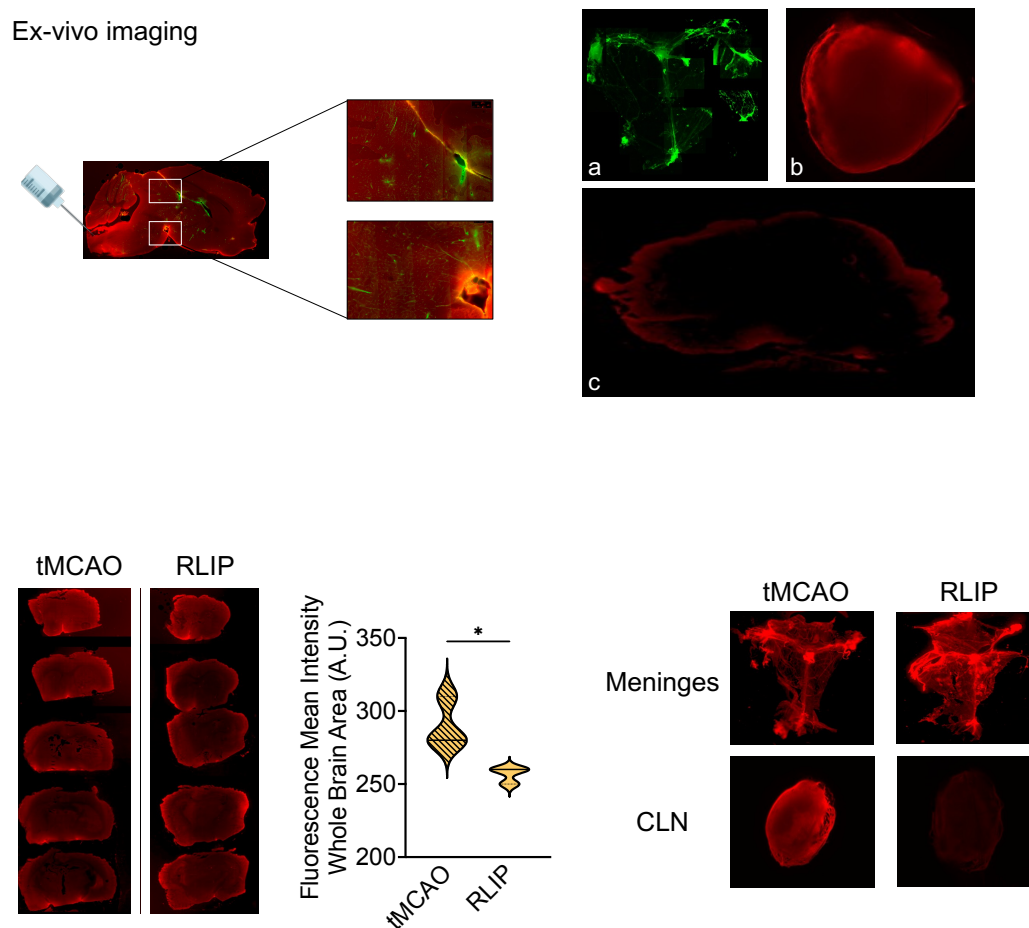


Figure 4.8 Ex-vivo analysis of glymphatic clearance

Representative immunofluorescence of dye distribution after cisterna magna (CM) injection in brain, meninges and cervical lymph nodes (CLN). Under stereotaxic instrument, mice subjected to tMCAO or RLIP were injected with Texas Red-Dextran 3kDa. Dyes were allowed to circulate 30 min. CLN, and meninges were collected and whole-mounted in glass cover. Brains were sectioned in 60 μ m coronal section and visualized under laser scanning fluorescence microscopy. RLIP significantly reduces dye accumulation in the brain parenchyma after stroke injury (lower panel).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$; $n = 3$ to 5/group.

4.9. MiR-130a as a potential biomarker of neuroprotection elicited by remote limb postconditioning

Blood was collected from mice subjected either to stroke or RLIP after 24 hours post-stroke. Given the importance of AQP4 in RLIP-mediated efficacy, we explored the possibility of identifying miRNAs that could selectively modulate its promoter. Previous studies have identified miR-130a as a strong modulator of the promoter of *AQP4* M1 (Sepramaniam et al., 2010). Thus, the relative expression of miR-130a-3p, a miRNA whose expression and activity are associated with astrocyte dynamics and neuronal survival, was evaluated with real-time PCR on the total RNA extracted from the whole blood. As shown in *Figure 4.9*, stroke induced a significantly increase in the expression of miR-130a-3p compared to RLIP.

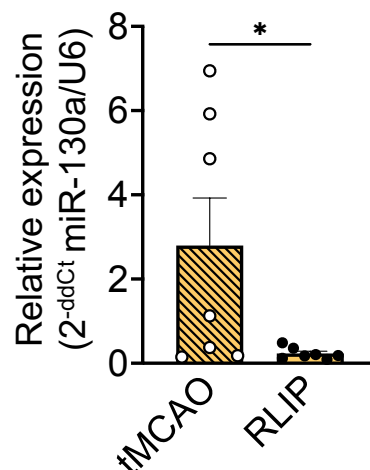


Figure 4.9 Evaluation of relative expression of mir-130a-3p as potential biomarker involved in RLIP neuroprotection

Total RNA was extracted from entire blood samples, according to the manufacturer's protocol. For miRNA quantification, reverse transcription was performed using the TaqMan™ MicroRNA Reverse Transcription Kit and real time PCR with TaqMan miRNA Assay. The relative expression of hsa- mir-130a-3p miRNA was normalized to the endogenous control, U6 snRNA using the 2^{-ddCt} method.

Results are expressed as mean ± SEM, Tukey t-test two tailed *p<0,05; n=7/group.

4.10. Circulating miRNAs as potential biomarkers of glymphatic dysfunction in patients with sleep apnea

Peripheral blood was collected from the peripheral vein of patients with disturbed sleep disorders.

A comprehensive miRNA screening was conducted to identify potential circulating biomarkers associated with glymphatic dysfunction in this population. A summary of the miRNA screening was plotted in the heat map (*Figure 4.10*) to highlight the differential expression of these miRNAs in the population analysed. Collectively, patient_1 showed a different pattern compared to the other patients that have a similar miRNA signature.

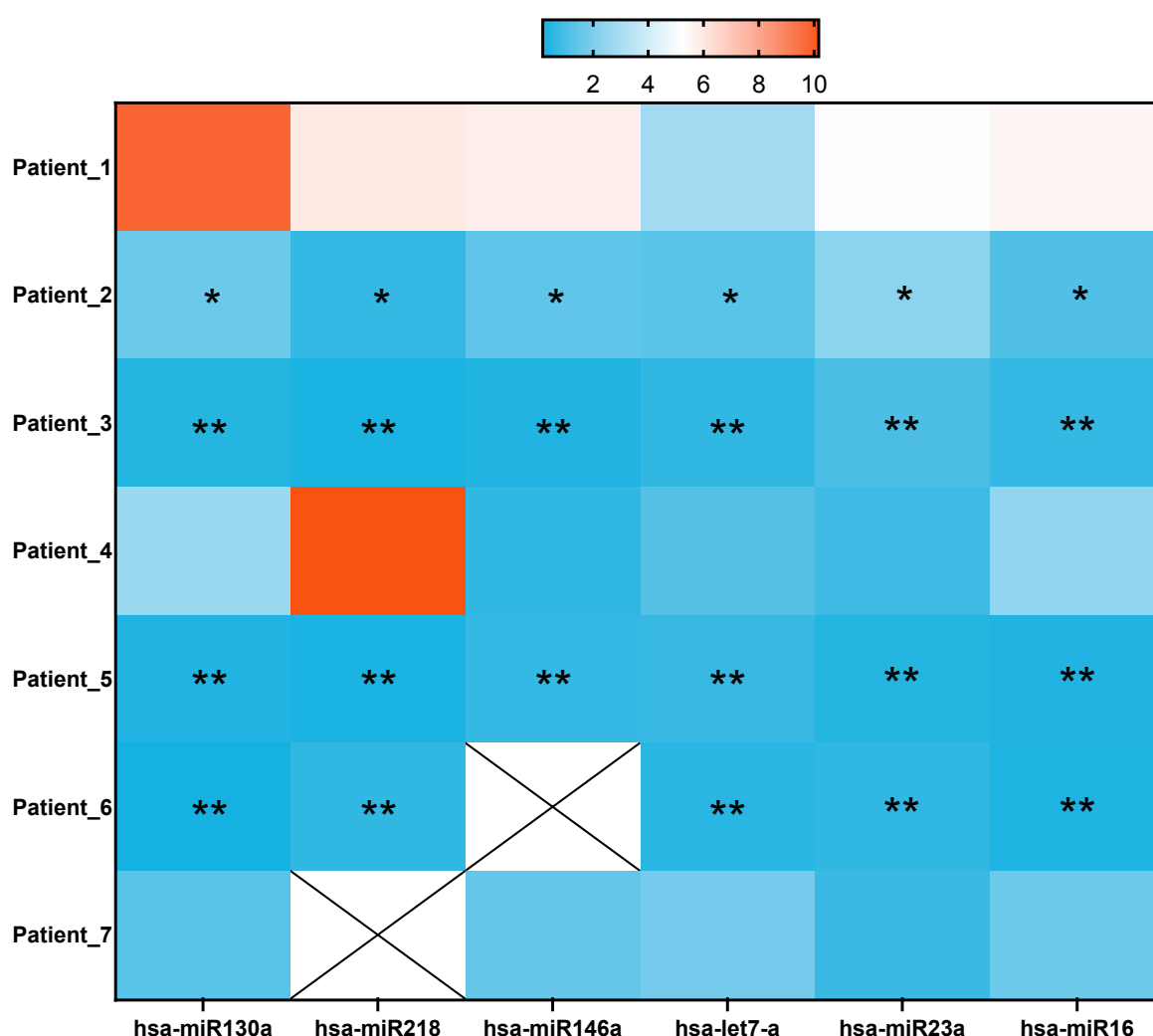


Figure 4.10 Differential expression of miRNAs in patients with sleep apnea disorders

Total RNA was extracted from whole blood samples, according to the manufacturer's protocol. For miRNA quantification, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit and real time PCR with TaqMan miRNA Assay. The relative expression of hsa-miR130a, hsa-miR218, hsa-miR146a, hsa-let7-a, hsa-miR23a, hsa-miR16 miRNAs was normalized to the endogenous control, U6 snRNA using the 2^{-ddCt} method.

2-way Anova with post hoc Tukey multiple comparison, * $p < 0,05$ ** $p < 0,01$ vs Patient_1

4.11. Circadian variation in metabolic profiles of muscle tissue

Metabolomic profiling was performed under physiological condition during the inactive (ZT3) or active (ZT15) phase in muscle tissue to investigate potential circadian variation in metabolism. The data revealed a time-of-day–dependent shift in muscle metabolism. Hierarchical clustering separated ZT13 and ZT15 samples, with most metabolites showing higher abundance at ZT15. Pathway enrichment analysis further identified an increased representation of amino acid–related pathways, particularly arginine and proline metabolism, glutamate metabolism, glutathione and NO metabolism. These results suggest that muscle tissue undergoes a biphasic state during the circadian cycle, highlighting ZT15 as a more metabolically active state characterized by enhanced amino acid turnover, redox activity and nitrogen metabolism (*Figure 4.11*).

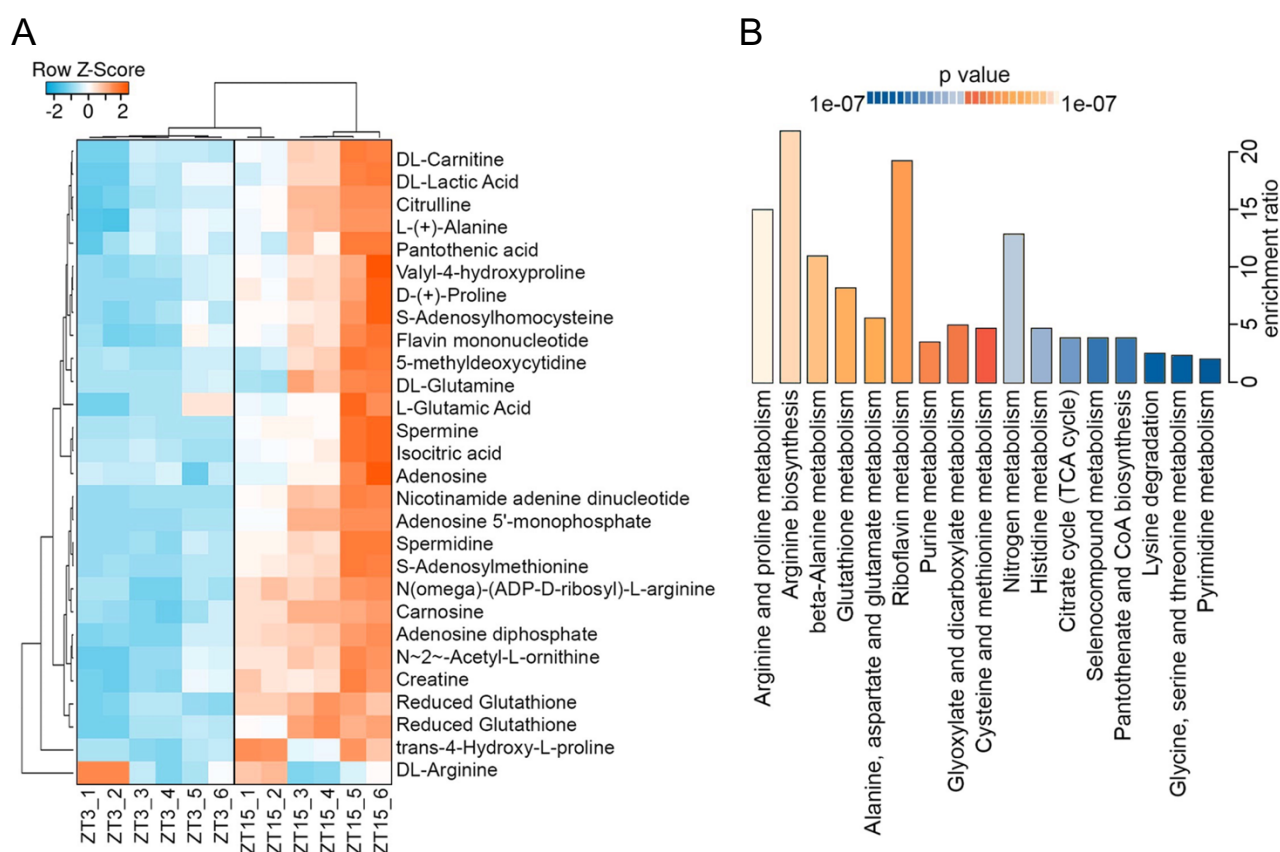


Figure 4.11 Circadian variation of muscle metabolic state under physiological condition

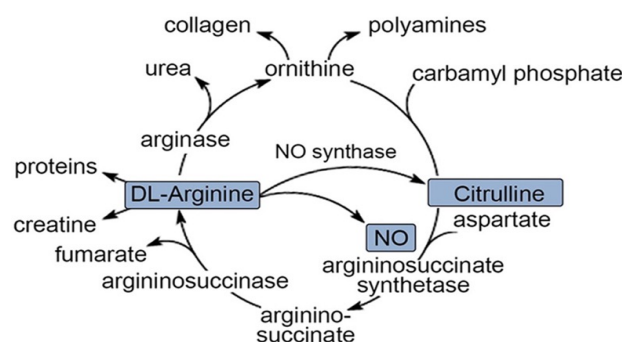
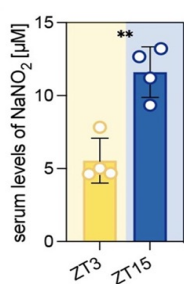
Metabolomic profiling was performed during the inactive (ZT3) and active (ZT15) phases to evaluate the diurnal variations in muscle metabolism. Hierarchical clustering showed two distinct phases between ZT3 and ZT15 samples, suggesting circadian differences in metabolic states. Pathway enrichment analysis identifies an increased representation of amino acid–related pathways, particularly arginine and proline metabolism, glutamate metabolism, glutathione and NO metabolism.

4.12. Circadian regulation of the arginine–citrulline–nitric oxide pathway

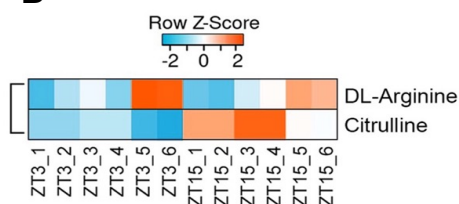
Based on the pathway enrichment analysis, NO may be involved in the circadian transition between the inactive and active phases. Thus, we further examined the arginine–citrulline–nitric oxide pathway, which could contribute to the higher metabolic rate during the active phase. Nitrite levels were measured in serum samples collected from mice during their inactive or active phase (*Figure 4.12A*). The results demonstrated a significant higher concentration of nitrite at ZT15 compared to ZT3. Consistently, metabolomic analysis showed an increase in citrulline levels along with a reduced arginine-to-citrulline ratio at ZT15 (*Figure 4.12 B, C*), suggesting an alteration in the activity of the NOS in the conversion of arginine to citrulline and consequently the release of NO.

Collectively, these data indicate that the shift from the inactive to the active phase is accompanied by an increase in NO production and arginine metabolism.

A



B



C

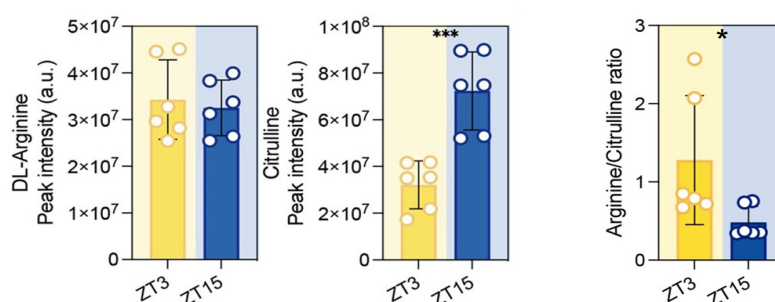


Figure 4.12 Circadian variation of the arginine–citrulline–nitric oxide pathway

Serum nitrite levels were measured during the inactive (ZT3) and active (ZT15) phases with the Griess Assay (**A**). Nitrite concentrations were higher at ZT15, indicating increased NO production during the active phase. Metabolomic profiling of muscle tissue showed elevated citrulline levels and a reduced arginine-to-citrulline ratio at ZT15, suggesting an increase of nitric oxide synthase activity with formation of NO and citrulline (**B-C**).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$; $n = 6$ /group.

4.13. Circadian disruption of the arginine–citrulline–nitric oxide pathway after focal ischemia

To investigate whether ischemic injury affects the biphasic regulation of the arginine–citrulline–NO pathway, mice were subjected to tMCAO during either the inactive (ZT3) or active phase (ZT15), and levels of arginine and citrulline were measured at 6 hours and 24 hours after stroke onset.

As illustrated in Figure 4.13, DL-Arginine levels increased at both 6 hours and 24 hours post-reperfusion at ZT3 and ZT15. Citrulline levels were reduced when tMCAO onset occurred during the active phase compared with controls. The arginine-to-citrulline ratio increased significantly at 6 hours and 24 hours both at ZT3 and ZT15, indicating a reduced activity of NOS after ischemia. These findings suggest that ischemia modifies the arginine–NO pathway in a circadian-dependent manner.

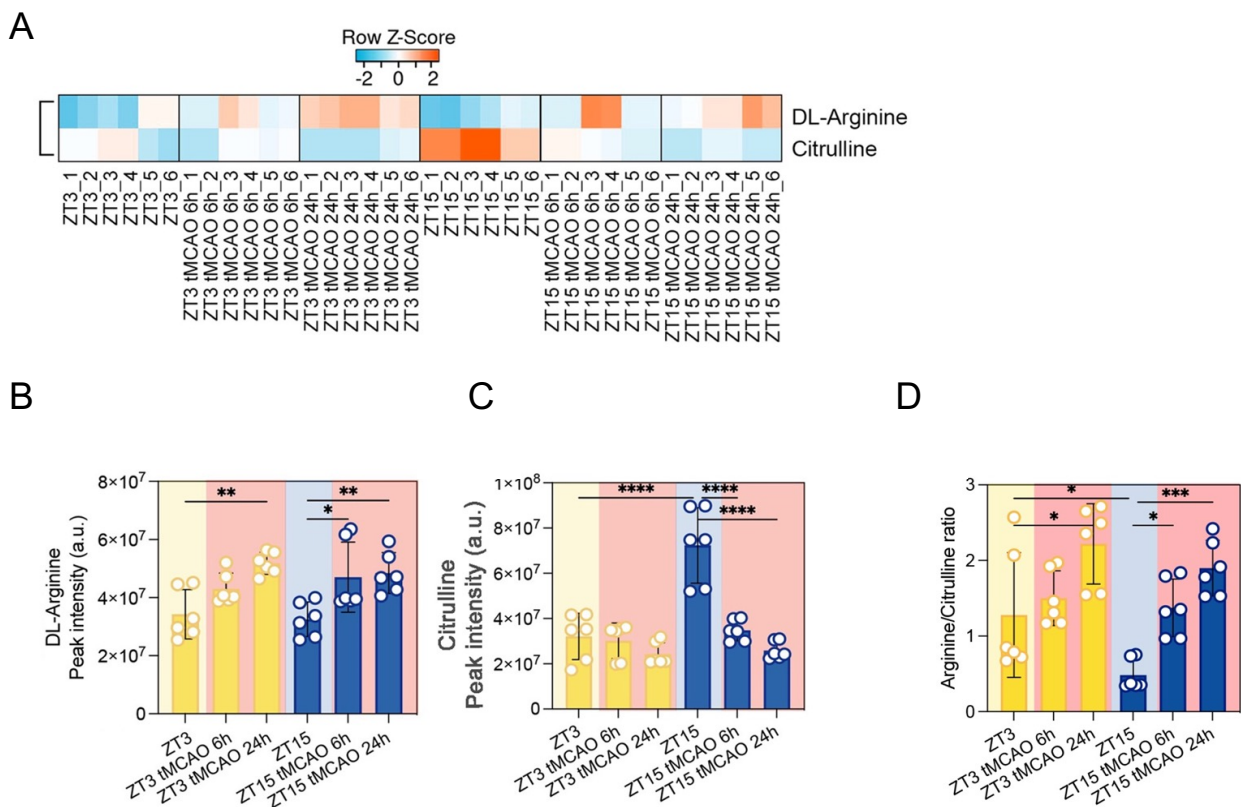


Figure 4.13 Circadian disruption of arginine–citrulline–nitric oxide pathway after focal ischemia

Mice were subjected tMCAO during the inactive (ZT3) or active (ZT15) phase. Levels of DL-arginine and citrulline were measured at 6 and 24 hours after reperfusion (**A**). DL-arginine levels significantly increased at 6h and 24h during both active and inactive phase (**B**). Citrulline levels were reduced when ischemia occurred during the active phase compared to baseline. No variation in citrulline levels when stroke onset is at the inactive phase (**C**). The arginine-to-citrulline ratio was significantly elevated at 6 and 24 hours at both ZT3 and ZT15, indicating a transient reduction in nitric oxide synthase activity following ischemic injury (**D**).

Results are expressed as mean $\pm$ SEM, 2-way Anova with post hoc Tukey multiple comparison, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$

4.14. Circadian variation of circulating microRNAs based on the time-of-day of stroke onset

Differential expression of miRNAs was evaluated in whole blood in human stroke patients (Figure 4.14). The term “day” includes patients with stroke onset at 6 a.m. – 6 p.m., the term “night” includes patients with stroke onset at 6 p.m. – 6 a.m. The relative expression of hsa-

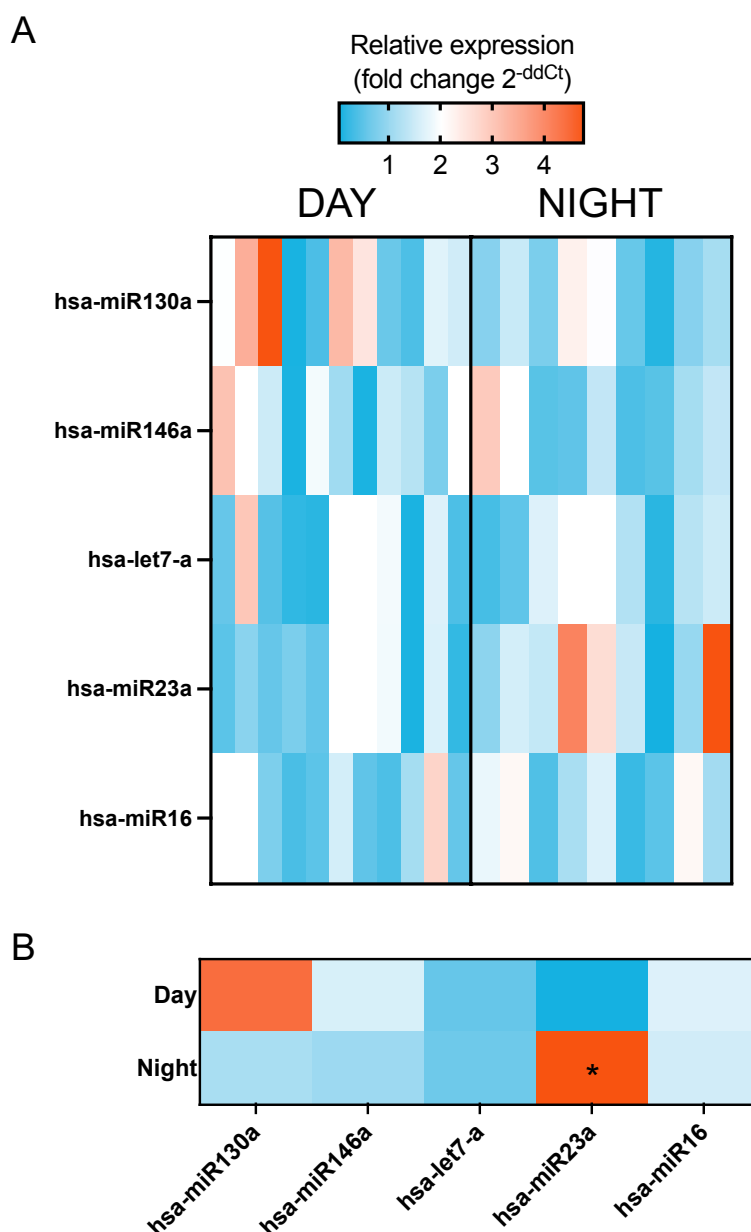


Figure 4.14 Circadian differential expression of microRNAs in patients with stroke

Differential expression of miRNAs was evaluated in whole blood in human stroke patients. Day includes patients with stroke onset at 6 a.m. – 6 p.m., night includes patients with stroke onset at 6 p.m. – 6 a.m. The relative expression of hsa-miR130a, hsa-miR146a, hsa-let7-a, hsa-miR23a, hsa-miR16 miRNAs was normalized to the endogenous control, U6 snRNA using the 2^{-ddCt} method. Colour intensity reflects relative expression levels: **blue** indicates low expression, and **red** indicates high expression. Panel **A** shows single patient data: the rows correspond to individual miRNA, while the columns correspond to the patients. Results in panel **B** are expressed as mean $\pm$ SEM, of n=9 to 11/group, Tukey t-test two tailed *p<0,05

miR130a, hsa-miR146a, hsa-let7-a, hsa-miR23a and hsa-miR16 was evaluated through real-time PCR. Based on the findings and as reported in the heat map, miRNA expression is differently modulated by time-of-day stroke onset. Among all the miRNAs analysed, miR-16 relative expression was significantly increased when stroke onset occurred during the awake phase (day) compared to the sleep phase (night). An opposite trend – not significant – was observed for miR-130a expression. No diurnal variations were observed for the other miRNAs analysed.

4.15. Remote limb postconditioning is neuroprotective only during the inactive phase of stroke onset

To investigate the time-of-day–dependent effects of remote ischemic limb postconditioning (RLIP), mice from inactive (ZT1–5) and active (ZT13–17) phases were subjected to 60 minutes of tMCAO, followed by either standard poststroke care or RLIP. As shown in *Figure 4.15*, TTC staining revealed that RLIP significantly reduced infarct volume when performed during the inactive phase (ZT1–5) but had no protective effects during the active phase (ZT13–17) at 24 hours (Day 1) and 72 hours (Day 3) post-stroke. Twenty-four hours marks an early time point for assessing the acute phase of post-stroke changes, whereas 3 days post-stroke represents a later stage of these changes, where the brain is vulnerable to further damage and rehabilitation may be started. These findings might indicate that the efficacy of RLIP is influenced by the time-of-day of stroke onset.

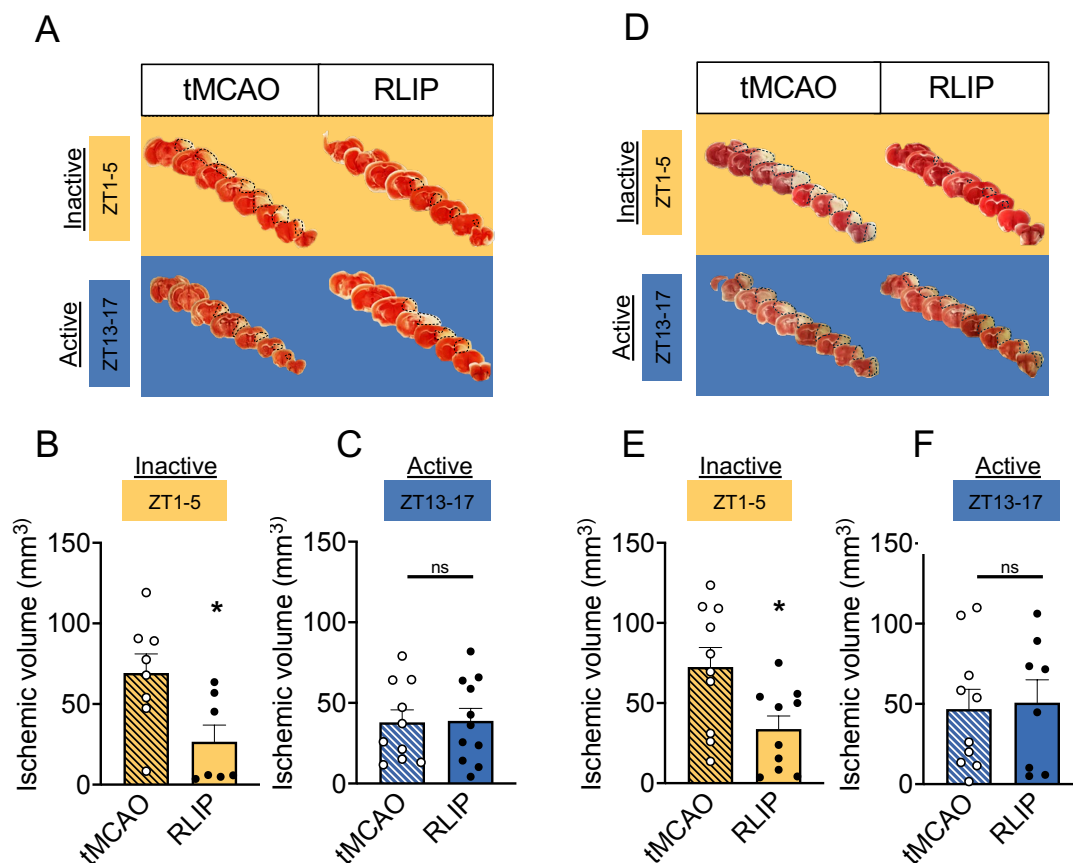


Figure 4.15 Neuroprotection elicited by remote limb ischemic postconditioning depends on time-of-day of stroke onset

Eight brain coronal sections were stained with TTC. The white region highlights the ischemic area (A, D). The lower panel shows the quantification of the infarct area. RLIP significantly reduces the ischemic volume compared to tMCAO during the inactive phase (**yellow plots**) both at day 1 and day 3 (B, E). RLIP did not reduce the infarct volume when applied during the active phase (**blue plots**) (C, F). Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$; $n = 7$ to 11/group.

4.16. Remote limb postconditioning improved neurological recovery only during the inactive phase

Neurological deficits were evaluated using the modified Neurological Severity Scores (mNSS), which include motor and sensory components, on both day 1 and day 3 post-stroke. RLIP significantly improved neurological recovery, as shown by the total mNSS during the inactive phase at both time points (*Figure 4.16a*). No differences were observed between RLIP-treated and stroke only group during the active phase.

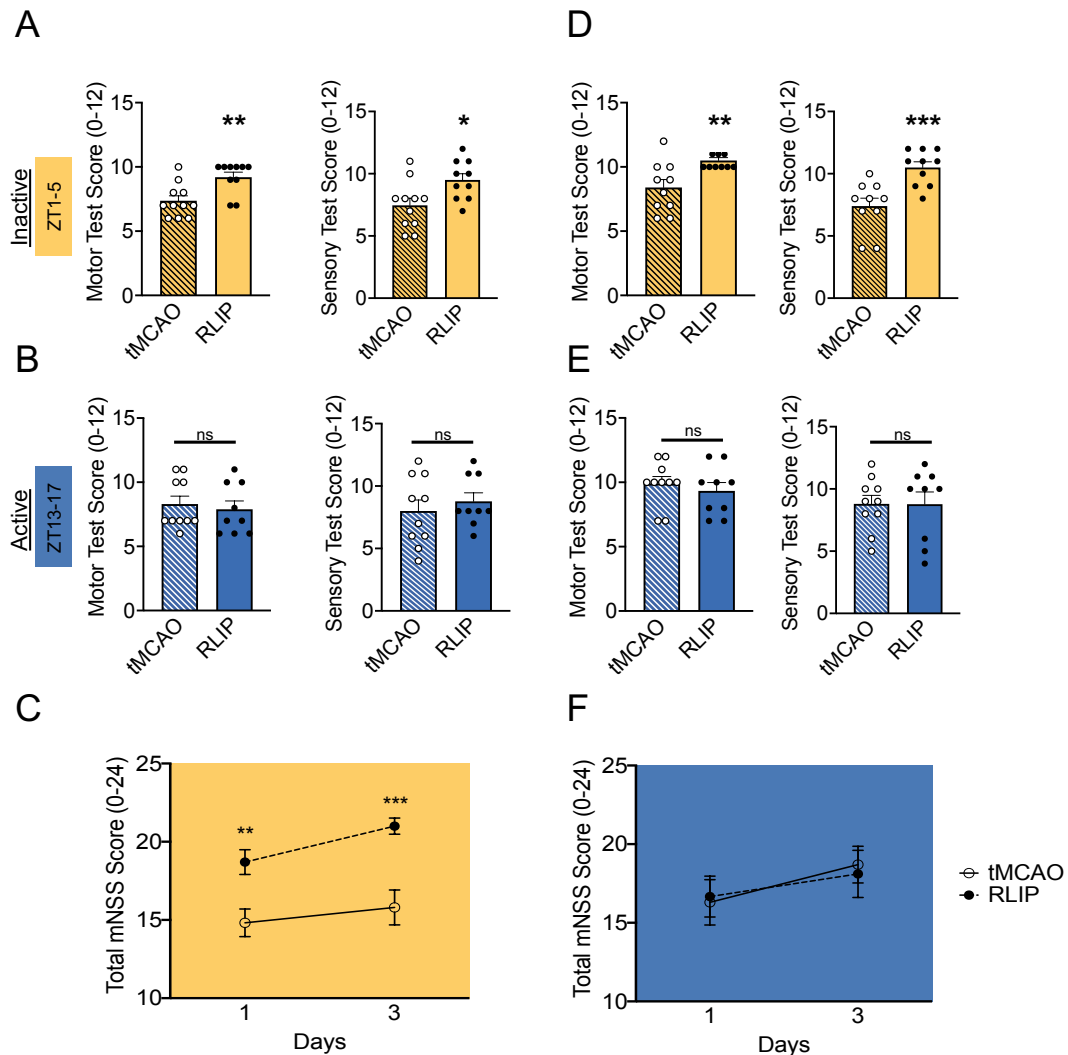


Figure 4.16a Remote limb ischemic postconditioning improves functional recovery during the inactive phase

Motor and sensory (summed in the total modified Neurological Severity Score [mNSS]) assessments were evaluated at day 1 (**A-C**) and at day 3 (**D-F**). A low score suggests moderate or severe defects, and a higher score indicates a mild defect. RLIP improves neurological outcomes during inactive phase at days 1 (**A**) and 3 (**D**). No difference between RLIP and tMCAO during active phase (**B**, **E**).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs tMCAO; $n = 7$ to 10 /group.

Motor coordination and skilled walking were assessed using the foot fault test on a walking grid (Figure 4.16b). RLIP significantly reduced foot faults only at day 3 during the inactive phase, while no benefits were observed in the active phase.

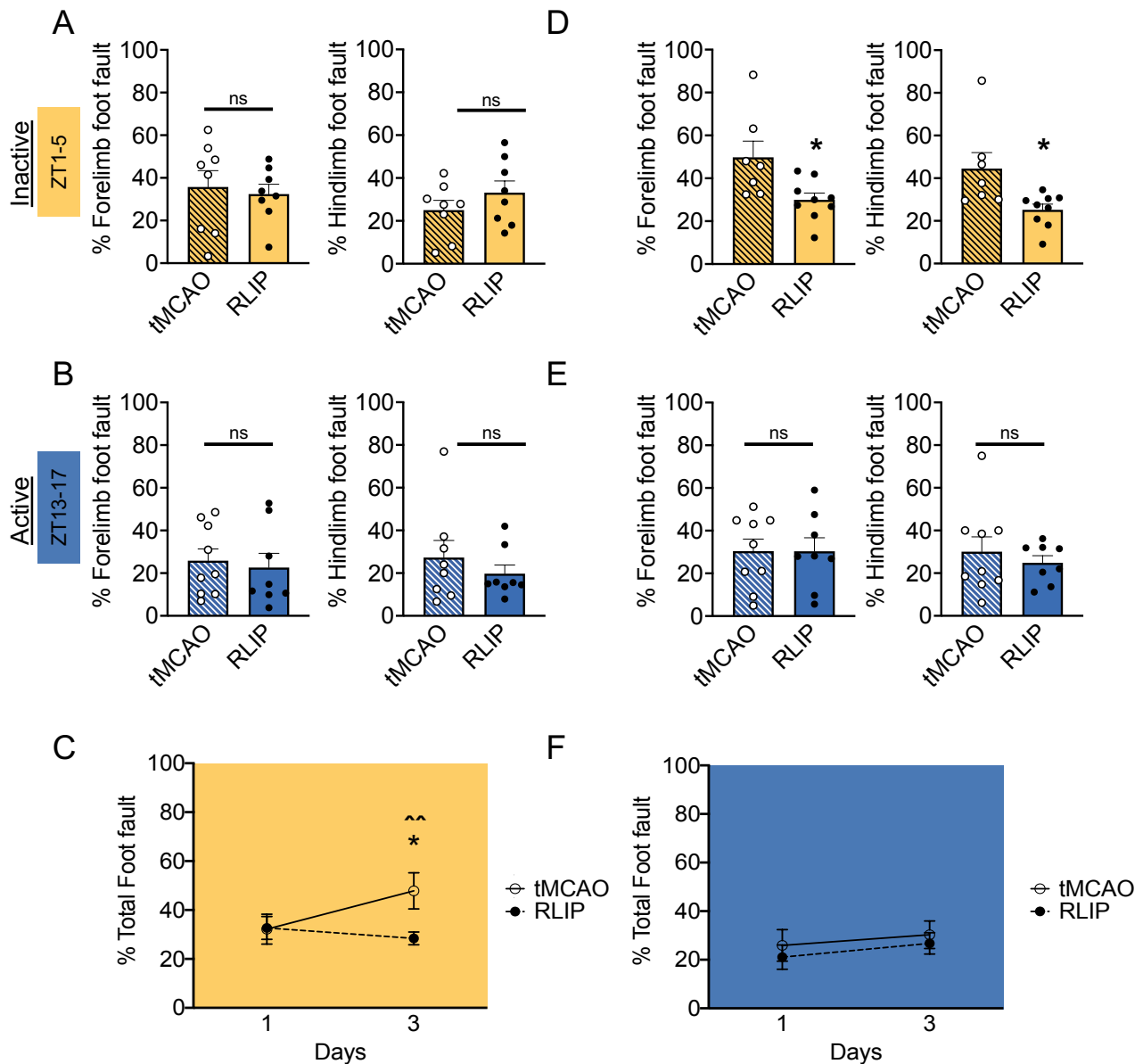


Figure 4.16b Remote limb ischemic postconditioning ameliorates motor outcome during inactive phase but not during active phase

Skilled walking was evaluated at days 1 (A-C) and 3 (D-F). RLIP has shown an improvement in the walking functional recovery after brain injury at day 3 during inactive phase (C-D); no effects during the active phase (B, E).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0,05$ RLIP vs tMCAO, ^^ $p < 0,01$ tMCAO day 3 vs tMCAO day 1: $n = 7$ to 10 /group.

Consistently, in the adhesive tape removal test, RLIP improved sensorimotor performance at day 3 only during the inactive phase (*Figure 4.16c_D*). No significant improvements were detected in this test at 24 hours (*Figure 4.16c_A*). Together, these findings support a circadian phase-dependent effect of RLIP in promoting post-stroke functional recovery.

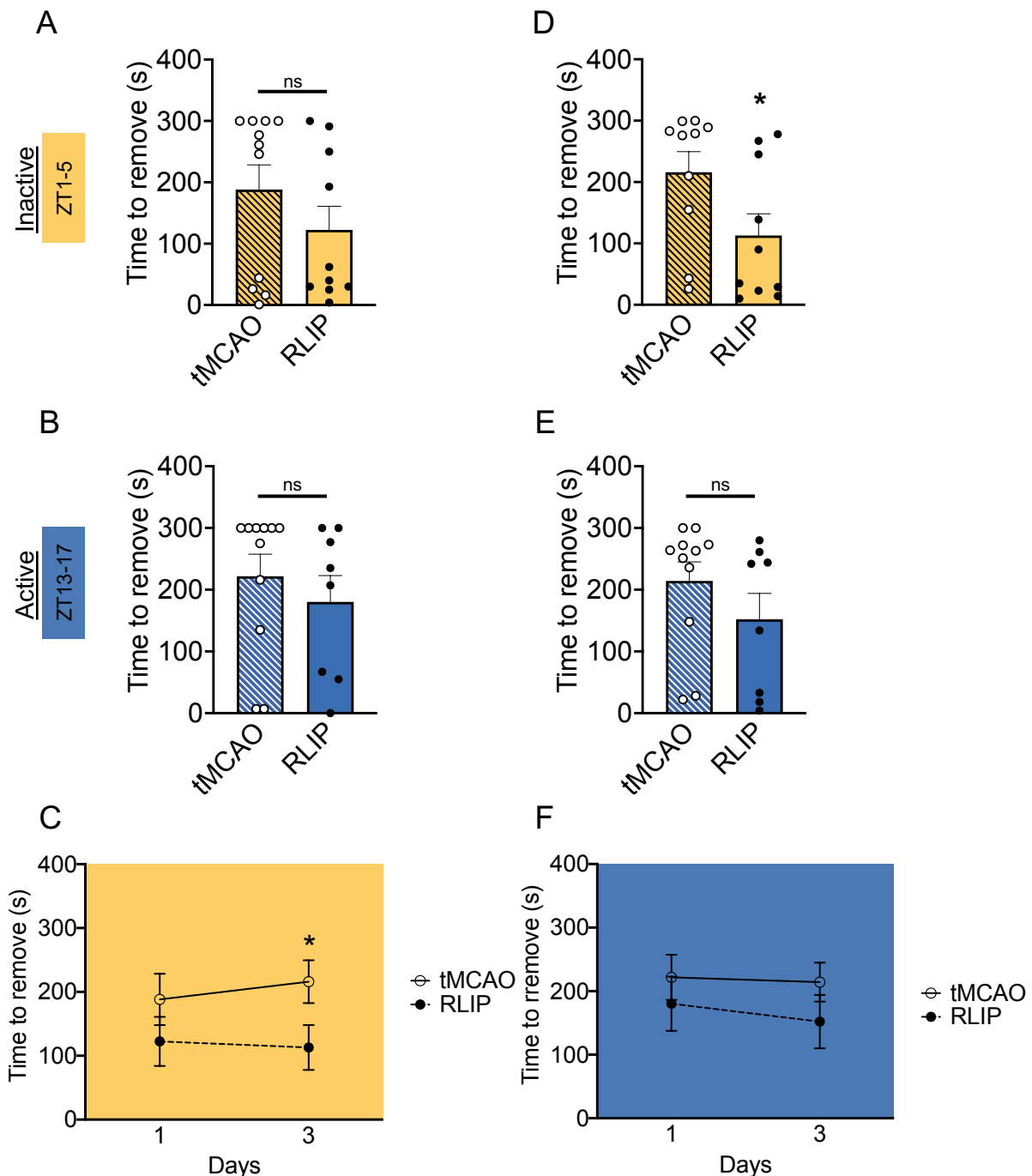


Figure 4.16c Remote limb ischemic postconditioning improves sensitivity recovery during the inactive phase but not during the active phase

RLIP has shown an improvement in the paw and mouth sensitivity recovery after brain injury at day 3 during inactive phase (**C, D**); no effects during active phase (**B, E**).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$ RLIP vs tMCAO, ^^ $p < 0.01$ tMCAO day 3 vs tMCAO day 1. N=7 to 10/group.

4.17. Circadian-phase–dependent regulation of PER2 and nNOS following remote limb ischemic postconditioning

During the inactive phase, as shown by Western Blot analysis, PER2 protein levels were significantly reduced in the RLIP group compared with stroke group (*Figure 4.17a_D, E*). This reduction was observed at both day 1 (*Figure 4.17a_D, E*) and 3 (*Figure 4.17b_D, E*). Conversely, nNOS protein levels were significantly elevated in the RLIP-treated mice compared with the tMCAO group during the inactive phase at day 1 (*Figure 4.17a_A, B*), with a similar trend up to day 3 (*Figure 4.17b_A, B*). No significant changes in nNOS levels were observed during the active phase. These results suggest a circadian phase–specific molecular response to RLIP, characterized by PER2 downregulation and nNOS upregulation during the inactive phase.

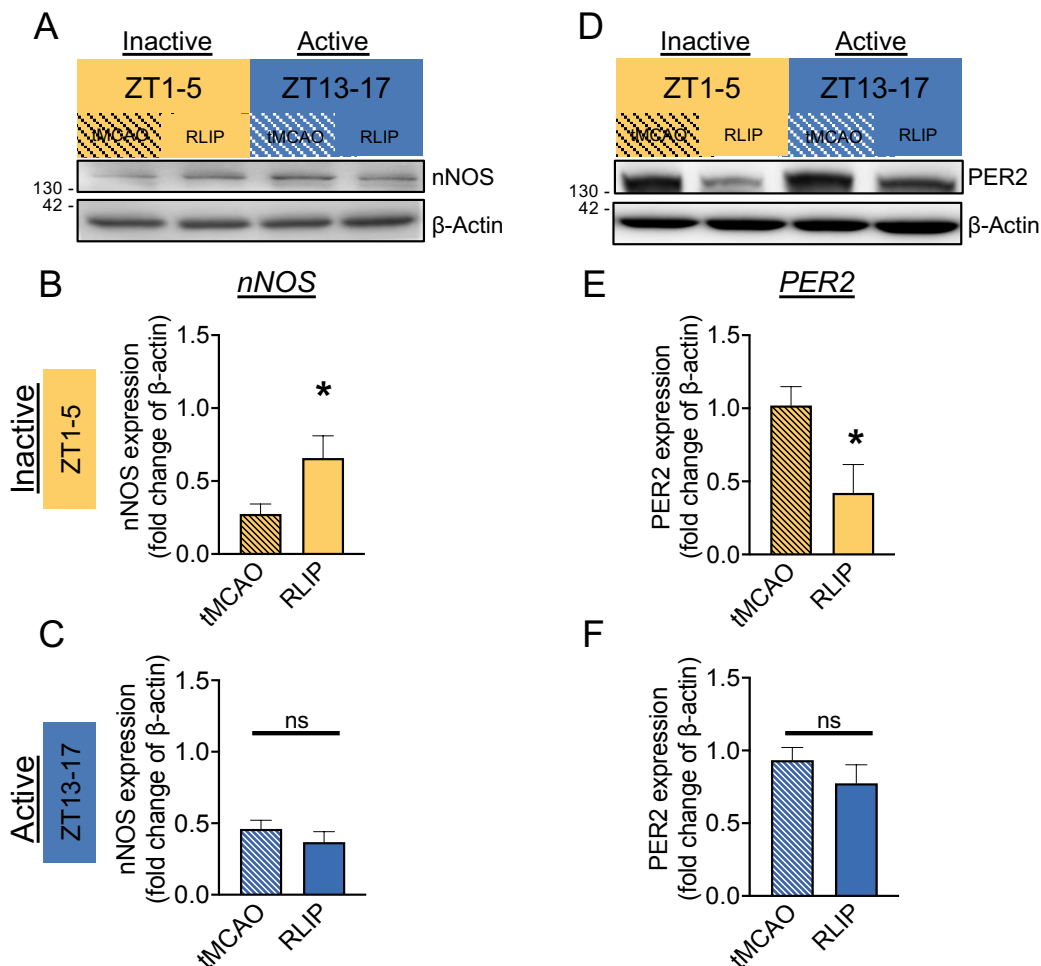


Figure 4.17a Representative Western blots of nNOS (neuronal nitric oxide synthase) and PER2 (period circadian regulator 2) at 24 hours after stroke

Representative Western Blot analysis of nNOS and PER2 protein levels at day 1 post stroke (**A, D**). Remote ischemic limb postconditioning induced a significantly reduction of PER2 expression compared with stroke group, during the inactive phase (**E**) but not during the active phase (**F**). nNOS increased after RLIP group when stroke onset was at ZT 1-5 (**B**) but not at ZT13-17 (**C**).

Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0.05$. N=3 to 5/group.

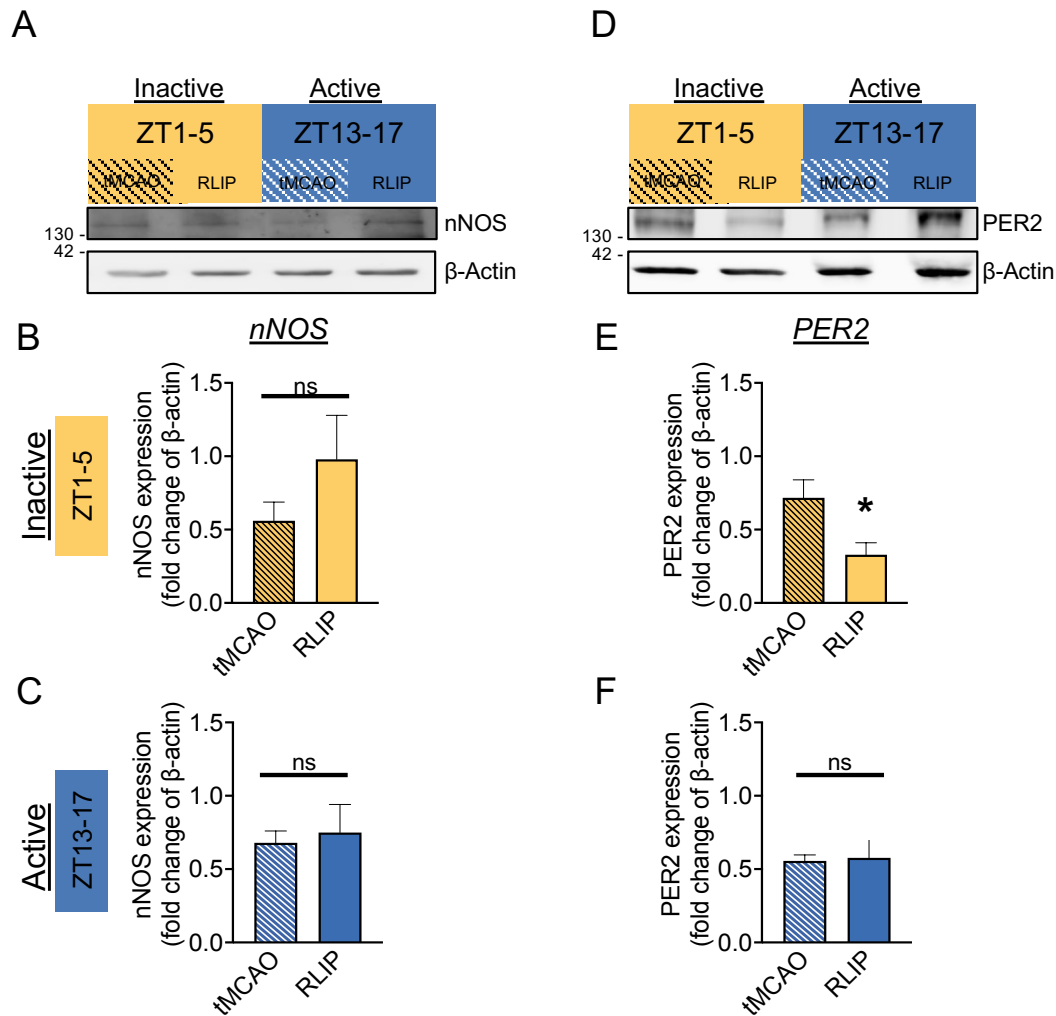


Figure 4.17b Representative Western blots of nNOS (neuronal nitric oxide synthase) and PER2 (period circadian regulator 2) at 72 hours after stroke

Representative Western Blot analysis of nNOS and PER2 protein levels at day 3 post stroke (**A, D**). Remote ischemic limb postconditioning induced a significantly reduction of PER2 expression compared with stroke group, during the inactive phase (**E**) but not during the active phase (**F**). nNOS shows a slight increased expression, but not significant, after RLIP group when stroke onset was at ZT 1-5 (**B**). Results are expressed as mean $\pm$ SEM, Tukey t-test two tailed * $p < 0,05$. N=3 to 5/group.

4.18. nNOS activity is required for PER2 modulation by remote limb ischemic postconditioning

To determine whether nNOS activity contributes to the modulation of PER2 expression after RLIP, mice underwent tMCAO or RLIP and were treated with either the selective nNOS inhibitor 7-nitroindazole (25 mg/kg) or vehicle (dimethyl sulfoxide) 30 minutes after stroke onset (*Figure 4.18A*). Western blot analysis performed 24 hours later at ZT1-5 confirmed that nNOS protein levels were drastically reduced under nNOS inhibition (*Figure 4.18B*, left panel). In addition, under this condition no significant difference in PER2 expression between the tMCAO and RLIP groups were observed (*Figure 4.18B*, right panel). Collectively, these results suggest that modulation of PER2 by RLIP requires nNOS activity.

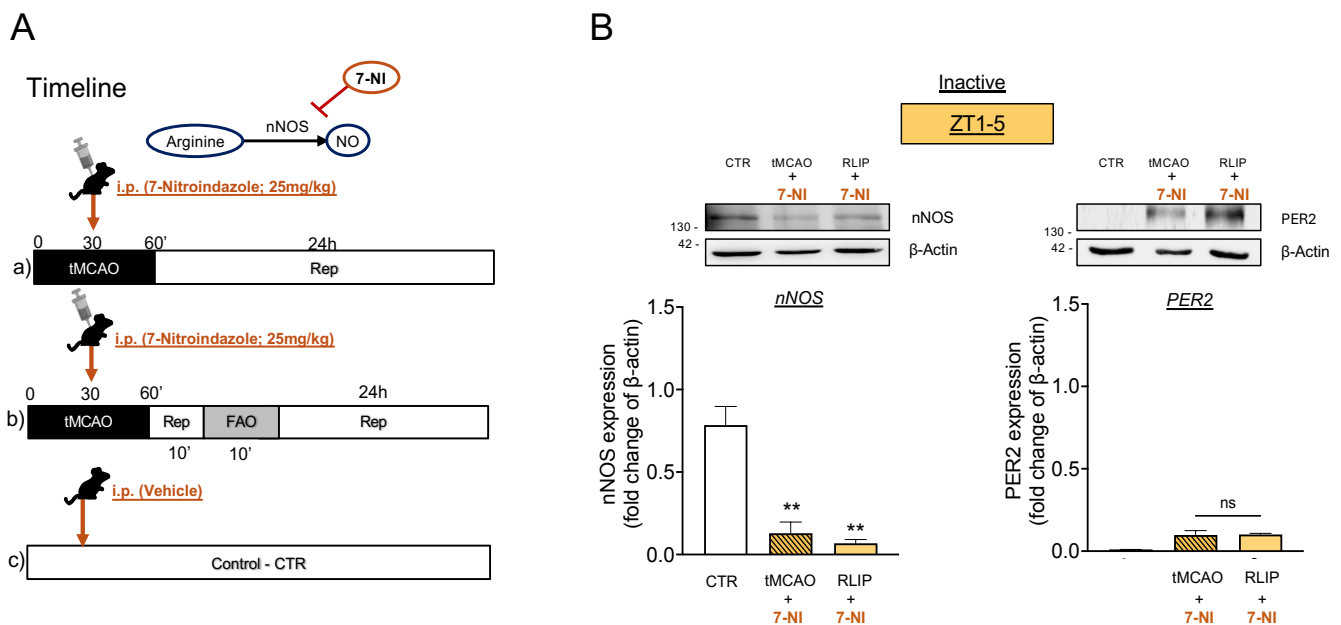


Figure 4.18 nNOS (neuronal NO synthase) activity is required for PER2 (period circadian regulator 2) modulation during the inactive phase by remote limb ischemic postconditioning

Experimental design (**A**): mice at ZT1-5 subjected to tMCAO or RLIP were administered with 7-nitroindazole (7-NI; nNOS inhibitor; 25 mg/kg) or Veh (dimethyl sulfoxide) 30 minutes after tMCAO induction. Western blot analysis was performed 24 hours later. Under nNOS inhibition, nNOS levels were drastically reduced (**B**, left panel), and no variation of PER2 expression between tMCAO or RLIP groups was observed (**C**, right panel).

Results are expressed as mean $\pm$ SEM of $n=3$ /group. One-way ANOVA and post hoc Tukey test for multiple comparison, ** $p<0.01$ vs CTR.

5. DISCUSSION

The present work provided evidence of the potential neuroprotective role of remote limb postconditioning against focal ischemia. In particular, the study has explored two specific aspects of the mechanistic function of remote conditioning in an experimental model of transient occlusion of the middle cerebral artery: the modulation of the glymphatic system and the influence by the circadian rhythms.

5.1. Remote conditioning as an endogenous modulator of glymphatic system

The study provides new mechanistic insights into the role of remote conditioning, as an endogenous neuroprotection strategy against stroke. Growing evidence has demonstrated that remote conditioning can reduce infarct size compared to controls (Cuomo et al., 2024; Pignataro, 2021; Vinciguerra et al., 2018), with neuroprotective effects not only within the ischemic penumbra but also throughout the brain, by enhancing anti-apoptotic, antioxidant, and anti-inflammatory responses (Basalay et al., 2020; Beretta et al., 2025; Torres-Querol et al., 2021). Our data show that the remote limb ischemic postconditioning (RLIP), performed through a transient occlusion of the femoral artery, induces a significant reduction of ischemic volume compared to stroke group (*Figure 4.1*). This neuroprotection is characterized by increased expression of the water channel protein AQP4, abundantly expressed in astrocytic end-feet (Salman et al., 2021) (*Figure 4.2*). During the acute phase of neuroinflammation in post-stroke injury (H. Guo et al., 2025), the early mobilization of astrocytes expressing AQP4 in the infarcted area may contribute to the protective effects observed with RLIP. The localization of AQP4 to astrocytic vascular end-feet promotes CSF movement through the brain (Díaz-Castro et al., 2023). Several studies have reported a dual role of AQP4 expression, often correlating with edema post-stroke (Vandebroek & Yasui, 2020). However, given the complex spatial and temporal patterns of AQP4 expression, it is still unclear whether increased AQP4 expression exacerbates brain edema or promotes fluid clearance (Manley et al., 2009; C. Sun et al., 2022; Tang & Yang, 2016). During the initial phase of ischemic injury, energy failure leads to an osmotic gradient – ionic imbalance and osmotic swelling – that drives water flow from the blood into brain parenchyma, causing cytotoxic edema through AQP4 channels, typically within the first 24 hours (Badaut et al., 2007; Papadopoulos & Verkman, 2013). At a later phase, vasogenic edema develops, and in this phase, excess water flows through the brain parenchyma transcellularly via AQP4, into perivascular spaces (vein side), from where it is cleared into cervical lymphatics (S. Li et al., 2015).

On the other hand, AQP4 is a fundamental player of glymphatic system, which activity is correlated with CSF dynamics (Mestre et al., 2018). It has been shown that reduction in AQP4 polarization, in turn, prevents CSF/ISF exchange by reducing the interstitial space volume promoting dysfunction of the glymphatic flow (Verkman et al., 2006). The polarization of AQP4 at the perivascular astrocytic end-feet facilitates glymphatic fluid movement and A β clearance in rodents (Mestre et al., 2018; Simon et al., 2022).

In the current study, increased values of AQP4 expression were found in the striata of RLIP-treated mice at 24 hours that may likely reflect the transition from cytotoxic to vasogenic edema. Moreover, RLIP counteracted AQP4 mislocalisation following ischemic injury, thereby improving AQP4 polarization at the astrocytic vascular end-feet, as demonstrated by immunofluorescence analysis (*Figure 4.3*). This effect appears to depend on syntrophin-dependent anchoring of AQP4 at astrocytic end-feet, which stabilizes the channel in the perivascular membrane domain (Amiry-Moghaddam et al., 2003; Cohen-Salmon et al., 2025) (*Figure 4.3*). The increase in Collagen-IV expression further supports an improvement in blood–brain barrier integrity following RLIP, as previously reported (Yonezawa et al., 2010). These findings are further supported by the data obtained by silencing AQP4 (*Figure 4.5*), which completely abolished the neuroprotective effects correlated with RLIP, confirming a mechanistic role for AQP4 in mediating RLIP effects. *Ex vivo* imaging of fluorescent tracer clearance showed that RLIP promoted the clearance of small-molecular-weight dyes from the brain parenchyma and meninges, suggesting restored glymphatic flow following stroke (*Figure 4.8*).

Taken together, these results support RLIP as a promising non-invasive strategy to elicit neuroprotection, by restoring AQP4 polarization at astrocytic end-feet and improving glymphatic clearance, thus counteracting glymphatic impairments post-stroke.

5.2. Neuroprotection elicited by remote conditioning is influenced by circadian rhythms

In the second part of the study, we asked whether the time-of-the-day may influence the efficacy of RLIP-induced neuroprotection. This hypothesis arises from a simple observation: since most of the stroke literature on neuroprotective strategies is based on experimental data collected during the day - when rodents are typically inactive - could this latter point represents an additional factor that explains the translational failure from bench to bedside? On the other hand, clinical trials generally recruit patients who had a stroke during the day (Broderick et al., 2022; Ferreira et al., 2019), and establishing the time of onset is often fundamental (Esposito et al., 2020, 2022). Based on these premises, we aimed to elucidate

whether endogenous neuroprotective strategy elicited by remote limb ischemic postconditioning may be influenced by the time of the day of stroke onset.

As reported in the literature, circadian rhythms have a strong influence on physiological processes, including stroke pathophysiology (Leng et al., 2019; Y. Shen et al., 2023). We hypothesized that the timing of experiments might impact the efficacy of RLIP.

Our data showed that RLIP is particularly effective in promoting post-stroke recovery when applied at a specific time of the day, specifically during the inactive phase. In particular, RLIP reduces the ischemic volume at both acute (1 day post-stroke) and later (3 days post-stroke) time points, but only during the inactive phase, referred to as Zeitgeber time 1-5 (ZT 1-5) (*Figure 4.15*). Twenty-four hours marks an early time point for assessing the acute phase of post-stroke changes, whereas 3 days post-stroke represents a later stage of these changes, where the brain is vulnerable to further damage and rehabilitation may be started.

As described in the paragraph 1.1.4, the interval between 48 and 96 hours following ischemia corresponds to the transition from the acute phase to the early reparative phase, during which secondary injury processes—such as reactive astrogliosis, neurovascular unit dysfunction, and alterations in AQP4 polarization—become fully established. Evaluating both time points allows the investigation of early-phase changes (day 1) as well as later-stage alterations (day 3), providing a comprehensive timeline for assessing RLIP-mediated modulation of AQP4 dynamics and neuroinflammatory responses during this critical stage of post-ischemic evolution.

Specifically, the reduction of the ischemic volume is associated with an improvement in neurological score, both sensory and motor tests (*Figure 4.15a-c*), as evaluated through the mNSS scores, foot-fault, and adhesive tape removal tests (Licastro et al., 2025). No improvements in post-stroke recovery were observed during the active phase.

These results are consistent with recent studies showing that three well-known neuroprotectants that work when applied during the inactive phase, failed to provide neuroprotection during the active phase in different rodent models of stroke, suggesting that circadian cycles may influence the translational failure (Esposito et al., 2020).

Similarly, evidence from a clinical study demonstrated circadian variations in both ischemic core volume and core growth after stroke (Reidler et al., 2023). In particular, patients with stroke onset around midnight showed larger infarct core and faster infarct progression compared with patients with onset around noon. The authors suggested that for nocturnal stroke (patients experiencing stroke during sleep/inactive phase), the therapeutic window may be significantly narrower than for diurnal strokes (patients experiencing stroke during

awake phase). Consequently, minimizing delays to EVT and IVT could be particularly crucial for night-onset patients to achieve treatment outcomes comparable to those with daytime onset (Reidler et al., 2023).

As is well known, rodents are nocturnal animals with nighttime being their active phase. Data obtained from metabolomic profiling indicate that the active phase is a more metabolically active state (*Figure 4.11*), characterized by enhanced amino acid turnover, redox activity, and nitrogen metabolism (*Figure 4.12 and Figure 4.13*). In particular, the arginine-to-citrulline pathway appears to be highly activated, suggesting high activation of nitric oxide synthase to produce citrulline and NO. The nitric oxide has a dual role in the biological process, which can be either advantageous or harmful to cellular function. In particular, NO acts as a potent vasodilator and an angiogenic stimulator, playing an important role in mediating blood flow and cell death mechanisms after stroke (Garry et al., 2015; Steinert et al., 2010). Although nNOS has traditionally been recognized as a harmful enzyme in the pathogenesis of cerebral ischemia (Samdani et al., 1997), it has been reconsidered as a neuroprotective marker involved in the endogenous neuroprotective mechanisms of brain preconditioning (Iadecola et al., 2011; Pignataro, Esposito, et al., 2013). It is now considered a key player in the conditioning-based medicine, especially after RLIP therapy.

In this work, we observed an increased expression of nNOS accompanied by a reduction of PER2 levels during the inactive phase, but not in the active phase (*Figure 4.17*). Pharmacological inhibition of nNOS abolished PER2 differences observed during the inactive phase, suggesting that these two proteins might be implicated in RLIP neuroprotection in a time-of-day–dependent manner (*Figure 4.17*). Previous studies have demonstrated a close interaction of the circadian gene *Per2* and nNOS. In particular, mutations in the circadian gene *Per2* in mice are associated with decreased NO production, while light-induced activation of NOS has been shown to decrease PER2 monomer levels (Viswambharan et al., 2007). Taken together, our findings indicate that there exists a circadian phase–specific molecular response elicited by RLIP, in which PER2 and nNOS are tightly linked, exerting a fundamental role in mediating time-of-day dependent signalling pathways in post-ischemic recovery. The absence of this response during the active phase further underscores the importance of considering the timing of stroke onset in therapeutic interventions (Licastro et al., 2025).

Clinically, most ischemic strokes occur in the morning hours, with emerging data suggesting a second peak in the evening (around 8 p.m.) (Elliott, 1998; Peter-Derex & Derex, 2019; Ryu et al., 2022). This latter subgroup population might benefit from RLIP-based strategies,

tailoring a subset of patients who experience stroke during a particular time of day, such as the inactive (rest) phase. In a population study, the authors reported that the recurrence of ischemic stroke was higher during the interval 6:00 a.m. to 12:00 p.m. compared with other times of the day (X. Wang et al., 2022). However, patients experiencing stroke during the awake phase were associated with better outcomes at three months (X. Wang et al., 2022). As previously reported, this may be a consequence of circadian variation in infarct core size. Consistently, our preclinical data simulated the evolution of the ischemic volume, which increases during the inactive phase (sleep phase for human) and is reduced during the active phase (awake phase for human), during which RLIP interventions exhibit no efficacy on stroke (*Figure 4.15*).

Similarly, in a retrospective study, the evaluation of time-of-day effects in EVT demonstrated a correlation between positive outcomes and daytime treatment (Burbano et al., 2023). These data further support our study, suggesting how the observed effects may derive from the circadian influence on neuronal susceptibility to stroke and the related mechanisms, such as reperfusion and CSF flow. Additionally, EVT shows diurnal efficacy in patients with stroke onset at specific times of the day (Sarraj et al., 2023). Conversely, one recent study reported a favourable outcome at 90 days for patients treated with mechanical thrombectomy during nighttime hours, compared with those treated during the daytime (Benali et al., 2021). To advance understanding of the pathophysiologic basis of human diurnal biology in stroke, consensus recommendations are now available to guide researchers and clinicians in the design, interpretation, and imaging of circadian/stroke population studies (Saver et al., 2023).

All these findings align with recent emerging evidence in the use of chronopharmacology (Hermida et al., 2016; Nahmias & Androulakis, 2021), drawing attention to the need to align the therapeutic interventions taking into account individual's biological clocks.

Future directions may include modulation of the circadian machinery to enhance neuroprotection, for instance, by extending the therapeutic window to strokes occurring during the active phase. For example, melatonin, which is well known to regulate circadian rhythms and modulate nNOS activity, could serve as a potential adjuvant to enhance RLIP-neuroprotection (J. G. Lee et al., 2019).

Overall, these findings provide new insights for identifying molecular targets, biomarkers, and novel therapeutic strategies to improve neuroprotection and recovery after stroke.

5.3. MicroRNAs as potential circulating biomarkers of remote conditioning

Growing evidence has established that microRNAs can be released into the blood and carried by vesicles and other carriers to transmit a signal from the periphery to the brain – a strategy known as remote conditioning (Pignataro, 2021). Previous works from our laboratory have provided miRNA expression profiles in rats subjected to remote conditioning, identifying a subset of miRNAs whose expression was significantly modulated following ischemic injury and post-conditioning treatment (Cepparulo et al., 2021; Cuomo et al., 2024; Vinciguerra et al., 2020). The hypothesis is that miRNAs are released in the bloodstream to communicate with other tissues, and to regulate gene expression of proteins related to pathophysiological mechanisms. Because of their ability to target multiple genes, it's often difficult to define the specific miRNA roles. However, data collected from literature help to clarify their possible functions.

Specifically, our data indicate that in mice, stroke increases the expression of miR-130a-3p, which is partially restored after RLIP (*Figure 4.9*). Previous studies have identified miR-130a as a strong modulator of the promoter of *AQP4* M1 (Sepramaniam et al., 2010). The miR-130a-3p has been implicated in astrocytic function (W. Li et al., 2022) and neuronal survival (Zhu et al., 2021), and its upregulation likely exacerbates injury after ischemic insult, whereas its inhibition exerts neuroprotection (Neag et al., 2022). These functions are highly relevant to the mechanisms explored in this work, particularly *AQP4* dynamics, glymphatic impairment, and neuroinflammatory modulation, which represent central themes of the thesis. Thus, the observed reduction of miR-130a-3p after RLIP may reflect a shift toward protective molecular signalling, and indicate the miR-130a-3p as potential candidate biomarker associated with neuroprotective mechanisms elicited by RLIP.

Preliminary human data obtained from patients affected by sleep apnea disorders, showed a similar circulating miRNA signature among all the patients, except for one patient who has a distinct pattern (*Figure 4.10*). The sample size was determined on the basis of feasibility and the availability of eligible participants during the recruitment period: the sleep apnea cohort used to investigate glymphatic dysfunction–associated microRNAs, and the circadian-stroke cohort employed to assess time-of-day–dependent variations in circulating miRNA profiles. Although a small cohort of subjects was analysed, this number allows to detect expression trends and to generate hypotheses for future investigation. Specifically, this approach enabled an initial characterization of a microRNA signature relevant to the thesis objectives, especially in relation with glymphatic impairment and circadian modulation in the context of acute ischemic stroke.

Previous profiling studies have identified differentially expressed circulating miRNAs, such as miR-21, miR-146a, miR-155 and miR-210, which have been reported during intermittent hypoxia with inflammation and hypoxia-responsive signalling pathways (Santamaria-Martos et al., 2019). Changes in the circulating levels of specific miRNAs may reflect both the extent of tissue injury and the activation of endogenous repair programs. Interestingly, several studies have demonstrated that miRNAs also regulate the expression and function of core circadian clock genes (Cheng et al., 2007; McNeill & Van Vactor, 2012), and that sleep deprivation alters the amplitude of blood transcriptome oscillations (de Assis & Oster, 2021). In healthy volunteers deprived of sleep overnight, plasma levels of miRNAs were significantly elevated the following morning, suggesting a link between circadian disruption, miRNA regulation, and neurodegenerative risk (Hijmans et al., 2019; van den Berg et al., 2020; L. Zhang et al., 2025). Diurnal variations in circulating miRNA profiles have also been confirmed in humans (Heegaard et al., 2016), emphasizing that time-of-day-dependent miRNA expression may influence susceptibility to ischemic injury and recovery potential. Within the stroke context, differential miRNA expression can allow the stratification and prediction of stroke subtypes and severity (*Figure 4.14*). Among the miRNAs identified, let-7a-5p appears to regulate the expression of proteins involved in inflammation, neurogenesis, cell cycle and cell proliferation (Rong et al., 2021). Moreover, suppression of let-7a gene has been associated with protection against cerebral ischemia/reperfusion injury (Z. K. Wang et al., 2016). Similarly, miR-146a targets molecules of the NF- κ B pathway and toll-like receptor signalling (Taganov et al., 2006), modulating the pathogenesis of neuroinflammatory disorders (H. Wang et al., 2019). Alterations in other miRNAs – including miR-320a-3p, miR-320c, miR-204-3p, miR-17-5p, miR-16-5p, miR-32-3p, miR-106b-5p, and miR-532-5p – have been documented in both experimental stroke models and patient samples from blood or cerebrospinal fluid (Bejleri et al., 2021; Jiang et al., 2022; P. Li et al., 2014; Petracci et al., 2025). The pathway enrichment analyses showed that genes predicted to be regulated by these miRNAs were significantly enriched in several processes, including axon guidance, MAPK and mTOR signalling, and ErbB receptor pathways (P. Li et al., 2014). Bioinformatic analyses showed that the potential target genes of miR-16 are mainly involved in programmed cell death and the p53 signalling pathways (Lezina et al., 2013; J. Shen et al., 2023). Furthermore, miR-23a-3p expression has been found to be increased at 4 hours after reperfusion in the peri-infarction area, and significantly elevated in the infarct core at both 4 hours and 24 hours of reperfusion (Manley et al., 2009; Rungta et al., 2015; C. Sun et al., 2022; H. Zhao et al., 2014). These changes

suggest that miR-23a-3p contributes to neuroprotection by suppressing oxidative stress and mitigating cerebral ischemia–reperfusion injury.

Overall, our findings highlight how microRNAs can be useful as molecular biomarkers as well as players in the neuroprotective mechanisms triggered by remote conditioning.

6. CONCLUSIONS

The data presented in this thesis highlight how circadian rhythms modulate the neuroprotective efficacy of remote conditioning in an experimental model of focal cerebral ischemia. The timing of intervention represents a critical factor for therapeutic outcomes, emphasizing the importance of considering individual circadian biology when developing and optimizing neuroprotective strategies. The identification of circadian modulation as a key determinant of RLIP efficacy underscores the importance of time-tailored and personalized therapeutic approaches in stroke management. Chronopharmacology has emerged as a key factor that may help bridge the translational gap between preclinical and clinical studies. A deeper understanding of how circadian rhythms influence remote conditioning could facilitate the identification of novel therapeutic targets, the discovery of biomarkers such as miRNAs, and the development of more effective treatment strategies for treating stroke. Moreover, this study identifies a potential link between RLIP-induced neuroprotection and the modulation of the glymphatic system. These findings support the hypothesis that RLIP exerts its neuroprotective effects, at least in part, by restoring glymphatic function—an essential mechanism for maintaining cerebral homeostasis during post-ischemic recovery. From a translational perspective, remote postconditioning represents an attractive therapeutic strategy due to its non-invasive nature and potential applicability in clinical settings.

LIMITATIONS OF THE STUDY

Despite these new insights, several limitations should be addressed in future studies. For example, the efficacy of RLIP across the full circadian cycle remains to be explored. Sex- and age-related factors, including comorbidities such as diabetes and age-associated diseases, which may influence both circadian regulation and neuroprotective responses, need to be assessed in future studies. The use of advanced live imaging *in vivo* to clarify the pathways connecting RLIP, glymphatic function, and blood–brain barrier recovery will be essential for promoting the development of personalized, time-tailored neuroprotective strategies for stroke. Moreover, future studies should focus on investigating the molecular interactions between circadian regulation and glymphatic–lymphatic dynamics under remote conditioning. Understanding the precise influence of circadian rhythms on CSF production and clearance mechanisms could reveal novel therapeutic targets for chronotherapy.

For the observation population study, we expect to increase the number of cases to further improve data accuracy and to better integrate miRNA profiling with clinical and polysomnographic parameters which could improve personalized risk stratification and early

prevention strategies in patients affected by sleep disorders, and potentially identify risk factors of ischemic stroke in OSAS population.

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