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TITOLO TESI

Characterization of Store-Operated Calcium Entry in the neuroimmune response evoked by ischemic preconditioning in mice subjected to middle cerebral artery occlusion

Settore Scientifico Disciplinare BIO 14-Farmacologia

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ABSTRACT

Cerebral ischemia is one of the leading causes of death and long-term disability worldwide. Currently approved therapies for ischemic stroke are limited to reperfusion through mechanical recanalization and/or pharmacological thrombolysis; however, only a small percentage of eligible patients may benefit from this treatment due to its contraindications and, furthermore, it does not provide neuroprotective effects. In this context, several studies have highlighted the potential of inducing ischemic tolerance by stimulating endogenous neuroprotection. To this aim, brain ischemic preconditioning (PC), namely a sublethal ischemic event able to increase the resistance of the brain against a subsequent, more intense ischemic insult, has been considered as a useful experimental paradigm to investigate the mechanisms implicated in brain tolerance. A deep comprehension of endogenous neuroprotection elicited by ischemic PC represents a promising approach to identify novel targets that can be translated into stroke therapy.

One of the main factors involved in the progression of neuronal damage during cerebral ischemia is the alteration of cellular Ca^{2+} homeostasis. Indeed, cytosolic Ca^{2+} overload due to increased membrane permeability or to its leak from intracellular organelles could result in neuronal demise. Detrimental effects involve the activation of a series of Ca^{2+}-dependent enzymes that degrade cellular components or activate death pathways, and the formation of cytotoxic products that cause irreversible mitochondrial damage and cellular demise.

The main objective of the present research work was to investigate the involvement of store-operated calcium entry (SOCE) in brain ischemia and ischemic preconditioning in mice subjected to focal cerebral ischemia. Following an ischemic insult and depletion of Ca^{2+} stores, the endoplasmic reticulum Ca^{2+} sensor stromal interaction molecule (STIM)1 interacts with the Ca^{2+} selective plasmamembrane channel Orai1, to promote SOCE, that may protect neurons by re-establishing Ca^{2+} homeostasis or could also be the source of excessive Ca^{2+} influx, thus causing non-excitotoxic neuronal death. This Ca^{2+} influx is regulated by SOCE-associated regulatory factor (SARAF) that associates with STIM1 and promotes a slow Ca^{2+}-dependent inactivation of SOCE, or directly interacts with Orai1 to promote SOCE

activation in the absence of STIM1. Furthermore, SOCE represents the main source of Ca^{2+} in immune cells, regulating several of their critical functions. Besides the pivotal role played by immune mediators in the evolution of cerebral ischemic damage, it has been demonstrated that the innate immune system is also an essential component of the delayed ischemic tolerance elicited in the brain by ischemic PC. Therefore, we investigated whether central and peripheral innate immune responses contribute to PC-induced ischemic tolerance and if modulation of different SOCE components occurs in ischemic damage (1h middle cerebral artery occlusion, MCAo, followed by 24h of reperfusion) and/or in neuroprotection conferred by ischemic PC (15 min MCAo, 72h before) in C57BL/6J adult male mice.

Ischemic PC significantly reduced histological damage and neurological deficits produced in mice by a more severe ischemia of 1h. Western blot analysis revealed that Orai1 expression is not affected by the ischemic insult preceded or not by the PC stimulus in the frontoparietal ischemic cortex. However, Orai1 expression was detected in neurons, but also in $Ly6B.2^{+}$ myeloid cells infiltrating the ischemic hemisphere. By contrast, STIM1 and SARAF expression, mainly found in $NeuN^{+}$ neurons, was significantly reduced in the ischemic cortex. Interestingly, ischemic PC prevented SARAF downregulation in the ischemic cortex, thus suggesting that this regulatory factor may play a crucial role in SOCE-mediated tolerance.

To assess the immunomodulatory effects of ischemic PC, we performed ELISA assay to demonstrate that cerebral damage was associated with increased protein levels of the proinflammatory cytokine IL-1β in the ischemic cortex, while this effect was prevented by the PC stimulus. Regarding alternatively-activated phenotypes, western blot analysis revealed a significant elevation of the expression of Ym1, marker of M2-polarized microglia/macrophages, in the ischemic cortex as compared to contralateral tissue. Interestingly, ischemic PC further increased Ym1 expression in the ipsilateral cortex as compared to MCAo group. Immunohistochemical analysis revealed that the majority of $Ym1^{+}$ cells are mainly amoeboid $CD11b^{+}$ myeloid cells, very likely monocytes/macrophages infiltrating from blood vessels. Thus, elevated brain infiltration of these phenotypes is very likely involved in the protective effects of ischemic PC.

The involvement of the peripheral immune response was confirmed by the evidence that the 70% increase in spleen weight observed after 1h MCAo was abolished in mice pre-exposed to PC. Accordingly, flow cytometry analysis revealed that PC significantly attenuates elevation of neutrophil counts (Ly-6G^{+} events) induced by 1h MCAo in blood. Since the Ca^{2+}-selective plasmamembrane channel Orai1 is crucial in the recruitment of immune cells during inflammation, we have analysed its expression in the whole population of circulating leukocytes and in neutrophils, demonstrating that the number of Orai1^{+} cells, mainly corresponding to Ly-6G^{+} neutrophils, was significantly enhanced in the blood after the ischemic insult, as compared to sham, regardless of whether mice received or not ischemic PC.

In conclusion, this research project reaffirms that cerebral ischemic tolerance induced by PC involves both central and peripheral modulation of the innate immune system, further underscoring the relevance of exploiting immunomodulatory approaches for the development of effective stroke therapies and originally demonstrates that preventing SARAF downregulation could represent an important neuroprotective mechanism aimed at preserving SOCE functions, making SARAF a valuable target to protect neurons from the ischemic damage.

SOMMARIO

L'ischemia cerebrale è una delle principali cause di morte e disabilità a lungo termine al mondo. Attualmente le terapie approvate per l'ictus ischemico si limitano alla riperfusione mediante ricanalizzazione meccanica e/o trombolisi farmacologica; tuttavia, solo una piccola percentuale di pazienti eleggibili può beneficiare di questo trattamento a causa delle sue controindicazioni e, inoltre, questi interventi non possiedono effetti neuroprotettivi. In tale contesto, il precondizionamento (PC) ischemico cerebrale, ovvero un evento ischemico subletale capace di potenziare la resistenza del cervello verso un evento ischemico successivo più intenso, è stato considerato come un utile modello sperimentale per studiare i meccanismi implicati nella tolleranza ischemica che si sviluppa a livello cerebrale. Una profonda comprensione della neuroprotezione endogena indotta dal PC ischemico rappresenta un approccio promettente per identificare nuovi bersagli traslabili alla terapia dell'ictus.

Uno dei fattori chiave coinvolto nella progressione del danno neuronale conseguente ad ischemia cerebrale è l'alterazione dell'omeostasi cellulare del Ca^{2+}. Infatti, il sovraccarico di Ca^{2+} citosolico, dovuto all'incremento della permeabilità di membrana o alla sua fuoriuscita dagli organuli cellulari, potrebbe condurre alla morte neuronale. Gli effetti deleteri includono l'attivazione di una serie di enzimi Ca^{2+}-dipendenti che degradano i componenti cellulari o attivano segnali di morte, e la formazione di prodotti citotossici che causano danno mitocondriale irreversibile e morte cellulare.

Il principale obiettivo del presente lavoro di ricerca è stato lo studio del coinvolgimento della corrente del calcio regolata dall'accumulo (SOCE) nell'ischemia cerebrale e nel precondizionamento ischemico in topi sottoposti a ischemia cerebrale focale. A seguito di un insulto ischemico e dell'esaurimento dei depositi di Ca^{2+}, il sensore del Ca^{2+} del reticolo endoplasmatico STIM1 (*stromal interaction molecule 1)* interagisce con il canale di membrana selettivo per il Ca^{2+} Orai1, per innescare SOCE, che può proteggere i neuroni ristabilendo l'omeostasi del Ca^{2+} o potrebbe anche essere la fonte dell'eccessivo influsso di Ca^{2+} alla base della morte neuronale non eccitotossica. Quest'influsso di Ca^{2+} è regolato dal fattore di

regolazione di SOCE, SARAF, che si associa con STIM1 e promuove una lenta inattivazione di SOCE dipendente dal Ca^{2+}, o interagisce direttamente con Orai1 per attivare SOCE in assenza di STIM1. Inoltre, SOCE rappresenta la principale fonte di Ca^{2+} nelle cellule immunitarie, di cui regola molte delle funzioni principali. Oltre al ruolo chiave giocato dai mediatori immunitari nell'evoluzione del danno ischemico, è stato dimostrato che il sistema immunitario innato è anche un componente essenziale della tolleranza ischemica ritardata indotta nel cervello dal PC. Pertanto, abbiamo valutato se le risposte immunitarie centrale e periferica contribuiscono all'instaurazione della tolleranza ischemica indotta dal PC e se avviene una modulazione dell'espressione dei componenti di SOCE nel danno ischemico (1h di occlusione dell'arteria cerebrale media, MCAo, seguita da 24h di riperfusione) e/o nella neuroprotezione conferita dal PC ischemico (15 min di MCAo, 72h prima) in topi maschi adulti del ceppo C57BL/6J.

Il PC ischemico ha significativamente ridotto il danno istologico e i deficit neurologici prodotti nei topi da un'ischemia più grave di 1h. L'analisi di western blot ha rivelato che l'espressione di Orai1 non è influenzata dall'insulto ischemico preceduto o meno dallo stimolo precondizionante nella corteccia ischemica frontoparietale. Tuttavia, l'espressione di Orai1 è stata rilevata nei neuroni, ma anche nelle cellule mieloidi $Ly6B.2^{+}$ infiltranti l'emisfero ischemico. Al contrario, l'espressione di STIM1 e SARAF, localizzata prevalentemente nei neuroni $NeuN^{+}$, è risultata significativamente ridotta nella corteccia ischemica. È interessante notare che il PC ischemico ha prevenuto la downregulation di SARAF nella corteccia ischemica, suggerendo così che questo fattore di regolazione potrebbe giocare un ruolo cruciale nella tolleranza mediata da SOCE. Gli effetti immunomodulanti del PC ischemico sono stati valutati attraverso il saggio ELISA, dimostrando che il danno cerebrale è associato ad un aumento dei livelli proteici della citochina proinfiammatoria IL-1β nella corteccia ischemica, mentre quest'effetto è prevenuto dallo stimolo precondizionante. Per quanto riguarda i fenotipi alternativi, l'analisi di western blot ha rilevato un aumento significativo dell'espressione di Ym1, marcatore di microglia e macrofagi del fenotipo M2, nella corteccia ischemica rispetto al tessuto controlaterale e, soprattutto, il PC ischemico incrementa ulteriormente l'espressione di Ym1 nella corteccia ispilaterale rispetto al gruppo MCAo. L'analisi

immunoistochimica ha rivelato che la maggior parte delle cellule $Ym1^+$ sono prevalentemente cellule mieloidi $CD11b^+$ di forma ameboide, con ogni probabilità monociti/macrofagi infiltranti dai vasi sanguigni. Pertanto, l'elevata infiltrazione cerebrale di questi fenotipi è verosimilmente coinvolta negli effetti neuroprotettivi del PC ischemico.

Il coinvolgimento del sistema immunitario periferico è stato confermato dall'evidenza che l'incremento del 70% del peso della milza osservato dopo 1h di MCAo è abolito nei topi pre-esposti al PC. Di conseguenza, l'analisi citofluorimetrica ha rivelato che il PC attenua significativamente l'aumento della conta neutrofilica (eventi Ly-$6G^+$) indotto da 1h di MCAo nel sangue. Considerando che il canale selettivo per il Ca^{2+} Orai1 gioca un ruolo cruciale nel reclutamento delle cellule immunitarie durante l'infiammazione, abbiamo analizzato la sua espressione nella popolazione leucocitaria circolante totale e nei neutrofili, dimostrando che il numero di cellule $Orai1^+$, corrispondenti prevalentemente a neutrofili Ly-$6G^+$, era significativamente elevato nel sangue dopo l'insulto ischemico, rispetto al gruppo sham, indipendentemente dal fatto che i topi abbiano ricevuto o meno il PC ischemico.

In conclusione, il presente progetto di ricerca riafferma che la tolleranza ischemica cerebrale indotta dal PC coinvolge la modulazione centrale e periferica del sistema immunitario innato, evidenziando ulteriormente l'importanza di sfruttare gli approcci immunomodulanti per lo sviluppo di terapie efficaci per l'ictus e dimostra, per la prima volta, che prevenire la downregulation di SARAF potrebbe rappresentare un importante meccanismo neuroprotettivo volto a preservare le funzioni di SOCE, rendendo SARAF un valido obiettivo per proteggere i neuroni dal danno ischemico.

Table of contents

List of figures and tables

List of abbreviations

AHA/ASA: American Heart Association/American Stroke Association
AMP: adenosine monophosphate
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK: AMP-activated protein kinase
Arg 1: arginase 1
ATP: adenosine triphosphate
BAIPC: bilateral arm ischemic preconditioning
BBB: blood brain barrier
BDNF: brain-derived neurotrophic factor
BSA: bovine serum albumine
CBF: cerebral blood flow
CCA: common carotid artery
CCR2: C-C motif chemokine receptor type 2
CD: cluster of differentiation
CNS: central neurvous system
COX: cyclooxygenase
CSD: cortical spreading depolarization
CTID: C-terminal inhibitory domain
CX3CL1: chemokine (C-X3-C motif) ligand 1, fractalkine
CX3CR1: chemokine receptor for fractalkine
DALYs: disability-adjusted life-years
DAMPs: damage-associated molecular patterns
DAPI: 4',6'-diamidino-2-phenylindole
DAWN: Clinical Mismatch in the Triage of Wake Up and Late Presenting Stroke Undergoing Neurointervention With Trevo
DEFUSE-3: Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution
DWI: diffusion-weighted imaging
ECA: external carotid artery
ECASS III: European Cooperative Acute Stroke Study III
ECM: extracellular matrix
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
FDA: Food and Drug Administration
FLAIR: fluid-attenuated inversion recovery
GABA: gamma-aminobutyric acid
GFAP: glial fibrillary acidic protein
HIF-1α: hypoxia inducing factor-1α
HRP: avidin-biotin peroxidase complex
Iba1: ionized calcium-binding adaptor molecule 1
ICA: internal carotid artery
ICD-11: Interantional Classification of Diseases-11
I_{crac}: Ca^{2+} release-activated Ca^{2+} current
IFN-γ: interferon-γ

IGF-1: insulin-like growth factor
IL: interleukin
IL-1Ra: interleukin-1 receptor antagonist
iNOS: inducible nitric oxide synthase
$InsP_3$: inositol-1,4,5-triphosphate
$InsP_3R$: inositol-1,4,5-triphosphate receptor/Ca^{2+} channels
IPerC: ischemic perconditioning
IPostC: ischemic postconditioning
IQR: interquartile range
IRF: interferon regulatory factor
I_{SOC}: Ca^{2+} selective store-operated Ca^{2+} current
LPS: lipopolysaccharide
Ly-6C: lymphocyte antigen 6 complex locus C1
MCA: middle cerebral artery
MCAo: middle cerebral artery occlusion
MHC II: major histocompatibility complex II
mK^+_{ATP}: mitochondrial ATP-sensitive K^+ channels
MMPs: matrix metalloproteasis
MR WITNESS: Study of Intravenous Thrombolysis With Alteplase in MRI-Selected Patients
MR: magnetic resonance
MyD88: myeloid differentiation factor 88
NADPH: nicotinamide adenine dinucleotide phosphate
NETs: neutrophil extracellular traps
NF-κB: nuclear factor-κ B
NGF: nerve growth factor
NINDS: National Institute of Neurological Disorders and Stroke
NMDA: N-methyl-D-aspartate
nNOS: neuronal nitric-oxide synthase
NO: nitric oxide
NOX: NADPH oxidase
Nrf2: nuclear factor (erythroid-derived 2)-like 2
OGD: oxygen-glucose deprivation
PBS: posphate buffered saline
PC: preconditioning
PF: paraformaldehyde
PKC: protein kinase C
PLA_2: phospholipase A_2
PLC: phospholipase C
PM: plasma membrane
PPAR-γ: peroxisome proliferator-activated receptor-γ
RECAST 1: Remote Ischemic Conditioning After Stroke Trial 1
RIPC: remote ischemic preconditioning
RIPerC: remote ischemic perconditioning
RIPostC: remote ischemic postconditioning
RNS: reactive nitrogen species
ROCs: receptor-operated channels
ROS: reactive oxygen species

rt-PA: recombinant tissue-plasminogen activator
SARAF: SOCE-associated regulatory factor
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM: standard error of the mean
SERCA: sarcoplasmic/endoplasmic Ca^{2+}-ATPase
SIRT1: Sirtuin 1
SOAR: STIM1 Orai1 activation region
SOCE: store-operated calcium entry
STAT3: signal transducers and activators of transcription 3
STIM: stromal interaction molecule
TBS-T: tween-20 tris-buffered saline
TGF-β: transforming growth factor-β
TIA: transient ischemic attack
TLRs: toll like receptors
TMB: tertramethyl-benzidine
TNF-α: tumor necrosis factor-α
TRPCs: transient receptor potential channels
UPR: unfolded protein response
VEGF: vascular endothelial growth factor
VOCCs: voltage-operated Ca^{2+} channels
WAKE-UP: Efficacy and Safety of MRI-based Thrombolysis in Wake-up Stroke
WHO: World Health Organization

Chapter 1 - Introduction

1.1 - Ischemic stroke

Stroke is a cerebrovascular disease characterized by a neurological deficit associated with an acute focal injury of the central nervous system (CNS) of vascular origin. Despite being a major cause of death and disability worldwide, the definition of the term "stroke" is not consistent in clinical practice and research, or in assessments of the public health (Coupland *et al.*, 2017).

The World Health Organization (WHO) defined stroke as "a clinical syndrome characterized by a rapid and sudden onset of focal or global cerebral deficit, lasting 24 hours or longer or leading to death, due to a vascular cause" (WHO MONICA Project Principal Invest, 1988). However, the increased knowledge of brain anatomy and pathology and the advances in brain imaging, have highlighted the need for a new definition of stroke that shifts from a clinical to a tissue-based determination. The Stroke Council of the American Heart Association/American Stroke Association (AHA/ASA) proposed in 2013 an updated definition of stroke for the 21st century, which incorporates both clinically evident and silent ischemic or haemorrhagic cerebral lesions. This broad definition includes any condition of permanent brain, spinal cord or retinal injury caused by vascular dysfunction and diagnosed through neuroimaging, neuropathological or other objective evidence of infarction, regardless of the presence of manifest clinical symptoms (Sacco et al., 2013).

By contrast, the WHO, the European Stroke Organization and the World Federation of Neurology adopted the definition conceived by the International Classification of Diseases-11 (ICD-11), which considers a stroke solely in the presence of manifest acute neurological dysfunctions (Norrving *et al.*, 2013). Thus, they maintained the original definition for stroke, while recognizing silent infarcts and haemorrhages as a diverse category of cerebrovascular diseases. The main symptoms of ischemic stroke typically include: numbness or weakness of face, leg or arm, paralysis, speech difficulty, trouble seeing, sudden severe headache and confusion.

1.1.1 - Incidence and risk factors

Stroke is the second cause of death globally and a leading cause of long-term disability, with an increasing incidence in developing countries (Campbell *et al.*, 2019). According to the WHO, 15 million people suffer of stroke worldwide each year; of these, 5 million die and another 5 million remain permanently disabled.

Based on an estimate of the Italian Ministry of Health, in Italy stroke is the third cause of death, providing 10-12% of all the deaths per year and representing the leading cause of disability. Every year, about 196,000 strokes occur in Italy, 20% of which are recurrent attacks. 10-20% of people affected by stroke die within a month and another 10% within the first year from symptoms onset. Only 15% of patients survive a stroke and recover completely, while about 45% survive with some forms of disability and a half of them develops serious deficits until the loss of self-sufficiency.

Stroke can occur at any age, but the risk of having a stroke doubles each decade after the age of 55. 75% of all strokes occur in people over 65 and the prevalence in people aged between 65-84 years is 6.5% (Italian Ministry of Health, Cardiovascular diseases, last update 2020).

Stroke is a multifactorial disorder regulated by modifiable and non-modifiable risk factors (table 1.1). Unmodifiable risk factors include age, sex, race and genetic disorders. Indeed, a family history of cerebrovascular diseases, older age, male sex and Hispanic and Black race are associated with higher stroke occurrence (Boehme *et al.*, 2017).

Data from the Global Burden Disease Study reveal that 91% of the stroke risk could be attributed to modifiable risk factors, and these account for almost half of stroke-related mortality, which highlights the need of improving their early detection and appropriate clinical management (Virani *et al.*, 2020). Modifiable risk factors include hypertension, obesity, hyperglycemia, hyperlipidemia and renal dysfunction, and behavioural risk factors, like smoking, sedentary lifestyle and unhealthy diet (Benjamin *et al.*, 2019).

Globally, hypertension has remained the leading modifiable predictor of stroke mortality since 1990, followed by poor dietary habits (i.e. a diet low in fibre, fruits, vegetables, legumes, whole grains, nuts and seeds, milk, calcium, or seafood, and

high in red meat, eggs, processed meat, sugar-sweetened beverages, trans-fatty acids, or sodium), impaired glucose tolerance, obesity, tobacco smoking, air pollution, alcohol use, hypercholesterolemia and low physical activity (Avan *et al.*, 2019). The evidence that a remarkably high proportion of stroke-related disability-adjusted life-years (DALYs) is potentially avoidable, together with the continuously increasing global stroke burden, highlights the urgent need to improve primary stroke prevention strategies worldwide (Feigin *et al.*, 2020).

Since the single most important risk factor for stroke globally is represented by high systolic blood pressure, interventions should be implemented to guarantee availability and proper use of antihypertensives, associated with other population-based strategies aimed at reducing concomitant detrimental factors (e.g., salt reduction, tobacco and alcohol control, physical activity and healthy diet) (Feigin *et al.*, 2020). Many stroke risk factors have profound effects on the structure and function of blood vessels, by promoting the formation of atherosclerotic plaques, stiffening the arteries and inducing narrowing and thickening of arterioles and capillaries (Hu, X. et al., 2017).

MODIFIABLE RISK FACTORS	**NON-MODIFIABLE RISK FACTORS**
Hypertension	Age
Cardiac diseases: atrial fibrillation, heart failure, valvular defects, heart chambers enlargement	Gender
Diabetes mellitus	Ethnicity
Hyperlipidaemia	Family history of stroke
Cigarette smoking	
Alcohol consumption	
Behavioural factors: obesity, physical inactivity, unhealthy diet	
Migraine	

Table 1.1 – Major modifiable and non-modifiable stroke risk factors.

The brain has limited energy stores and its integrity depends on a continuous supply of oxygen and energy substrates, thus pathologic conditions such as hypertension, diabetes and hypercholesterolemia impair the adaptive mechanisms that ensure proper cerebral perfusion and keep cerebral blood flow (CBF) stable during blood pressure reduction (cerebrovascular autoregulation) (Moskowitz *et al.*, 2010). These vascular alterations compromise the ability of the endothelium to regulate microvascular flow, thus impairing the development of collateral flow arising from adjacent non-ischemic territories, essential for the survival of perinfarct zone, and increasing the vulnerability of the brain to ischemia after an arterial occlusion (Hu, X. *et al.*, 2017).

1.1.2 - Pathobiology of ischemic stroke

Stroke is a pathological condition caused by the reduction or interruption of blood supply to the brain. Based on the cause of the interruption of CBF, stroke can be classified into two major types: ischemic and haemorrhagic (figure 1.1). Haemorrhagic stroke occurs when an artery in the brain bursts, bleeding into the surrounding tissue, or in the subarachnoid space, thus causing an intracerebral or subarachnoid haemorrhage, respectively. Ischemic stroke is the most common type of stroke, accounting for about 87% of all stroke cases (Benjamin *et al.*, 2019). It is caused by a thrombotic or embolic occlusion of a major cerebral artery, most often the middle cerebral artery (MCA).

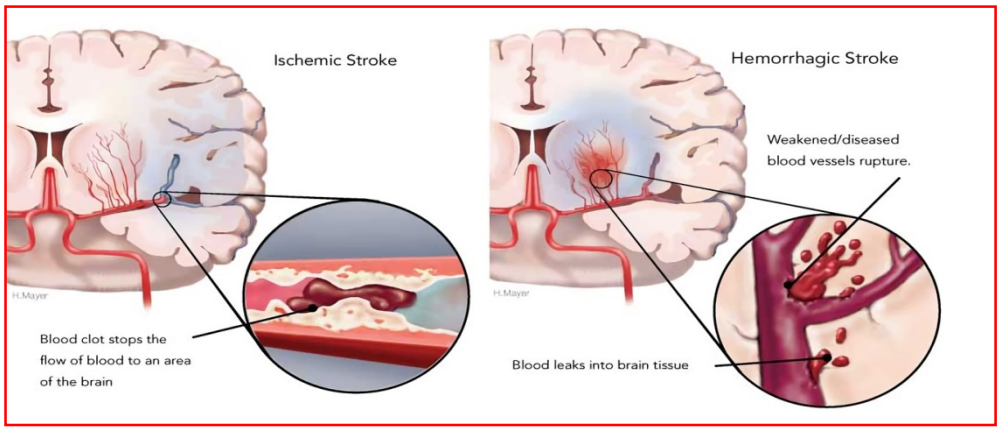

Figure 1.1 – Ischemic and haemorrhagic stroke.

Thrombosis, generally resulting from conditions such as atherosclerosis or hypercoagulability, is characterized by the formation of a thrombus in a vessel, which reduces blood flow or blocks it completely *in situ*. The formation of thrombi usually affects carotid bifurcation, the origin of the MCA or the terminal branches of the basilar artery. Embolism, instead, can be considered as a complication of thrombosis: the embolus is a fragment originated from the main thrombotic mass (for example an atherosclerotic plaque), which flows into the bloodstream until it blocks distally a smaller blood vessels in the brain.

Although the brain accounts for only 2% of total body weight, it receives about 15% of the total cardiac output and consumes about 20% of the delivered oxygen, in order to sustain its high metabolic activity.

The brain is totally dependent on continuous oxidative metabolism for the maintenance of its functional and structural integrity, and considering its limited energy stores, it requires a constant supply of oxygen and glucose. For this reason, the brain is particularly vulnerable to the ischemic insult and even a temporary deficit in oxygen supply may cause irreversible damage.

The adequate supply of oxygenated blood to the brain is provided by the circle of Willis (figure 1.2), an arterial network located at the base of the skull, formed by the anastomosis of the internal carotid arteries with the vertebral arteries, that provide respectively the anterior and posterior circulation to the brain.

The anterior circulation supplies blood to the majority of the cerebral hemispheres, including the frontal lobes, parietal lobes, lateral temporal lobes and anterior part of deep cerebral hemispheres, like the striatum; the posterior circulation supplies brainstem, cerebellum, occipital lobes, medial temporal lobes and posterior part of the deep hemisphere, like the thalamus (Rosner *et al.*, 2018).

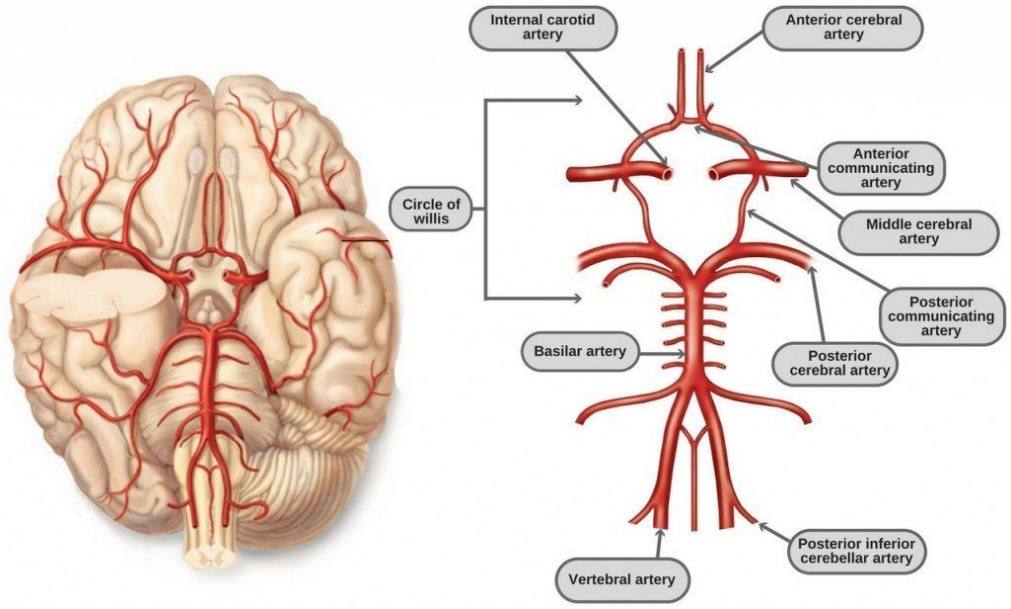

Figure 1.2 – Circle of Willis. Schematic representation of the arterial network that provides cerebral perfusion.

In terms of blood flow distribution, ischemic stroke is often focal, thus characterized by the reduction of CBF in a specific area of the brain (Xing *et al.*, 2012).

The brain region directly supplied by the occluded vessel, the infarct core, is characterized by more than 80% reduction of blood flow levels (Lo, 2008), leading to severe disruption of metabolic processes, cellular energy supply, ion homeostasis and a consequent loss of cellular integrity. The infarct core is structurally and functionally irreversibly damaged and evolves toward death within minutes (Sommer, 2017). The meta-stable region surrounding this core was originally identified by Astrup et al in 1981 as the ischemic penumbra and defined as functionally impaired but still viable tissue (Astrup *et al.*, 1981) (figure 1.3), due to collateral circuits that provide residual circulation. The ischemic penumbra is characterized by a significant reduction in perfusion levels (60-65%), just sufficient to sustain basal adenosine triphosphate (ATP) levels, oxygen metabolism and normal ionic gradients in the presence of electrical activity inhibition and suppressed protein synthesis (Moskowitz *et al.*, 2010). The disruption of cellular homeostasis in the penumbra leads to a slowly progressing cell death, mainly due to apoptotic and inflammatory cascades. Thus, the penumbra represents a potentially salvageable tissue and its recovery is the main goal of neuroprotective approaches. However,

unless perfusion is re-established within hours or cells are made more resistant to injury, the penumbra evolves towards a structural irreversible lesion and therapeutic opportunities are lost (Liu *et al.*, 2012; Baron, 2019).

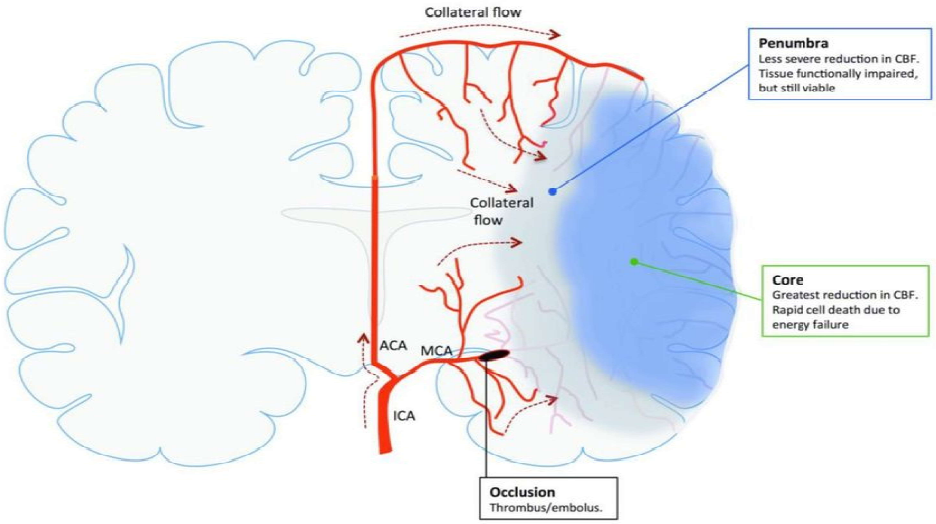

Figure 1.3 – Ischemic core and penumbra. Illustration of ischemic core and penumbra after middle cerebral artery occlusion (Jackman and Iadecola, 2015).

1.1.3 - Acute ischemic stroke treatment

The main goal of acute therapies is the recovery of the penumbra as fast as possible, in order to avoid core expansion and to limit neurological impairments. In patients, magnetic resonance (MR) brain images have highlighted that every minute of MCA occlusion causes the death of 1.9 million neurons and the destruction of 14 billion synapses (Kamal *et al.*, 2018). Currently, the approved therapy for acute ischemic stroke is limited to blood flow restoration in the brain through intraarterial mechanical thrombectomy and/or pharmacological thrombolysis with recombinant tissue-plasminogen activator (rt-PA).

The thrombolytic rt-PA is the only pharmacological agent approved for the treatment of acute ischemic stroke; its efficacy was first proven in 1995 in NINDS (National Institute of Neurological Disorders and Stroke) trial, that has shown an improvement of about 30% in the outcome of patients at three months (NINDS Study Group, 1995). rt-PA is a serine protease naturally found in vascular endothelium which

catalyses the conversion of the zymogen plasminogen into the active proteolytic enzyme plasmin, responsible for the breakdown of blood clots. As demonstrated by the ECASS III (European Cooperative Acute Stroke Study III) trial, rt-PA must be administered intravenously within 4.5 hours from symptoms onset to be effective (Hacke *et al.*, 2008). More recently, two important trials, MR WITNESS (Study of Intravenous Thrombolysis With Alteplase in MRI-Selected Patients) and WAKE-UP (Efficacy and Safety of MRI-based Thrombolysis in Wake-up Stroke), demonstrated that quantitative mismatch between diffusion-weighted imaging (DWI) and fluid-attenuated inversion recovery (FLAIR) can be effectively used to identify stroke patients with an unknown time of onset that will benefit from intravenous rt-PA administered in an extended time-window (Schwamm *et al.*, 2018; Thomalla *et al.*, 2018). However, less than 10% of hospitalized patients may receive this treatment due to its limited therapeutic window (McDermott *et al.*, 2019). In addition, thrombolytic therapy is generally associated with a high risk of cerebral haemorrhage (Whiteley *et al.*, 2016), ineffectiveness for large thrombi (Riedel *et al.*, 2011; Strbian *et al.*, 2014), incomplete recanalization in about 70% of patients (Lum *et al.*, 2006), infarct expansion due to reperfusion injury and residual neurological deficits in patients (Bang, 2017).

Intraarterial mechanical thrombectomy involves the use of endovascular devices to remove the blood clot from an occluded artery in a minimally invasive surgical procedure. This approach has some benefits compared to endovascular administration of thrombolytic agents, since it acts more rapidly, allowing to achieve recanalization within few minutes, is more effective in case of large blood clots in proximal vessels and displays a lower risk of intracerebral haemorrhage (Arnaout *et al.*, 2012). After the initial disappointing results with early generation thrombectomy devices, in 2014 endovascular thrombectomy was shown to be safe and effective for treating acute ischemic stroke (Berkhemer *et al.*, 2015) and, in 2015, it became standard-of-care for patients with a large vessel occlusion presenting within 6 hours of symptom onset (Campbell *et al.*, 2015; Goyal *et al.*, 2015; Saver *et al.*, 2015). In 2018, the AHA/ASA released updated ischemic stroke guidelines taking into account the results from the DAWN (Clinical Mismatch in the Triage of Wake Up and Late Presenting Stroke Undergoing Neurointervention With Trevo) and DEFUSE-3

(Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution) randomized clinical trials, investigating endovascular thrombectomy using Trevo or any Food and Drug Administration (FDA) approved device, respectively. In selected patients with acute ischemic stroke, within 6 to 24 hours of last known normal, who have large vessel occlusion in the anterior circulation and meet imaging and other eligibility criteria, mechanical thrombectomy is recommended (Albers *et al.*, 2018; Nogueira *et al.*, 2018). In particular, significant amelioration of outcomes is mainly observed in patients with small infarct core volumes (i.e., with slow early DWI growth rate).

Although blood flow restoration is the only therapeutic intervention applied to avoid core expansion, it has been demonstrated that, not only the sole reperfusion is ineffective in completely block cell death progression, but in addition it potentiates detrimental mechanisms, thus promoting the progression of ischemic damage in the penumbra, in a paradoxical phenomenon known as 'reperfusion injury'. Indeed, rapid reperfusion after a period of ischemia provides an excessive oxygen availability, leading to an intense production of reactive species, that cause oxidative stress, endothelial dysfunction, DNA damage and local inflammation, resulting in cell death via different mechanisms (Wu *et al.*, 2018). This results in the exacerbation of neurological impairment in stroke patients.

Thus, the effect of thrombolytic agents and endovascular devices is limited to cerebral blood flow restoration, whereas, the aim of neuroprotective approaches is to target the detrimental molecular and cellular processes occurring during the ischemic cascade, in order to prevent the expansion of the lesion and to promote functional recovery of patients (Amantea and Bagetta, 2017). However, despite promising results obtained in preclinical studies, most of the potentially neuroprotective agents tested in the last decades (glutamate receptors antagonists, calcium channels blockers, free radicals scavengers, anti-inflammatory, anti-apoptotic or regenerative drugs) have failed in clinical trials due to toxicity or lack of neuroprotective efficacy in stroke patients (Kikuchi *et al.*, 2014).

The causes of this clinical failure are mainly linked to the lack of information regarding drugs pharmacology, target specificity, binding affinity, molecular selectivity, inadequate brain penetration capacity and to the differences between the

homogeneity of ischemic damage entity and response to treatment in animal models and the lack of reproducibility in patient population with comorbidities (Savitz *et al.*, 2019).

Therefore, considering the high incidence of ischemic stroke and its severe consequences, it is extremely important to find new targets for the development of effective neuroprotective therapies that can improve the quality of life of stroke patients. To this end, a better understanding of the mechanisms underlying ischemic brain injury is of pivotal importance.

1.1.4 - Mechanisms underlying ischemic neuronal injury

The complex spatiotemporal sequence of pathophysiological events occurring in the brain after vascular occlusion is known as 'ischemic cascade' (Campbell *et al.*, 2019). The evolution of ischemic brain injury starts within minutes of vessel occlusion and continues for hours and even days, depending on the severity and duration of CBF reduction and the vulnerability of the specific brain region affected (Moskowitz *et al.*, 2010).

Brain is almost exclusively dependent on the continuous supply of glucose and oxygen to perform oxidative phosphorylation for energy production, since it has no energy stores in form of lipids or carbohydrates and lacks myoglobin as oxygen reserve. The first consequence of CBF reduction is the depletion of substrates for the production of ATP that leads to intensification of anaerobic glycolysis, with consequent acidosis that promotes free radicals production and exacerbates injury (Xing *et al.*, 2012).

Energy failure leads to alterations of the Na^+/K^+-ATPase and Ca^{2+}/H-ATPase pumps, resulting in reversal of the function mode of Na^+-Ca^{2+} transporter (Phan *et al.*, 2002) and inability to maintain the membrane potential and ionic gradients across the plasma membrane (PM). These events cause an abnormal Na^+ and Ca^{2+} influx and K^+ efflux, resulting in neuronal depolarization and cytotoxic oedema (Khoshnam *et al.*, 2017).

Other major pathogenic mechanisms of the ischemic cascade that ultimately lead to cell death include excitotoxicity, peri-infarct depolarization, mitochondrial

dysfunctions, oxidative stress, blood brain barrier (BBB) disruption and inflammation (figure 1.4 A-B).

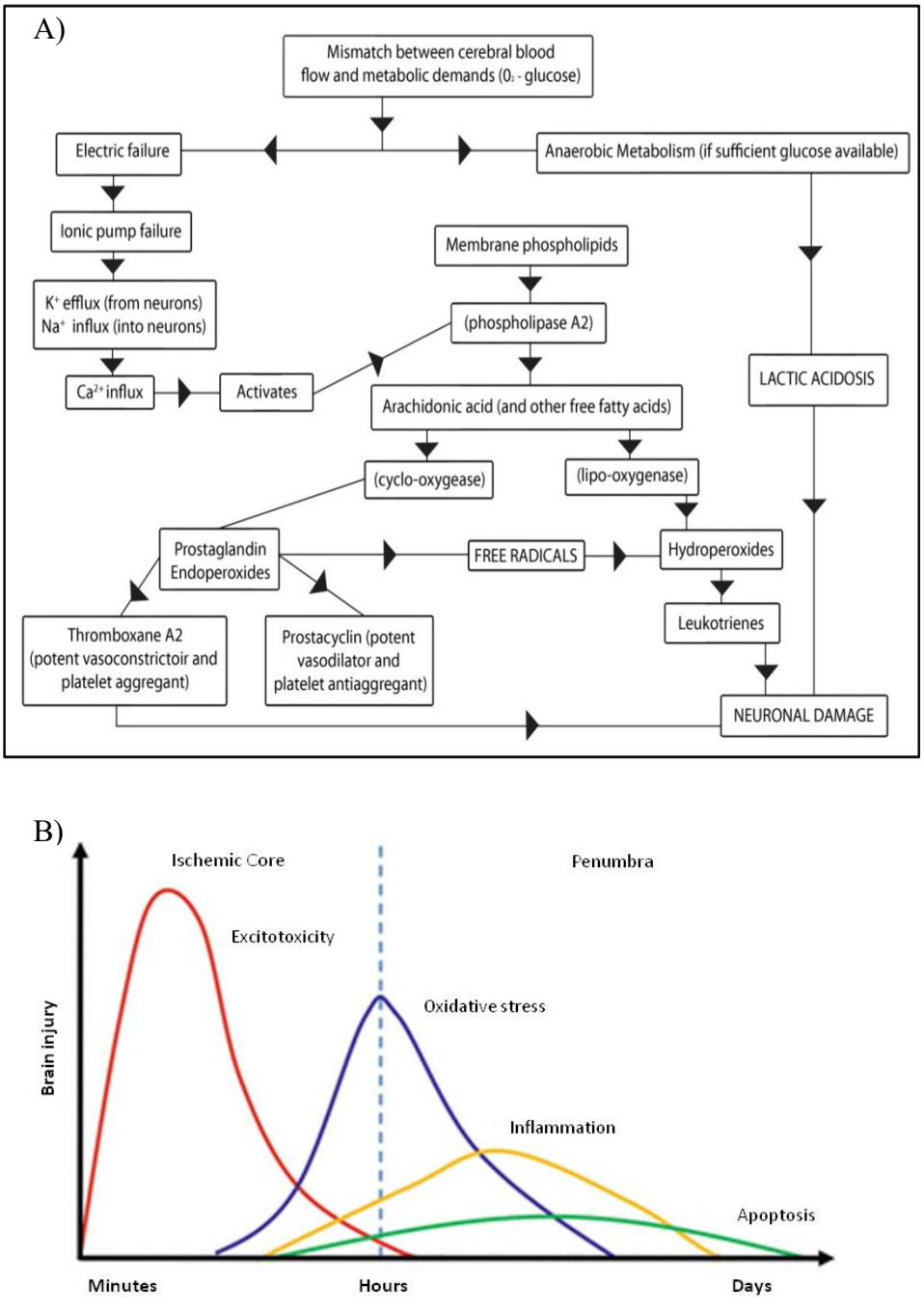

Figure 1.4 – Pathophysiological events occurring in the brain after ischemic stroke. A) Diagram illustrating the ischemic cascade. B) Spatiotemporal evolution of the mechanisms activated in the brain following ischemic damage (Velly *et al.*, 2017).

- **Excitotoxicity and Calcium dysregulation**

Excitotoxicity and calcium overload are pivotal mechanisms contributing to the early stages of ischemic cell death (figure 1.5). Reduced energy supply determines elevation of Ca^{2+}-dependent and non-exocytotic (i.e., through transporter reversal mode) release and reduced neuronal and glial uptake of glutamate, thus resulting in extracellular elevation of this excitatory neurotransmitter at toxic levels. Excess glutamate causes prolonged stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, further enhancing the influx of calcium and sodium into neurons (Campbell *et al.*, 2019).

The excessive influx of Na^+ and water causes cell swelling, oedema and extracellular space shrinkage, that lead to cell necrosis via osmotic lysis (Xing *et al.*, 2012), primarily observed in the infarct core.

The intracellular build-up of Ca^{2+} is further sustained by its release from intracellular stores, like mitochondria, endoplasmic reticulum (ER) and Ca^{2+}-binding proteins (Durukan and Tatlisumak, 2007; Moskowitz *et al.*, 2010); moreover, other channels and ion pumps activated during ischemia, are responsible for a further Ca^{2+} accumulation. These include failure of Ca^{2+} efflux mechanisms especially the Na^+/ Ca^{2+} exchanger (Bano *et al.*, 2007), and by the activation of acid-sensing ion channels (Xiong *et al.*, 2004) stimulated by the pH range commonly found in ischemic tissue following acidosis, and transient receptor potential channels (TRPCs) (Aarts and Tymianski, 2005). Detrimental effects of calcium overload involve activation of several Ca^{2+}-dependent enzymes, including protein kinase C (PKC), phospholipase A_2 (PLA_2), phospholipase C (PLC), cyclooxygenase (COX), neuronal nitric-oxide synthase (nNOS), calpain and various proteases and endonucleases (Belov Kirdajova *et al.*, 2020). The result is the activation of a series of catabolic processes that degrade cellular components, and the formation of cytotoxic products such as arachidonic acid metabolites and free radicals, that cause irreversible mitochondrial damage and cellular demise, finally leading to necrotic or apoptotic cell death (Rama and García, 2016).

With the aim to mitigate excitotoxicity, numerous approaches have been studied, including the inhibition of glutamate release or the blockade of its receptors. Among

these, NMDA receptors represent the most calcium-permeable ionotropic receptors, thus their blockade reduces calcium influx and consequently ischemic neuronal damage (Lai *et al.*, 2014). The attenuation of glutamate-mediated excitotoxicity resulted effective in animal models of stroke, however targeting NMDA receptors in clinical trials has not led to improved neurological deficits in patients and was often associated with excess toxicity (Domercq and Matute, 2019). Indeed, based on their location and subunit composition, NMDA receptors mediate both physiological and pathological activities, thus their non-selective blockade may cause side effects, leading to cognitive impairment, hallucinations and even coma (Lipton, 2007; Soria *et al.*, 2014).

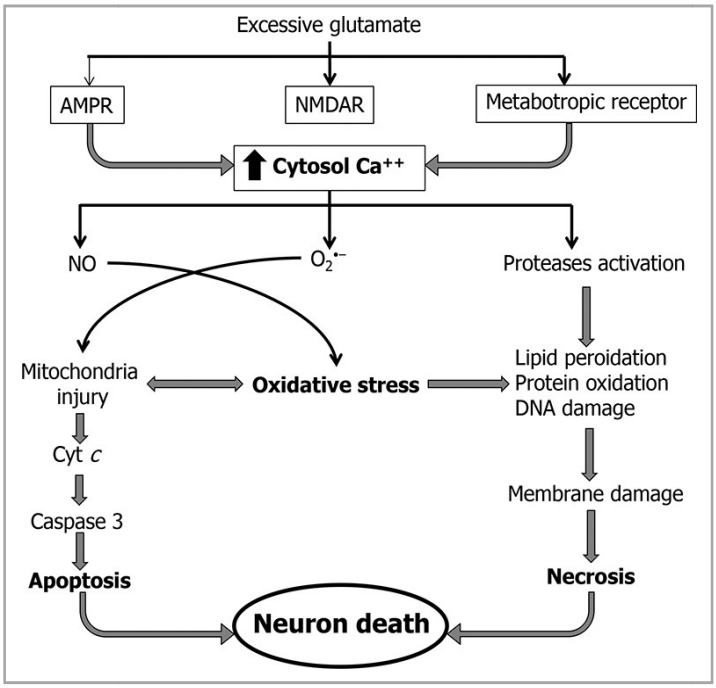

Figure 1.5 – Glutamate-mediated excitotoxicity. Overview of the main cellular mechanisms induced by glutamate-mediated excitotoxicity and calcium overload in neurons (Rama and García, 2016).

- **Oxidative and nitrosative stress**

The brain is particularly susceptible to free radicals attack because of its high concentration of peroxidable lipids, limited antioxidant defences and high oxygen

consumption (De Silva and Miller, 2016; Grochowski *et al.*, 2018). Following cerebral ischemia and, more importantly, after reperfusion, an intense production of reactive oxygen (ROS) and nitrogen species (RNS) occurs in neurons. This is a crucial factor for the development of reperfusion injury, the paradoxical response observed in the ischemic tissue after the restoration of blood flow (Granger and Kvietys, 2015). The oxygen provided by reperfusion supports neuronal viability but can also be used by mitochondria and pro-oxidant enzymes to produce more free radicals (Rama and García, 2016) (figure 1.6). Moreover, the acidic environment, commonly found in the ischemic brain, promotes a pro-oxidant effect by increasing H^+ concentration and enhancing the rate of production of ROS (Khoshnam *et al.*, 2017).

ROS are mainly generated by mitochondria during electron transport chain and oxidative phosphorylation. During ischemia, the increase of free-Ca^{2+} concentration may overload the mitochondrial proton circuit, thus causing increased ROS production and the release of pro-apoptotic molecules such as cytochrome *C* (Sims and Anderson, 2002; Lin and Beal, 2006; Murphy, 2009).

Thus, free radicals may indirectly trigger reactions that culminate in cell death, including peroxidation of mitochondrial membranes leading to mitochondrial dysfunction (Mukherjee *et al.*, 2019), cross-linking of macromolecules that lose the ability to perform their functions (Li *et al.*, 2019), impairment of endothelial cells resulting in enhanced BBB permeability (Wang *et al.*, 2019), exacerbation of inflammatory reactions through the stimulation of cytokines and adhesion molecules expression (Xu *et al.*, 2018).

Other important sources of ROS are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and some Ca^{2+}-dependent enzymes such as PLA2 and COX (Rama and García, 2016).

Nitrosative stress is caused by an increased production of RNS, able to nitrosilate and alter protein structures and functions (Ridnour *et al.*, 2004). RNS derive from nitric oxide (NO) that reacts with superoxide and hydrogen peroxide to form hydroxyl radical and peroxynitrite, that exacerbate neuronal ischemic damage (Ballarin and Tymianski, 2018).

In the context of ischemic stroke treatment, antioxidant therapy has been widely investigated, through strategies aimed at inhibiting ROS production or potentiating the mechanisms of scavenging. Although promising results were obtained in experimental models of ischemic stroke, the use of exogenous antioxidants has failed to provide significant neuroprotection in clinical trials due to low specificity of the effects, narrow therapeutic window and occurrence of side effects (Yang *et al.*, 2019).

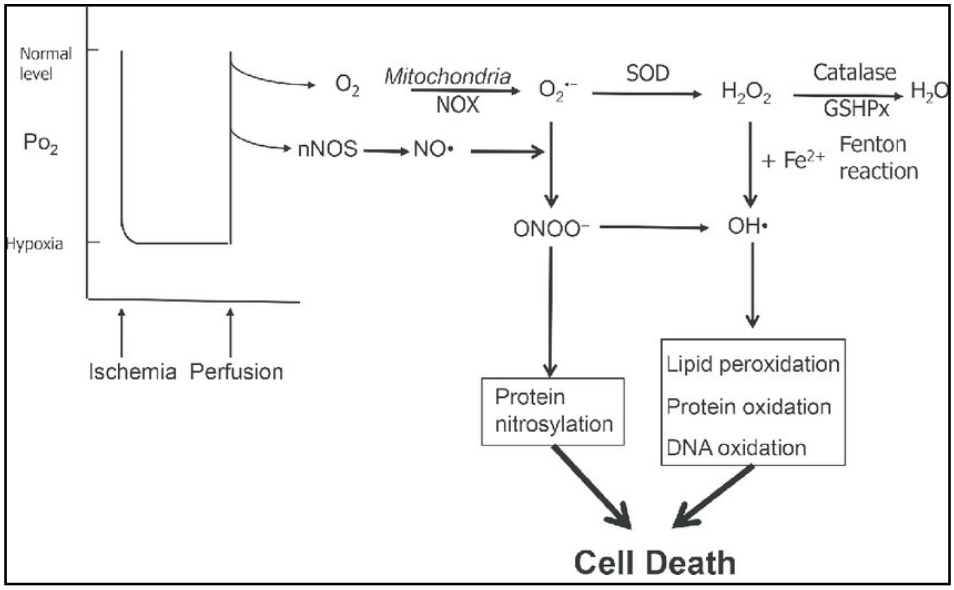

Figure 1.6 – Oxidative and nitrosative stress. Main sources of ROS and RNS during cerebral ischemia and reperfusion (Rama and García, 2016).

- **Cortical spreading depolarization**

During the ischemic cascade, high levels of extracellular glutamate and K^+ trigger a high energy consumption phenomenon, known as cortical spreading depolarization (CSD), consisting in an intense depolarization of neurons and glia slowly propagating into gray matter by way of chemical contiguity (Chung *et al.*, 2016). This phenomenon was first observed by Leao in 1944 and defined as 'cortical spreading depression', namely the spreading of loss of ionic homeostasis and depression of electrical activity (Leao, 1944). These waves of CSD originate within the infarct core and gradually invade peri-ischemic tissue, causing a major metabolic

demand into the penumbra and thus expanding the volume of infarction (Campbell *et al.*, 2019). The consequences are breakdown of ion gradients, neuronal swelling and distortion of dendritic spines, with concomitant oligemia due to alteration of homeostatic vascular response (Kramer *et al.*, 2016).

- **Neuronal death mechanisms**

After ischemic stroke, neuronal death mainly occurs by necrosis or apoptosis, depending on duration and severity of CBF reduction and on the residual ability of neurons to produce energy (Rama and García, 2016) (figure 1.7). Moreover, recent findings have highlighted the existence of other types of cell death, including autophagy, necroptosis and pyroptosis, often coexisting in the ischemic tissue. Neuronal death is triggered by multiple mechanisms, such as Ca^{2+} overload, oxidative stress and mitochondrial dysfunction. In particular, mitochondria are considered as one of the main contributor in activating pathways leading to ischemic neuronal death (Vosler *et al.*, 2009). Indeed, neuron viability is closely linked to mitochondria for Ca^{2+} homeostasis and ATP production, thus alterations in mitochondrial bioenergetic functions and membrane potential, occurring following ischemia, play a pivotal role in defining the specific cell death pathway activated (Liu *et al.*, 2018). The maintenance of mitochondrial structure and functions and the elimination of impaired mitochondria are fundamental ways to mitigate oxidative stress and cell death during an ischemic insult. A study conducted by Lemasters in 2005 described a particular type of autophagy defined as 'mitophagy', consisting in removing impaired mitochondria to keep stable cellular homeostasis (Lemasters, 2005). Several studies suggest that mitophagy is protective in the context of ischemic stroke by suppressing mitochondria-mediated cell death signalling cascades (Zhang *et al.*, 2013; Li *et al.*, 2014; Di *et al.*, 2015; Qi *et al.*, 2015).

Necrosis rapidly occurs in the ischemic core, where there is a severe reduction of blood flow (Sommer, 2017). Necrotic neurons are characterized by cell swelling, disruption of cell organelles and plasma membrane rupture, resulting in extracellular release of proteolytic enzymes and proinflammatory molecules.

By contrast, apoptosis, an energy-dependent process, mainly occurs in the ischemic penumbra, where a residual blood flow provides oxygen and glucose to keep the neurons metabolically active, and is enhanced by reperfusion, which allows energy production to be restored; neurons in the ischemic penumbra may undergo apoptosis after several hours or even days (Radak *et al.*, 2017). The apoptotic cascade can be triggered by the activation of membrane receptors (e.g., CD95R) or through the release of proapoptotic molecules such as cytochrome *c* by damaged mitochondria (Rama and García, 2016). Both pathways converge on the activation of proteolytic enzymes, the caspases, that degrade selected target proteins, and induce specific molecular and morphological changes, including cell shrinkage, membrane blebbing, nuclear pyknosis, chromatin condensation and genomic fragmentation.

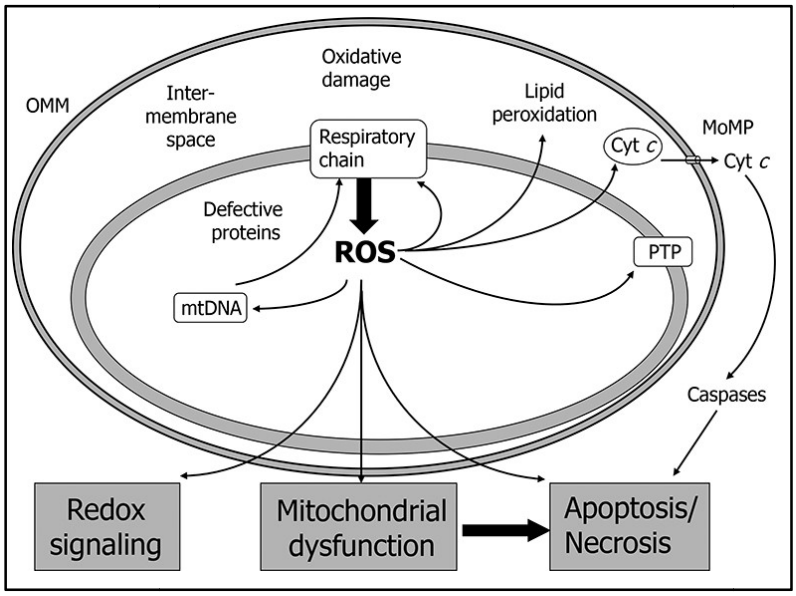

Figure 1.7 – Neuronal death mechanisms. Excessive ROS production in mitochondria alters mitochondrial structure and functions leading to necrosis and apoptosis of ischemic neurons (Rama and García, 2016).

1.1.5 - Role of immune responses and inflammation

The progression of ischemic cerebral damage is severely affected by the innate and adaptive immune responses, triggered immediately after the insult and lasting for several days, involving soluble mediators and specialized cells localized in the brain

or recruited from the periphery (Amantea *et al.*, 2015; Qin *et al.*, 2020). Within 1-2 hours from stroke onset, brain resident microglia and astrocytes are activated by damaged neurons, compromising the integrity of the BBB and promoting the infiltration of circulating cells, such as neutrophils, monocytes/macrophages and T cells, that exacerbate ischemic injury (Jayaraj *et al.*, 2019; Qin *et al.*, 2020). The early non-specific inflammatory reaction occurs in a few hours after the ischemic event, during the acute phase (Gelderblom *et al.*, 2015; Amantea *et al.*, 2018). This represents the input for the subsequent activation of adaptive immunity involving T and B cell-mediated inflammatory and humoral effects, which occurs within 24 h following injury and persists for several days (Qin *et al.*, 2020) (figure 1.8).

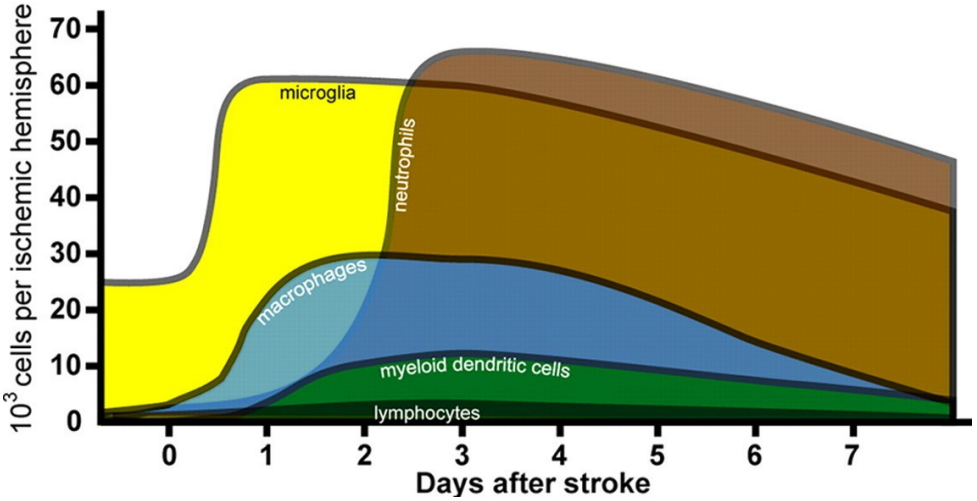

Figure 1.8 – Temporal dynamics of inflammatory cells recruitment in the ischemic brain. Number of immune cells observed in the ischemic hemisphere of mice after an ischemic insult of 1 h (Gelderblom *et al.*, 2009).

Recent studies showed that an higher leukocytes count in the blood of stroke patients, within 24 h from symptoms onset, associates with worse functional impairment and unfavourable clinical outcomes, such as recurrent ischemic event and death (Quan *et al.*, 2019). In particular, it has been demonstrated that high neutrophil-to-lymphocyte ratio underlies a worse clinical outcome at 3 months, in stroke patients treated with rt-PA (Maestrini *et al.*, 2015; Guo *et al.*, 2016).

Although targeting the immune system is an attractive therapeutic approach in the context of stroke therapy, the use of immunosuppressant drugs has not shown

effective results in the clinical settings. The lack of translational power is due to the dualistic role played by immune mediators on the progression of ischemic brain damage, exerting both detrimental and beneficial functions. Indeed, brain resident and blood-borne immune cells recruited from the periphery contribute to exacerbate ischemic brain damage by prompting inflammatory reactions, but also participate to reparative processes that provide tissue recovery (figure 1.9) (Amantea *et al.*, 2018).
Post-ischemic inflammation is characterized by a specific sequence of events, involving the brain, its vessels, the blood and lymphoid organs, and starts in the intravascular compartment immediately after arterial occlusion, with the activation of platelets and endothelial cells (Anrather and Iadecola, 2016). In this context, excessive ROS production enhances the expression of integrins on leukocytes and corresponding adhesion molecules (i.e., intercellular adhesion molecule 1, P-selectin and E-selectin) on endothelial cells surface, thus activating a prothrombotic and proinflammatory state and promoting the recruitment of circulating leukocytes, that combined with fibrin clots and reduced NO availability, lead to further microvascular occlusions (Crack and Wong, 2008; De Meyer *et al.*, 2016; Mo *et al.*, 2020). Due to BBB disruption, infiltrating leukocytes are able to extravasate into the injured brain parenchyma, where they release neurotoxic substances including pro-inflammatory cytokines, chemokines and free radicals (Simats *et al.*, 2016). In addition, the release into the circulation of damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines by dying neurons and brain resident immune cells, promotes the activation of a transient systemic inflammatory syndrome, characterized by elevated serum levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 released by circulating and splenic immune cells, both in animal models and stroke patients (Iadecola *et al.*, 2020). This intense inflammatory response is followed by a strong immunosuppressive state in the periphery, characterized by lymphopenia and reduced T-cell responsiveness in patients, thus promoting recurrent infections, a major determinant of morbidity and mortality in stroke patients (Anrather and Iadecola, 2016; Miró-Mur *et al.*, 2016).

Astrocytes. Within few minutes from stroke onset, the proinflammatory environment observed in the ischemic brain activates the pericapillary astrocytes, inducing changes in their morphological and functional characteristics, in a process known as

"reactive astrogliosis" (Pekny *et al.*, 2019). Reactive astrocytes start to proliferate and show hypertrophy of their cellular processes and overexpression of glial fibrillary acidic protein (GFAP), the main component of astrocyte cytoplasmic intermediate filaments (Haim *et al.*, 2015). Within days after stroke, astrocytes migrate toward the peri-infarct region, where the release of extracellular matrix (ECM) molecules leads to the formation of a glial scar at the infarct border, that helps to delimit the ischemic area from the healthy tissue, thus restricting the diffusion of neuroinflammation (Pekny *et al.*, 2019). Furthermore, reactive astrocytes contribute to the production of pro-inflammatory mediators (i.e. TNF-α, IL-1α, IL-1β and interferon (IFN)-γ, free radicals and matrix metalloproteasis (MMPs) that participate to BBB disruption, oedema formation and further recruitment of circulating immune cells (Hennessy *et al.*, 2015; Rempe *et al.*, 2016).

In particular, ATP released by damaged cells in the ischemic brain activates P2Y1 receptors on astrocytes stimulating the production of proinflammatory cytokines and chemokines, via the nuclear factor-κ B (NF-κB) pathway, a key regulator of the inflammatory responses (Kuboyama *et al.*, 2011). The activation of this signalling pathway in astrocytes can culminate in neuronal damage after stroke and its inhibition has been demonstrated to be neuroprotective by reducing the expression of proinflammatory genes and, consequently, the infiltration of circulating leukocytes (Dvoriantchikova *et al.*, 2009).

Astrocytic response to stroke may exacerbate cerebral ischemic damage, but also exerts beneficial effects. Indeed the glial scar may hinder axonal regeneration and neuronal reconnection during the recovery phase after stroke, thus worsening functional outcome of patients, but concomitantly this physical barrier protects the viable tissue in the acute phase, by avoiding the diffusion of soluble factors released by damaged cells in the infarct core (Liu and Chopp, 2016). Astrocytes also contribute to reuptake of extracellular glutamate, thus reducing excitotoxicity and release neurotrophic factors that, in turn, promote neurogenesis, axonal remodelling, angiogenesis and BBB rebuilding, thus supporting the functional recovery of patients (Barreto *et al.*, 2012; Xu *et al.*, 2020).

Microglia. Under physiological conditions, in the brain, resting microglia are characterized by a small cell body, ramified morphology and low proliferative

activity (Michell-Robinson *et al.*, 2015). By contrast, pathological conditions that alter brain homeostasis, such as cerebral ischemia, induce a rapid activation of microglial cells, that start to proliferate, migrate toward the site of injury and show a modified morphology and gene expression (Zhang *et al.*, 2020). The interaction between neurons and microglia in the brain is critical to keep these immune cells in a resting state. Under normal conditions, CX3CL1 (fractalkine), a membrane-bound chemokine expressed on neurons, interacts with its receptor CX3CR1 on microglia, and prevent their activation (Iadecola and Anrather, 2011), but after ischemic neuronal damage, loss of CX3CL1 results in enhanced microglial activation and neurotoxicity (Cardona *et al.*, 2006).

In experimental models of ischemic stroke, microglia are rapidly activated within few minutes following neuronal insult and accumulate in the peri-infarct region after 30 min (Eyo *et al.*, 2015; Yu *et al.*, 2020); then microglial reaction reaches the peak after 48-72 h from stroke onset and may last for several weeks (Guruswamy and Elali, 2017). Activated microglia are characterized by upregulation of cell surface markers, such as cluster of differentiation (CD) 11b, CD68 and ionized calcium-binding adaptor molecule 1 (Iba1) (Benakis *et al.*, 2015).

In the ischemic brain, microglia undergo radical morphological changes, including deramification and enlargement of cell bodies to finally acquire an amoeboid macrophage-like phenotype, enhancing their phagocytic and migratory abilities (Zhang, 2019). Based on the spatial evolution of the ischemic damage, microglia acquire different morphologies that reflect their activation state. In particular, in penumbral areas, during the post-acute phase after stroke, microglia show hypertrophic cell bodies and rare and short processes; on the contrary, completely deramified round microglia with a phagocytic behaviour, corresponding to the most active state, are mainly found in the ischemic core between 3 and 7 days after stroke (Perego *et al.*, 2011; Anttila *et al.*, 2017). Microglia in this activation state acquire the ability to perform some typical functions of macrophages, like migration, phagocytosis and antigen presentation through major histocompatibility complex (MHC) II (Iadecola and Anrather, 2011). The phagocytic behaviour is helpful in clearing cell debris and other immune cells, and participate to the resolution of inflammation; however, phagocytosis could also contribute to neuronal loss by

engulfing potentially salvageable neurons in the ischemic penumbra (Amantea, 2016). Furthermore, microglia can also phagocyte endothelial cells, destroying vascular integrity and facilitating the entry of serum components in the brain (Xiong *et al.*, 2016). The morphological modifications are accompanied by an increased production of proinflammatory cytokines and chemokines, through several pathways. DAMPs released by damaged neurons stimulate a strong inflammatory reaction through the activation of toll like receptors (TLRs); in particular, in the brain, TLR4 is mainly expressed on microglial cells and its enhanced expression observed after ischemic injury activates the NF-κB pathway, that lead to the secretion of proinflammatory cytokines, such as TNF-α and IL-6, sustaining neuronal damage (Zhao *et al.*, 2017). Moreover, enhanced levels of ATP in brain parenchyma lead to the activation of P2X7 receptors on microglia, stimulating further release of inflammatory molecules (Iadecola and Anrather, 2011). Microglia also participate to BBB disruption through the release of MMPs, such as MMP9 (Dudvarski Stankovic *et al.*, 2016), and may enhance the expression of adhesion molecules on endothelial cells, thus promoting the recruitment of circulating leukocytes (da Fonseca *et al.*, 2014).

Once activated, microglia is able to switch toward a multitude of phenotypes based on the specific microenvironmental stimuli received, thereby exerting both detrimental and reparative functions. In particular, they may polarize toward the classical proinflammatory M1 phenotype or the alternative anti-inflammatory M2 phenotype, that represent the two extremes of their activation state (Kanazawa, Ninomiya, *et al.*, 2017). The M1 phenotype is characterized by the expression of surface markers such as CD16 and CD68 (Shin *et al.*, 2014) and release cytotoxic molecules including TNF-α, IL-1β, IL-6, IFN-γ, ROS and RNS, that worsen cerebral ischemic damage (Zeng *et al.*, 2018). The alternatively-activated M2 microglia mainly show arginase (Arg) 1 and CD206 as surface antigens and promote tissue repair, angiogenesis and resolution of inflammation, through the release of anti-inflammatory molecules and growth factors, including IL-4, IL-10, IL-13, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 (Xiong *et al.*, 2016; Zhang *et al.*, 2020). Polarization of microglia is a dynamic process

evolving over time after ischemic stroke. It has been demonstrated in animal models that M2 polarized microglia first appear in the early stages after stroke, with the aim to contain the progression of the damage, reaching their maximal levels after 3-5 days, and then gradually shift toward the M1 detrimental phenotype, peaking around 14 days after ischemic injury (Hu, X. *et al.*, 2012; Suenaga *et al.*, 2015). Several factors orchestrate the process of microglial polarization. For example, the activation of NF-κB or Notch pathways lead to the acquisition of M1 inflammatory phenotype(Liu *et al.*, 2017; Wu *et al.*, 2018). By contrast, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, such as rosiglitazione or pioglitazone, and the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway, are mechanisms driving microglial transition toward the M2 reparative phenotype (Hasegawa-Moriyama *et al.*, 2013; Ballesteros *et al.*, 2014; Xu *et al.*, 2015).

Monocytes/macrophages. Monocytes recruitment into the ischemic brain starts within 24 h from stroke onset and reach a peak around day 4; then these cells may develop morphological and functional characteristics of tissue macrophages and persist in brain parenchyma for weeks (Planas, 2018). Peripheral immature inflammatory monocytes, mainly originating from the spleen, primarily infiltrate cerebral tissue (Miró-Mur *et al.*, 2016). There are two main subsets of monocytes mobilized after ischemic stroke, that exert inflammatory or patrolling functions. The inflammatory type express high levels of lymphocyte antigen 6 complex locus C1 (Ly-6C) and C-C motif chemokine receptor type 2 (CCR2) ($CCR2^{+}Ly\text{-}6C^{high}$), while patrolling monocytes are characterized by an elevated expression of chemokine receptor for fractalkine (CX3CR1) and show low levels of Ly-6C ($CX3CR1^{+}Ly\text{-}6C^{low}$) (Jian *et al.*, 2019). It has been demonstrated that $CCR2^{+}Ly\text{-}6C^{high}$ monocytes, displaying amoeboid morphology and phagocytic activity, firstly infiltrate brain parenchyma in the acute phase after stroke (at 3-5 days after middle cerebral artery occlusion, MCAo) and then differentiate into patrolling $CX3CR1^{+}Ly\text{-}6C^{low}$ macrophages during later stages (14-28 days post-MCAo), that retain amoeboid shape in the ischemic core but also acquire ramified or elongated morphology (Garcia-Bonilla *et al.*, 2016). This latter subset contributes to tissue repair mechanisms and to functional recovery after stroke. Accordingly, a recent work

showed that inhibiting monocytes recruitment using anti-CCR2 antibody during the first week after injury reduces the expression of anti-inflammatory genes in the brain and, as a consequence, impairs functional recovery (Wattananit *et al.*, 2016). Similar to microglia, both populations of monocytes/macrophages may be influenced by the cerebral inflammatory microenvironment to switch towards different phenotypes (Jian *et al.*, 2019). The classically activated M1 phenotype exacerbates the inflammatory reaction and worsens cerebral ischemic damage by releasing cytotoxic molecules, such as TNF-α, IL-1β, and ROS; by contrast, the alternative M2 phenotype secretes anti-inflammatory cytokines, like TGF-β, IL-4, IL-10 and growth factors, thus promoting angiogenesis and tissue repair (Amantea, 2016; Xiong *et al.*, 2016). These two different states of polarization are characterized by the expression of specific cell surface markers: CD16, CD32, MHC II and inducible nitric oxide synthase (iNOS) are typically expressed in M1 microglia/macrophages, while the alternative M2 state is mainly characterized by the expression of Arg-1 and Ym1 (Fumagalli *et al.*, 2015).

Studies in animal models of ischemic stroke revealed that the expression of M2 phenotype markers by microglia and macrophages starts at 12 hours, increases at 1 to 3 days and decreases several days after the ischemic insult (Perego *et al.*, 2011; Hu, X. *et al.*, 2012), whereas the markers of M1 phenotype are detectable during the first 14 days after ischemic stroke (Kanazawa, Ninomiya, *et al.*, 2017).

Neutrophils. During the post-ischemic inflammatory reaction, neutrophils are the first circulating cells attracted to the ischemic brain, detected within few hours in perivascular region and peaking in brain parenchyma at 1-3 days from the injury (Perez-de-Puig *et al.*, 2015). It has been demonstrated that elevation of neutrophil counts is detected in the blood of stroke patients within 24 h from stroke onset, and this correlates with worse functional outcomes and higher infarct size, similar to that observed in mice models of ischemic stroke (Cai *et al.*, 2020). In the acute phase, neutrophils exacerbate cerebral ischemic damage by causing further microvessel occlusions and by contributing to BBB disruption through the release of MMPs and reactive oxygen and nitrogen species (Malone *et al.*, 2019). Furthermore, neutrophils, by interacting with activated platelets, can produce neutrophil extracellular traps (NETs), that promote clot formation and reduce the effectiveness of thrombolytic

treatment (García-Culebras *et al.*, 2018), representing useful target to improve success of recanalization therapies (Peña-Martínez *et al.*, 2019). Neutrophils infiltration into brain parenchyma is promoted by BBB disruption and expression of endothelial adhesion molecules such as selectins and integrins, and contributes to worsening ischemic damage through the release of inflammatory cytokines, proteolytic enzymes and free radicals (Khoshnam *et al.*, 2017).

However, as well as microglia and macrophages, neutrophils can also switch between N1 pro-inflammatory phenotype and N2 protective state. N1 subset releases inflammatory substances like IFNγ and TNF-α, whereas N2 neutrophils exert anti-inflammatory effects (Hermann *et al.*, 2018). It has been demonstrated in rodent models that the stimulation of the PPAR-γ with its agonist rosiglitazone, supports the polarization of neutrophils toward the N2 reparative phenotype, expressing Ym1 and CD206 as surface markers, that increases neutrophils clearance and promotes the resolution of inflammation after permanent MCAo (Cuartero *et al.*, 2013). Furthermore, a recent work showed the critical role played by TLR4 in modulating neutrophil polarization, since its absence resulted neuroprotective in mice exposed to MCAo, by promoting neutrophil recruitment into the ischemic brain and their alternative N2 polarization (García-Culebras *et al.*, 2019).

Adaptive immune response. The activation of the adaptive immune system following ischemic stroke involves T and B cell-mediated proinflammatory or protective effects. T lymphocytes are rapidly recruited to the ischemic brain within few hours, reach higher level between 1 and 7 days and can be detected until 30 days after stroke onset (Jian *et al.*, 2019; Qin *et al.*, 2020). $CD4^+$ helper T cells in brain parenchyma may be stimulated by microglia/macrophages to differentiate toward Th1 proinflammatory cells or Th2 antiinflammatory subset (Dolati *et al.*, 2018). Th1 cells sustain ischemic damage by producing proinflammatory cytokines such as IL-2, IL-12 and IFN-γ, whereas Th2 cells protect the brain by secreting anti-inflammatory cytokines like IL-4, IL-10 and IL-13 (Filiano *et al.*, 2017). By contrast, cytotoxic $CD8^+$ T lymphocytes play a detrimental role by directly damaging neurons through the release of perforin/granzyme (Mracsko *et al.*, 2014) and promote further BBB damage through the release of inflammatory mediators such as IFN-γ and IL-16 (Qin *et al.*, 2020). After cerebral ischemia, disruption of cell membranes results in the

exposure of neural antigens, normally sequestered behind the BBB, that reach spleen and lymph nodes where they triggers the recruitment of circulating T and B lymphocytes and the production of auto-antibodies directed against CNS antigens (Iadecola *et al.*, 2020). Around 4-6 weeks after stroke, B cells start to accumulate in the ischemic brain and release IgA and IgG antibodies, which bind the Fc receptors and trigger the complement pathway leading to neuronal damage and subsequent cognitive impairment (Jian *et al.*, 2019). Furthermore, regulatory B cells, as well as T lymphocytes, may participate in the resolution of inflammation by releasing anti-inflammatory cytokines, including IL-10 and TGF-β (Doyle *et al.*, 2015).

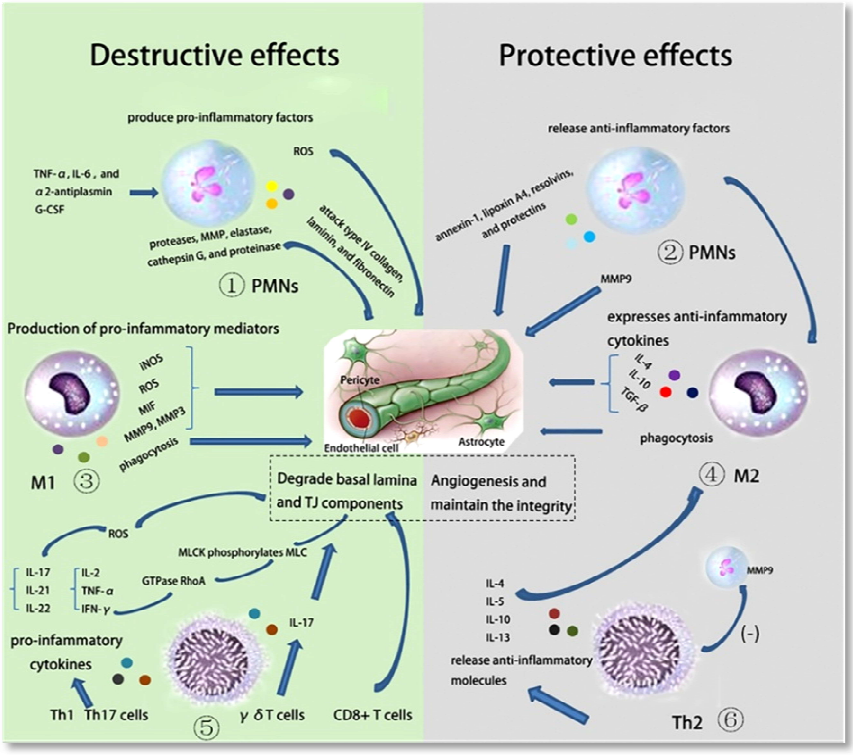

Figure 1.9 – Dualistic role of immune mediators in the evolution of ischemic brain damage (Li *et al.*, 2018).

Thus, considering the need of effective neuroprotective therapies and the crucial role played by inflammatory mediators in the progression of ischemic brain damage, an interesting approach in the context of ischemic stroke treatment is to target the post-ischemic immune reaction. In particular, the goal is to achieve neuroprotection by blocking the harmful response of innate immune cells and by promoting their

polarization toward M2 and N2 reparative phenotypes (Amantea *et al.*, 2018). Furthermore, since post-ischemic inflammation may last for several weeks after arterial occlusion, this approach has the potential to extend the therapeutic window for the treatment of stroke patients.

1.2 - Role of Store-Operated Calcium Entry (SOCE) in cerebral ischemia

One of the most significant factors involved in neuronal death during ischemic stroke is the progressive intracellular accumulation of Ca^{2+}; in addition to the deleterious effects of excessive Ca^{2+} influx from the extracellular space, also the release of Ca^{2+} from intracellular stores, such as mitochondria and ER, contributes to ischemic neuronal death. Moreover, during cerebral ischemia, complete depletion of ER Ca^{2+} stores leads to neuronal death by inducing mechanisms of ER stress (Secondo *et al.*, 2018). The ER is the biggest reservoir of Ca^{2+} in the cells and plays a central role in cellular Ca^{2+} signalling. Under physiological conditions, the concentration of Ca^{2+} in the ER lumen is significantly higher (10-100 μM) than the cytoplasmic concentration (100-300 nM) (Mattson *et al.*, 2000). This high Ca^{2+} concentration in the ER lumen is important for folding of newly synthetized proteins through a series of Ca^{2+}-dependent reactions; if this function is impaired, unfolded proteins accumulate into the ER and activate the unfolded protein response (UPR), which results in cell death via the mechanisms of ER stress (Rastogi and Srivastava, 2019).

Ca^{2+} is sequestered into ER by the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) and released through the inositol-1,4,5-triphosphate ($InsP_3$) receptor/Ca^{2+} channels ($InsP_3R$) or ryanodine receptor/Ca^{2+} channels. Alterations of Ca^{2+} concentration in the ER, as well as acute Ca^{2+} release can be apoptogenic (Pinton and Rizzuto, 2006).

During cerebral ischemia, the massive activation of Gq-linked receptors on the PM results in a significant release of Ca^{2+} from ER through $InsP_3R$ and this causes a considerable influx of Ca^{2+} from the extracellular space, in order to re-establish Ca^{2+} levels in the ER (Secondo *et al.*, 2018). In particular, the reduction of Ca^{2+} concentration in the ER lumen triggers the store operated Ca^{2+} entry (SOCE), through the opening of PM Ca^{2+} channels. Molecularly, SOCE is mediated by the interaction between the proteins stromal interaction molecule (STIM), an ER Ca^{2+}

sensor, and Orai, an high selective Ca^{2+} channel in the PM, responsible for Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}); in addition, STIM can also activate the less Ca^{2+} selective store-operated Ca^{2+}current (I_{SOC}), that involves the interaction between Orai and TRPCs (Desai *et al.*, 2015; Lopez *et al.*, 2016). The ER sensor protein STIM detects depletion of Ca^{2+} stores, oligomerizes and migrates to ER-PM junctions, where it binds the Ca^{2+} channel Orai promoting Ca^{2+} influx (Feske *et al.*, 2006; Peinelt *et al.*, 2006; Prakriya *et al.*, 2006).

STIM is a single spanning transmembrane protein mainly found in the ER (Roos *et al.*, 2005; Zhang *et al.*, 2005; Baba *et al.*, 2006). The N-terminal region is located in the ER lumen and contains the EF-hand motives, which bind Ca^{2+} and work as sensor (Rosado *et al.*, 2016). The C-terminal cytosolic region is constituted by 3 coiled-coil domains forming the CRAC modulatory domain, which comprises the STIM1 Orai1 activation region (SOAR) involved in the activation of Orai and the C-terminal inhibitory domain (CTID) (Lopez *et al.*, 2016). In vertebrates, two different forms of STIM protein have been identified, namely, STIM1 and STIM2 that are ubiquitously expressed in murine and human brain tissue (Kraft, 2015) and reach their highest levels of expression in cortex and hippocampus (Steinbeck *et al.*, 2011).

Orai is a tetraspanning PM protein, forming a ion-conducting pore highly selective for Ca^{2+}; N- and C-terminal domains are cytosolic and both are essential for STIM1 interaction and regulation (Palty and Isacoff, 2016). There are three isoforms of this PM channel, Orai1, Orai2 and Orai3, with different inactivation and permeability characteristics (DeHaven *et al.*, 2007; Lis *et al.*, 2007). All the three forms of Orai interact with STIM1 to activate SOCE, but Orai1 generates a quantitatively much higher current (Putney, 2017).

SOCE is negatively regulated by SOCE-associated regulatory factor (SARAF), which detects elevation of Ca^{2+} concentration and associates with STIM to promote a slow Ca^{2+}-dependent inactivation of SOCE, thus maintaining proper intracellular Ca^{2+} levels (Palty *et al.*, 2012). SARAF is an ER resident protein with a single transmembrane domain, an intraluminal domain which regulates SARAF activity and a cytosolic region responsible for the modulation of STIM1-Orai1 interaction (Palty *et al.*, 2012). Under resting conditions, the CTID of STIM1 mediates the association between the SOAR region and SARAF, in order to prevent spontaneous activation of

Orai1. Following ER stores depletion, SARAF decouples from STIM1 thus allowing the SOAR domain of STIM1 to interact with Orai1 and providing Ca^{2+} entry (Jha *et al.*, 2013). Recently, it has been demonstrated that SARAF may also be located in the PM and that it can mediate a direct activation of Orai1, in cells with low expression of STIM1 (Albarran *et al.*, 2016) (figure 1.10).

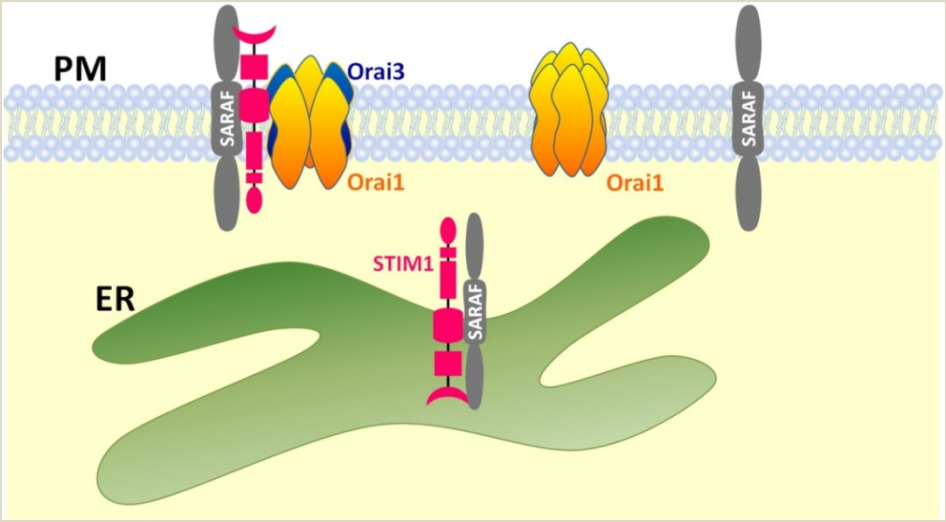

Figure 1.10 – Schematic representation of the interaction between the main molecular components of SOCE (modified from Lopez *et al.*, 2016).

Ca^{2+} influx mediated by SOCE, underlying the refilling of ER, is helpful in restoring Ca^{2+} homeostasis and has been suggested to prevent neuronal damage caused by ER-stress mechanisms after cerebral ischemia (Sirabella *et al.*, 2009; Parekh, 2010; Lang *et al.*, 2018). Accordingly, a recent study has demonstrated that reduced expression of STIM1 and Orai1 mediates hypoxic/ischemic neuronal death in rats undergone focal cerebral ischemia and in primary cortical neurons (Secondo *et al.*, 2019). By contrast, previous studies have shown an increased expression of STIM1 and Orai1 in the hippocampus of rats subjected to global cerebral ischemia, suggesting that SOCE could represent the source of Ca^{2+} overload in neurons, thus causing a non-excitotoxic neuronal death (Zhang, *et al.*, 2014). Furthermore, *in vitro* and *in vivo* studies in mice demonstrated that STIM2 deficiency significantly enhances neuronal survival under hypoxic conditions and reduces neurological deficits observed in mice after focal ischemia (Berna-Erro *et al.*, 2009).

These contrasting data confirm the involvement of SOCE in the evolution of ischemic brain damage, but further studies are required to fully understand its specific role.

1.2.1 - Physiological and pathological roles of SOCE

SOCE, providing Ca^{2+} refilling after ER stores depletion, is an ubiquitous process which generates Ca^{2+} signals in both excitable and non-excitable cells. In addition to its important role in maintaining Ca^{2+} homeostasis, a number of studies demonstrated that SOCE modulates several cell functions, such as the regulation of Ca^{2+}-dependent enzymes(Parekh, 2008), gene expression (Dragoni *et al.*, 2011; Kar *et al.*, 2012), cell cycle progression (Courjaret and Machaca, 2012; Moccia *et al.*, 2012) and apoptosis (Dubois *et al.*, 2014). This implies that SOCE impairment may be involved in different physiological and pathological processes including skeletal and smooth muscles contractility (Feldman *et al.*, 2017), platelet functions (Varga-Szabo *et al.*, 2008; Braun *et al.*, 2009; Ahmad *et al.*, 2011; Berna-Erro *et al.*, 2016; Nagy *et al.*, 2018), neuronal excitability (Moccia *et al.*, 2015), hypoxic/ischemic neuronal injury (Berna-Erro *et al.*, 2009; Zhang, *et al.*, 2014), cardiac hypertrophy (Bénard *et al.*, 2016) and carcinogenesis (Chen *et al.*, 2011; White, 2017).

In neurons, Ca^{2+} influx is mainly provided by voltage-operated Ca^{2+} channels (VOCCs) and receptor-operated channels (ROCs); however, it has been demonstrated that STIM1 and Orai1 are ubiquitously expressed in the brain (Klejman *et al.*, 2009), thus SOCE represents an important source of Ca^{2+} in resting neurons (Hooper *et al.*, 2014). In particular, this Ca^{2+} current in the central nervous system is involved in the regulation of neurotransmitters release and synaptic plasticity (Bollimuntha *et al.*, 2017). Many studies demonstrated that SOCE plays a critical and dualistic role in the modulation of different components of the neurovascular unit, since it is involved in both death and survival mechanisms; thus, alterations of SOCE in neurons, glia or hematopoietic cells may underlie different neurodegenerative disorders including ischemic stroke, Parkinson's, Alzheimer's and Huntington's diseases (Secondo *et al.*, 2018).

During ischemic stroke, beside its role in neuronal survival mechanisms, SOCE participates to endothelial repair by promoting the recruitment of circulating endothelial progenitor cells to the injured vessels (Moccia *et al.*, 2014). Moreover, it has been demonstrated in mice that Ca^{2+} signals generated by Orai1 and STIM1 in platelets regulate several functions as aggregation, adhesiveness, granule release and procoagulant activity; thus SOCE is crucial in hemostasis and arterial thrombus formation (Varga-Szabo *et al.*, 2008; Braun *et al.*, 2009; Gilio *et al.*, 2010; Van Kruchten *et al.*, 2012). As a consequence, Orai1 or STIM1 deficient mice showed a reduced thrombus formation with mild bleeding time prolongation (Braun *et al.*, 2009; Gilio *et al.*, 2010), and this results in an increased resistance to arterial thrombosis and brain ischemia.

1.2.2 - SOCE in immune cells

Besides the crucial role played by SOCE current in neuronal death/survival mechanisms, it is important to highlight its involvement in the modulation of immune cells functions, as this latter mechanism may underlie SOCE involvement in ischemic stroke pathobiology.

SOCE was first identified in mast cells and T lymphocytes (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993). The increase of cytoplasmic Ca^{2+} concentration represents the main signal transduction mechanism regulating a variety of functions in immune cells (Clapham, 2007; Feske, 2009; Vig and Kinet, 2009), and SOCE is the predominant source of Ca^{2+} in adaptive and innate immune cells in response to their engagement (Feske *et al.*, 2015; Demaurex and Nunes, 2016; Vaeth *et al.*, 2017).

In particular, the activation of immunoreceptors, such as Fc receptors on mast cells and macrophages, chemokine receptors on neutrophils and antigen receptors on lymphocytes, results in a small and transient increase of intracellular Ca^{2+} levels due to the opening of ER channels; therefore, the reduction of Ca^{2+} concentration in the ER lumen activates the highly selective CRAC channels in the plasma membrane, thus providing a more robust and sustained influx of Ca^{2+} from the extracellular space, required for proper functioning of immune cells (Feske, 2011) (figure 1.11).

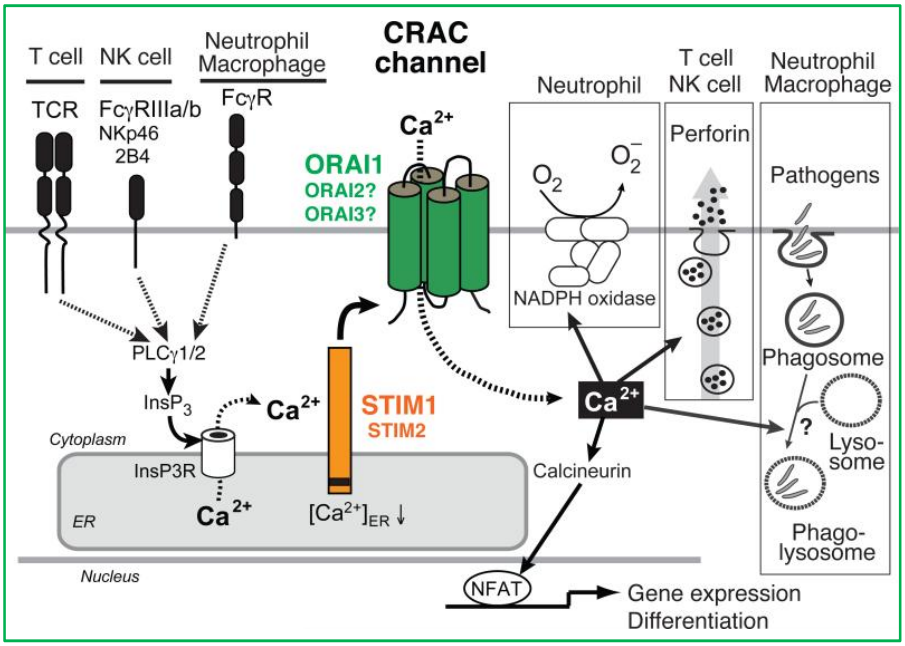

Figure 1.11 – Role of SOCE in immune cells. Overview of the functions regulated by Orai1/STIM1-mediated Ca^{2+} entry in immune cells (Feske, 2011).

The critical role played by SOCE in the regulation of immunity is confirmed by the evidence that, in patients, loss of function mutations in genes encoding Orai1 and STIM1 proteins cause a severe immunodeficiency, characterized by recurrent infections and autoimmunity (Lacruz and Feske, 2015). Immunodeficiency is mainly due to dysregulation of T lymphocytes, whose functions are dramatically compromised in the absence of SOCE; however, further clinical evidence, like frequent mycobacterial infections, also suggests the involvement of the innate immune response (Demaurex and Nunes, 2016).

Calcium signals generated by Orai-STIM interaction activates signalling molecules and transcription factors involved in several functions of innate immune cells, such as differentiation, maturation, proliferation, chemotaxis, secretion of cytokines and ROS and phagocytosis (Secondo *et al.*, 2018). Innate immune cells may express different isoforms of STIM and Orai proteins, but their specific contribution depends on the cell type and its activation state.

Human and murine neutrophils express all five isoforms of STIM and Orai proteins (Clemens and Lowell, 2019). In particular, Orai1 and Orai2 are the main CRAC channels expressed in neutrophils; indeed, Orai1-deficient neutrophils exhibit a

partially reduced calcium influx, whereas loss of both Orai1 and Orai2 completely abolishes SOCE, impairing critical functions such as phagocytosis, degranulation, and production of leukotriene and ROS (Grimes *et al*., 2020). Regarding STIM isoforms, it has been demonstrated that STIM1, but not STIM2, in neutrophils critically affects their functions like phagocytosis, degranulation and ROS production, while STIM2-deficient neutrophils show a reduction of cytokine production during an inflammatory reaction (Clemens *et al.*, 2017a). Indeed, STIM2 is critical for the activation of the transcription factor NF-kB and the subsequent expression of pro-inflammatory cytokines (Demaurex and Saul, 2018).

Similar to neutrophils, also monocytes and macrophages express different isoforms of STIM and Orai proteins. In blood monocytes Orai1, Orai2 and Orai3 channels are expressed in comparable proportions and they are important for ROS production and bacterial killing; in particular, it has been shown that Orai1 is the predominant isoform under resting conditions, whereas Orai3 is essential to prevent the oxidative inactivation of CRAC currents (Saul *et al.*, 2016). Interestingly, in inflammatory macrophages, the expression of Orai1 is significantly higher compared to alternatively activated macrophages or monocytes (Clemens and Lowell, 2019). On the contrary, it seems that STIM isoforms are not critical in the regulation of macrophage functions; indeed Vaeth et al. demonstrated that macrophages lacking both STIM1 and STIM2 proteins show no relevant defects in inflammasome activation, phagocytosis or cytokine production (Vaeth *et al.*, 2015a).

Although several recent studies have clarified the critical role played by SOCE components in innate immunity, their relevance in the modulation of immune cells phenotypes during cerebral ischemia has not been investigated yet. Thus, reprogramming the innate immune system by modulating SOCE components may represent an attractive neuroprotective strategy.

1.3 - Ischemic tolerance

In the context of ischemic stroke treatment, with the aim to reduce further clinical failures, recent studies have highlighted the potential of stimulating endogenous neuroprotection, namely the ability of the brain to develop tolerance in response to a

harmful stimulus. The achievement of endogenous neuroprotection in stroke patients is a very promising strategy, that would allow to reduce the risk of toxicity and to extend the time window in which neurons can survive until normal blood flow is restored (Phipps and Cronin, 2020). In particular, the potentially salvageable cells in the ischemic penumbra, that maintain a low metabolic activity, receive the maximal benefit from the activation of an ischemic tolerant state (Liu *et al.*, 2009). It has been documented that the brain has the ability to activate self-protecting mechanisms in response to injurious events like cerebral ischemia, thus recruiting its defences and potentiating its resistance against a future damage (Riepe *et al.*, 1997; Huber *et al.*, 1999).

In general, a harmful stimulus applied to a tissue or organ below the threshold of damage can stimulate endogenous protective mechanisms (Dirnagl *et al.*, 2009).

The phenomenon known as 'ischemic tolerance' implies that a sub-lethal ischemic event applied to the brain or other organs, is able to activate cellular pathways that makes the organ more resistant against a subsequent, more intense ischemic event (Yunoki *et al.*, 2017).

Ischemic tolerance can be stimulated through the ischemic conditioning that consists in inducing brief periods of ischemia and reperfusion prior, during or after a more severe injurious ischemic event (figure 1.12).

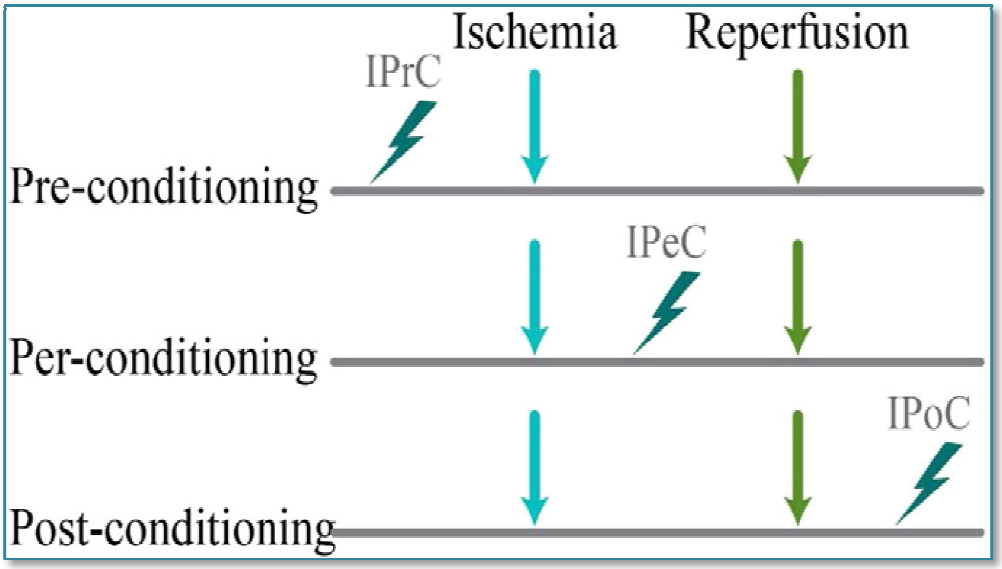

Figure 1.12 – Schematic representation of pre-, per- and post-conditioning (Wang *et al.*, 2015).

The investigation in animal models of the neuroprotective mechanisms activated by pre-, per- or post-conditioning is an helpful strategy to find new therapeutic targets that can be stimulated to prompt endogenous defences of the brain against ischemia, in order to prevent or repair neuronal damage (Wang *et al.*, 2015).

In addition to ischemic episodes, ischemic tolerance in the brain can be stimulated by several sub-lethal stressor stimuli, such as hypoxia, hypothermia, hyperthermia, seizures, exposure to anaesthetic drugs or proinflammatory substances (Garcia-Bonilla *et al.*, 2014; Sisalli *et al.*, 2015). Despite the different nature of these harmful stimuli, each of them can promote a cross-tolerance to another, thus their signalling pathways converge on common mechanisms that ultimately lead to a latent ischemia-tolerant phenotype (Bhuiyan and Kim, 2010). For example, the stimulation of the immune response through systemic administration of TLRs ligands, such as lipopolysaccharide (LPS), prior to focal cerebral ischemia significantly reduces cerebral damage in rodents (Rosenzweig *et al.*, 2004; Hua *et al.*, 2008; Stevens *et al.*, 2008; Marsh *et al.*, 2009; Leung *et al.*, 2012; Garcia-Bonilla *et al.*, 2018). TLRs stimulation induced a reprogramming of leukocytes that react to a subsequent insult by reducing the production of proinflammatory cytokines and potentiating their anti-inflammatory effects (Stevens *et al.*, 2014). Other examples of substances able to stimulate ischemic tolerance in the brain are inhalational anaesthetics. Many *in vivo* studies have demonstrated that animals exposed to different concentrations of isoflurane, prior to MCAo, show a marked reduction of cerebral infarct volume and neurological deficits (Zhu *et al.*, 2010; Li *et al.*, 2013; Sun *et al.*, 2015; Tong *et al.*, 2015). Mechanisms of actions of anaesthetics include the antagonisms of AMPA and NMDA receptors, the attenuation of apoptotic and inflammatory pathways, the activation of ATP-sensitive potassium channels and a dose-dependent increase of CBF (Wang *et al.*, 2016). Interestingly, anaesthetic preconditioning could be used during surgical procedures to prevent complications like perioperative stroke. Furthermore, it has been demonstrated that activation of Sirtuin 1 (SIRT1, a class of III NAD^+-dependent histone deacetylase) is involved in the delayed ischemic tolerance provided by ischemic preconditioning; thus a strong activator of SIRT1 like the polyphenol resveratrol could be used as neuroprotective agent for stroke therapy (Della-Morte *et al.*, 2009; Johnson *et al.*, 2011).

1.3.1 - Ischemic preconditioning

Brain ischemic preconditioning (PC) is a neuroprotective phenomenon used in experimental settings to induce tolerance through a sublethal ischemic stimulus able to increase the resistance of the brain against a subsequent more intense ischemic event (Yunoki *et al.*, 2017). The concept of ischemic PC was initially described in the rabbit heart by Murry et al in the 80's, which observed that a mild ischemic episode protects the organ against a subsequent lethal ischemia (Murry *et al.*, 1986); thereafter, the protective effects of ischemic PC were also demonstrated in the brain (Kitagawa *et al.*, 1990). Clinical data supporting this evidence have already shown a reduction in the negative effects of stroke and myocardial infarction in patients when they are preceded, respectively, by a recent transient ischemic attack (TIA) or angina episodes (Heusch and Gersh, 2017; Wang *et al.*, 2017). Moreover, patients who suffer of peripheral vascular disease, leading to chronic peripheral hypoperfusion, or sleep apnoea, that causes frequent episodes of nocturnal hypoxemia, may develop a natural ischemic tolerance (Koch *et al.*, 2007; Connolly *et al.*, 2013).

Ischemic PC consists in three phases: a priming phase in which the protective mechanisms are triggered, a refractive phase lasting 1-7 days, during which the organ is resistant to the ischemic injury, and a neuroprotective phase characterized by a reprogramming of the response to stroke that reduces injury (Stevens *et al.*, 2014). Thus, ischemic PC induces two temporal phases of ischemic tolerance, in which specific neuroprotective responses are stimulated in a defined range of time between the sublethal preconditioning stimulus and the lethal insult (Narayanan *et al.*, 2013) (figure 1.13). Early or rapid ischemic tolerance, mostly consisting in posttranslational protein modifications, occurs within minutes from exposure to a preconditioning stimulus and wanes in 1-2 hours; by contrast, delayed ischemic tolerance is a long-term response requiring genomic reprogramming and *de novo* protein synthesis, therefore it develops with a latency of about 1 day and may last for several days after the preconditioning. If the severe ischemic insult occurs between these time windows, from few hours until 24 hours after the preconditioning event, the brain does not result protected (Stetler *et al.*, 2014; Hao *et al.*, 2020). The preconditioning described by Murry et al. in the heart stimulates the rapid ischemic tolerance, while

delayed ischemic tolerance plays a major role in the brain and it has been shown to provide more robust and longer lasting neuroprotection (Narayanan *et al.*, 2013). Delayed ischemic tolerance is able to reprogram the transcriptional response to stroke and to stimulate the development of a neuroprotective state, by mitigating inflammatory and stress responses normally observed after stroke alone, thus a better understanding of the mechanisms elicited by ischemic PC may help to identify novel targets for stroke therapy (Dirnagl *et al.*, 2009; McDonough and Weinstein, 2018).

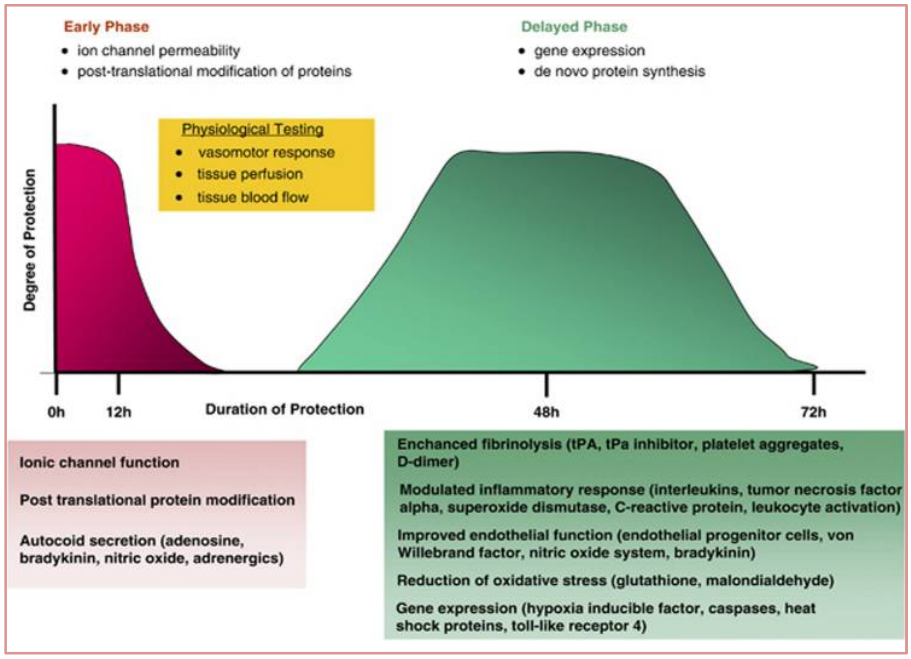

Figure 1.13 – Overview of early and delayed phase of ischemic tolerance elicited by ischemic PC (Koch *et al.*, 2014).

Currently, there are two methods to study the PC phenomenon: mechanical or pharmacological. Although the translation of mechanical ischemic PC in patients is difficult to achieve, the use of pharmacological agents able to activate critical pathways for cerebroprotection is clinically feasible (Wang *et al.*, 2015).

1.3.2 - Remote ischemic preconditioning

Considering the unpredictability of stroke onset and the high sensitivity of the brain to ischemic insults, most *in vivo* models of local ischemic PC, obtained by direct cerebral artery occlusion, have narrow safety margins and lack translational value (Meller and Simon, 2015). To overcome these problems, another form of ischemic PC, known as remote ischemic preconditioning (RIPC), has been developed. RIPC is a phenomenon that allows to obtain ischemic tolerance in a target organ, like the brain, by inducing a mild ischemic injury in other non-vital organs distant from the target (Yunoki *et al.*, 2017). In particular, RIPC is induced through repeated cycles of temporary block and restoration of blood flow in arms or thighs, able to induce the release of soluble mediators into the bloodstream that can reach the target organ and confer protection (Narayanan *et al.*, 2013). The efficacy of RIPC has been demonstrated in a rat model of focal cerebral ischemia, in which bilateral limb occlusion reduces ischemic brain damage (Hu, S. *et al.*, 2012). The neuroprotection conferred by RIPC is considered equivalent to that provided by local ischemic PC through brief focal ischemia (Sisalli *et al.*, 2015). Considering the relative simplicity, non-invasivity and safety of this technique, RIPC is an attractive strategy in the clinical setting for selected patients at high risk for cerebral ischemic events in the immediate future, such as patients with subarachnoid haemorrhage or subjected to invasive surgeries with cerebral ischemia as potential risk (McDonough and Weinstein, 2018).

Some clinical trials have already translated the RIPC in the clinical settings by using blood pressure cuffs to induce temporary reduction of blood flow and reperfusion in arms or thighs of patients (Kharbanda *et al.*, 2002; Keep *et al.*, 2014). Furthermore it has been reported that repetitive cycles of bilateral upper arm ischemic PC enhance brain perfusion and reduce the incidence of stroke in patients with intracranial arterial stenosis; the bilateral arm ischemic preconditioning (BAIPC) device patented by Xunming Ji used in this study, automatically performs regular cycles of ischemia/reperfusion (5 min of ischemia followed by 5 min of reperfusion) (Meng *et al.*, 2012). Thus RIPC is able to stimulate a robust neuroprotection that involves cellular, humoral and neural factors carried to the brain; the identifications of these

peripheral mediators may help in finding novel target for stroke therapy (Liu *et al.*, 2016).

1.3.3 - Ischemic per-conditioning

Ischemic per-conditioning (IPerC) is a neuroprotective phenomenon in which the tolerant state is obtained by the induction of brief periods of ischemia during the prolonged ischemic event (Wang *et al.*, 2015). The remote IPerC (RIPerC) is a particular type of IPerC that provides neuroprotection through the application of brief cycles of ischemia/reperfusion to a limb during an episode of severe ischemic stroke, and has the potential to protect the brain until reperfusion is achieved and from the subsequent reperfusion injury (Purroy *et al.*, 2020). Studies in animal models have demonstrated that RIPerC significantly reduces cerebral infarct in rats and mice after middle cerebral artery occlusion, as compared with the control group (Hahn *et al.*, 2011; Kitagawa *et al.*, 2018), and it was found to be effective alone or in combination with rT-PA administration in a mouse model of ischemic stroke (Hoda *et al.*, 2012). These results were confirmed by clinical evidence suggesting that RIPerC in patients provides immediate neuroprotective effects by reducing the cytotoxic oedema caused by reperfusion (Hougaard *et al.*, 2014). Thus, RIPerC is an interesting protective strategy that can be used in ischemic stroke patients, alone or in combination with other recanalization therapies (Purroy *et al.*, 2020).

1.3.4 - Ischemic post-conditioning

Ischemic post-conditioning (IPostC) in the brain is performed through cycles of brief ischemia induced ‘after’ an episode of ischemic stroke (Xie *et al.*, 2018). Based on the specific time window in which the cycles of ischemia are induced, IPostC can be classified in rapid or delayed. Rapid IPostC is performed from seconds to few minutes after reperfusion and it has been demonstrated to reduce infarct size in animal models (Zhao *et al.*, 2006; Pignataro *et al.*, 2008; Zhao, 2009). Delayed IPostC, instead, protects against focal ischemia if induced 3 to 6 hours after reperfusion onset (Ren *et al.*, 2008; Leconte *et al.*, 2009), and its therapeutic window

is extended until 2 days after global cerebral ischemia (Burda *et al.*, 2006). IPostC can be also induced remotely (RIPostC), to be translated in the clinical setting, by inducing cycles of limb ischemia after reperfusion. Its efficacy was first demonstrated in a study conducted by Ren et al., in which ipsilateral limb ischemia significantly reduced cerebral ischemic damage in rats (Ren *et al.*, 2009). The mechanisms underlying the neuroprotection evoked by IPostC include reduction of cerebral oedema, inflammation, oxidative stress and apoptosis, and improvement of cerebral blood flow (Xie *et al.*, 2018). Recently, the RECAST (Remote Ischemic Conditioning After Stroke Trial) 1 have assessed the efficacy of RIPostC in acute ischemic stroke patients demonstrating that it is safe, well tolerated and may improve neurological outcome in patients receiving four cycles of intermittent limb ischemia within 24 h from stroke onset (England *et al.*, 2017); furthermore, the safety and feasibility of RIPostC has been demonstrated also in stroke patients receiving intravenous thrombolysis with rt-PA (Che *et al.*, 2019).

1.3.5 - Neuronal mechanisms underlying cerebral ischemic tolerance

The robust delayed ischemic tolerance provided by ischemic PC requires the modulation of gene expression and *de novo* protein synthesis, leading to a reprogramming of the transcriptional response to stroke, which finally results in the development of an ischemia-tolerant phenotype in neurons (Hao *et al.*, 2020). There are marked differences between the pattern of genes expressed after stroke alone or stroke preceded by ischemic PC. Stroke induces a strong upregulation of genes that mediate stress and inflammatory pathways, increased metabolism and ion channel functions; by contrast, after ischemic PC a downregulation in the expression of these genes is observed (Sisalli *et al.*, 2015). PC-mediated neuroprotection in the brain acts through several biological mechanisms that involve not only neurons, but also vasculature, glial cells and the immune system (McDonough and Weinstein, 2018) (figure 1.14).

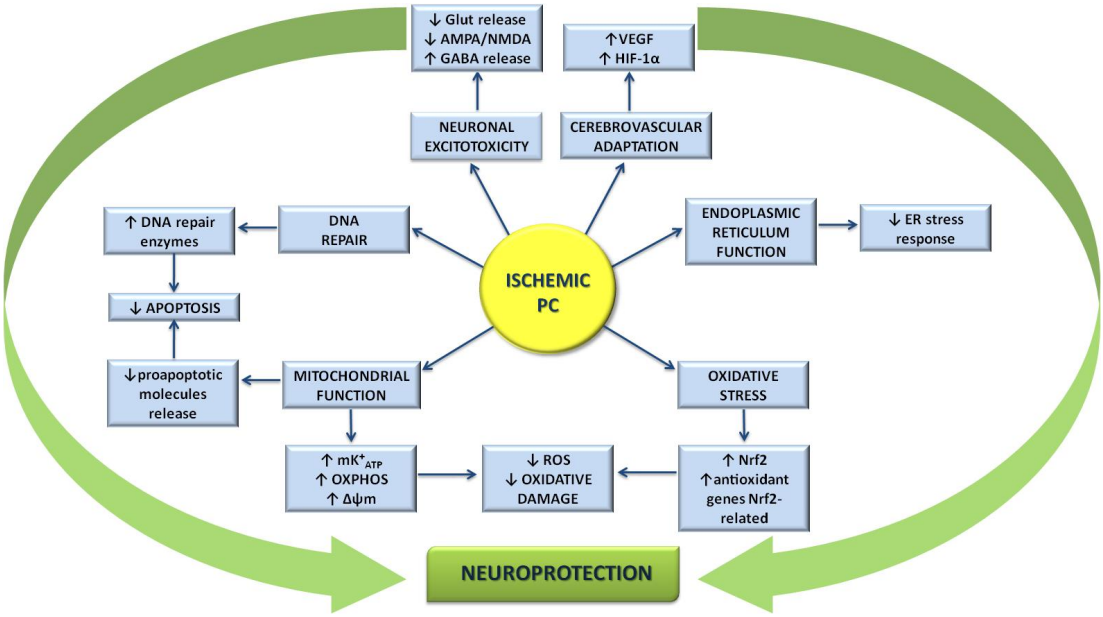

Figure 1.14 – Schematic representation of the main mechanisms leading to the establishment of cerebral ischemic tolerance after ischemic PC.

Mechanisms underlying PC-induced ischemic tolerance include:

- Modulation of neuronal excitotoxicity: ischemic PC mitigates excitotoxicity by reducing glutamate release, downregulating the expression of AMPA and NMDA receptors and potentiating the release and the activity of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Dave *et al.*, 2005; DeFazio *et al.*, 2009). Thus, ischemic PC makes neurons more resistant to the ischemic injury by mediating synaptic modifications that promote functional recovery after ischemic stroke (Narayanan *et al.*, 2013).

- Reduction of oxidative stress: ischemic PC reduces oxidative stress in the brain by mechanisms involving the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor regulating the redox state of the cells by inducing the expression of antioxidant enzymes. In particular, the preconditioning stimulus causes a mild oxidative stress in neurons through the generation of lipid electrophiles, followed by the activation of endogenous defences that involve the recruitment of Nrf2 and the following upregulation of antioxidant genes Nrf2-related (Yang *et al.*, 2020).

- DNA repair: ischemic PC promotes the activation of DNA repair and plasticity mechanisms, by enhancing the recruitment of DNA repairing enzymes and

neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Li *et al.*, 2006; Lehotský *et al.*, 2009).

- Modulation of mitochondrial functions: mitochondria are critically involved in PC-induced neuroprotection due to their role in energy production, Ca^{2+} homeostasis and apoptotic mechanisms (Wang *et al.*, 2015). Ischemic PC prevents the activation of mitochondria-dependent cell death pathways by preserving mitochondrial membrane potential, potentiating oxidative phosphorylation capacity, reducing the production of ROS from electron transport chain and the release of proapoptotic molecules (Liu *et al.*, 2002; Dirnagl and Meisel, 2008; Racay *et al.*, 2009; Correia *et al.*, 2010; Ding *et al.*, 2012). In particular, ischemic PC increases the expression of εPKC, which in turn stimulates the activation of mitochondrial ATP-sensitive K^+ channels (mK^+_{ATP}) that depolarize mitochondrial membrane potential and increase electron transport chain rate and ATP production (Wang *et al.*, 2015).
- Cerebrovascular adaptation: ischemic PC is able to increase microvessel formation and brain perfusion, by enhancing the activity of VEGF and hypoxia inducing factor (HIF)-1α (Lee *et al.*, 2017).
- Restoration of Ca^{2+} homeostasis: ischemic PC protects neurons from a severe ischemic insult by modulating the expression of proteins that play a role in maintaining the intracellular ionic homeostasis (i.e., Ca^{2+}-ATPase, acid sensing ion channels, Na^+/Ca^{2+} exchanger and Na^+/H^+ exchanger) (Cuomo *et al.*, 2015). In particular, two isoforms of the Na^+/Ca^{2+} exchanger, NCX1 and NCX3, have been identified as mediators of cerebral ischemic tolerance elicited by ischemic PC, since they are able to recover Na^+ and Ca^{2+} homeostasis, which is impaired by a severe episode of ischemia (Pignataro *et al.*, 2020). Indeed a recent study showed that the neuroprotection observed in preconditioned rats is linked to an upregulation in the expression of NCX1 and NCX3 in the preserved brain regions and, interestingly, the neuroprotective effect is reverted in animals lacking these transporters (Pignataro *et al.*, 2012). As observed *in vivo*, *in vitro* experiments performed in neurons exposed to OGD/reoxygenation confirmed that the neuroprotection conferred by preconditioning is related to an increase in NCX1 and NCX3 expression, and the treatment with siRNA directed against these transporters prevented this effect (Sisalli *et al.*, 2015). The involvement of genes regulating Ca^{2+} channels expression has been recently

confirmed through the analysis of transcript levels of a panel of genes encoding for several neuronal ion channels, which showed a downregulation in many Ca^{2+} channel genes under ischemic-tolerant state both in mice brains and in neuronal cultures (Hernandez-Encarnacion *et al.*, 2017).

Among the mechanisms underlying cerebral ischemic tolerance, previous studies have highlighted that ischemic PC reduces post-ischemic neuronal death by mitigating ER stress response (Hu, Y. Q. *et al.*, 2017); indeed, there are profound differences in the expression of ER stress genes in animals subjected to stroke alone or preceded by ischemic pre-treatment (Lehotský *et al.*, 2009; Pavlíková *et al.*, 2009; Urban *et al.*, 2009). Ca^{2+} influx mediated by SOCE, underlying the refilling of ER, is helpful in restoring Ca^{2+} homeostasis and could prevent neuronal damage caused by ER-stress mechanisms after cerebral ischemia (Sirabella *et al.*, 2009; Parekh, 2010; Lang *et al.*, 2018). In this regard, a recent study has shown that the stimulation of ER calcium refilling through SOCE is a critical mechanism by which PC induces ischemic tolerance in neurons; in particular, ischemic PC potentiates this mechanism by upregulating the expression of SOCE components, Orai1 and STIM1, thus reducing ER stress and mitigating the subsequent neuronal death (Secondo *et al.*, 2019). Nevertheless, further work is necessary to clarify the exact role of SOCE and its molecular components in the development of ischemic tolerance.

1.3.6 - Immune mechanisms involved in ischemic preconditioning

The innate immune system is critically involved in PC-mediated ischemic tolerance, as demonstrated by the evidence that activation of inflammatory pathways prior to ischemia, through administration of TLRs ligands, confers robust neuroprotection (Stevens *et al.*, 2014; McDonough and Weinstein, 2018). In particular, inflammation represents a critical component of delayed ischemic tolerance, considering that activation of transcription is the main mechanisms by which inflammatory responses are modulated. Ischemic stroke triggers a robust inflammatory response through the stimulation of TLRs and TNF receptors pathways, resulting in an enhanced production and release of proinflammatory molecules, such as TNF-α, IL-1β, iNOS

and COX2. Similar to stroke, the PC stimulus stimulate a mild inflammatory reaction via the NF-κB pathway, that is resolved during the early phase of ischemic tolerance, but simultaneously promotes a genomic reprogramming of innate immune system that will be able to react differently to a future episode of severe ischemia, by reducing the release of proinflammatory mediators and promoting the expression of anti-inflammatory cytokines such as IL-10, TGF-β and IFN (figure 1.15) (Garcia-Bonilla *et al.*, 2014). The mechanisms by which ischemic PC reduces the inflammatory response to stroke include the inhibition of TLR4/NF-κB and TLR4/myeloid differentiation factor 88 (MyD88) pathways and the potentiation of interferon regulatory factor (IRF)-dependent signalling (Pradillo *et al.*, 2009; Stevens *et al.*, 2011; Wang, P. F. *et al.*, 2015).

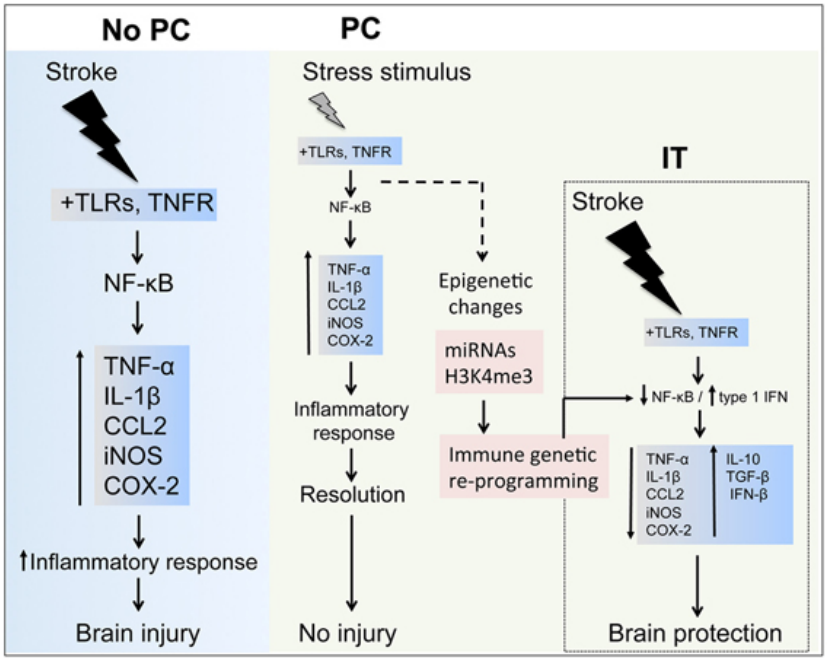

Figure 1.15 – Immune and inflammatory mechanisms in cerebral ischemic tolerance (Garcia-Bonilla *et al.*, 2014).

Cellular mediators of ischemic PC in the brain are resident microglia that, after exposure to a preconditioning stimulus, show a marked expression of type I interferon stimulated genes, required for the induction of ischemic tolerance (McDonough *et al.*, 2017). Moreover, an increased number of activated microglia is

observed in the preconditioned hemisphere due to CX3CR1-dependent proliferation, exerting beneficial functions (McDonough *et al.*, 2020). Also astrocytes, through the TLR3 signaling pathway, contribute to IPC-evoked ischemic tolerance by enhancing IFN secretion and reducing the release of IL-6 (Pan *et al.*, 2014). The role of peripheral immune cells in PC-mechanisms is not completely clarified, but a recent study has demonstrated that LPS-preconditioning in mice stimulates the release of splenic Ly6C^{hi} monocytes, that show upregulation of typical markers of alternative polarization such as IL-10 and arginase-1. These protective monocytes reach the ischemic brain where they suppress the production of proinflammatory cytokines and reduce neutrophil extravasation (Garcia-Bonilla *et al.*, 2018).

These findings strongly highlight that the immune system is critically involved in the ischemic tolerance elicited by different preconditioning stimuli, acting simultaneously as target and mediator of neuroprotection, and a deeper comprehension of this inflammatory pathways may help in the identification of novel therapeutic strategies for stroke patients (McDonough and Weinstein, 2018).

Chapter 2 - Aims of the study

Despite many advances have been made in stroke research, there is still a lack of effective strategies that can be used to prevent or limit the progression of ischemic damage. The main purpose of currently approved therapies for acute ischemic stroke is to restore blood flow, as fast as possible, in order to protect the brain from damage caused by the reduction in oxygen and nutrients supply. In addition, a major aim of acute interventions would be to attenuate all the factors responsible for the exacerbation of ischemic damage and/or to promote reparative mechanisms, thus preventing neurological deterioration of patients and promoting their functional recovery (Neuhaus *et al.*, 2017).

Currently, the acute therapy for stroke treatment is limited to blood flow restoration, through mechanical recanalization and pharmacological thrombolysis with rt-PA, or the combination of both, in eligible patients (Zerna *et al.*, 2018). The introduction of these treatments in clinical practice has revolutionised the management of stroke by significantly reducing the associated disabilities, however they can be used only in a small percentage of eligible patients, due to several contraindications, and, more importantly, they lack neuroprotective effects (Xiong *et al.*, 2018; Campbell, 2019). Therefore, considering the high incidence of ischemic stroke, the unpredictability of its onset and its severe consequences, it is extremely important to find new targets for the development of effective neuroprotective strategies. In the last decades, several neuroprotective strategies have been tested in animal models of stroke demonstrating promising results, but unfortunately almost all have failed to be translated into the clinical setting, due to lack of efficacy or toxicity in humans (Neuhaus *et al.*, 2017; Rajah and Ding, 2017).

With the aim to reduce further clinical failures, recent studies have highlighted the potential of the stimulation of endogenous neuroprotection, namely the ability of the brain to develop tolerance in response to an injurious stimulus like cerebral ischemia. In this context, brain ischemic preconditioning is used as preclinical model to induce tolerance, by a sublethal ischemic event able to increase the resistance of the brain against a subsequent, more intense ischemic insult (Yunoki *et al.*, 2017).

A deep comprehension of the mechanisms underlying cerebral ischemic tolerance elicited by ischemic PC is a promising approach to identify novel targets for stroke therapy.

The PC paradigm used in the present study is based on the establishment of delayed ischemic tolerance that has been shown to elicit a robust and long lasting neuroprotection in the brain. In particular, delayed ischemic tolerance is a long-term response, developing with a latency period of about one day, requiring genomic reprogramming and *de novo* protein synthesis, that mitigate post-ischemic inflammatory and stress responses normally elicited by an episode of severe stroke, leading to the establishment of an ischemic-tolerant state (Sisalli *et al.*, 2015; Hao *et al.*, 2020). Although the mechanisms underlying the neuroprotection evoked by ischemic PC have not been fully clarified, it is known that both neuronal and immune pathways participate in the establishment of ischemic tolerance (Garcia-Bonilla *et al.*, 2014).

A prominent contributor of neuronal ischemic damage is the alteration of ER functions, caused by Ca^{2+} stores depletion, while the neuroprotective state that characterizes ischemic tolerance is associated with limitation of ER stress (Paschen and Mengesdorf, 2005; Lehotský *et al.*, 2009). During cerebral ischemia, the reduction of Ca^{2+} concentration in the ER lumen triggers SOCE in order to re-establish Ca^{2+} homeostasis (Lopez *et al.*, 2016). Molecularly, SOCE is mediated by the interaction between the ER Ca^{2+} sensor, STIM1, and the Ca^{2+} selective PM channel Orai1, and is regulated by the negative regulatory factor SARAF that prevents Ca^{2+} overload and maintains proper intracellular Ca^{2+} levels (Palty *et al.*, 2012; Rosado *et al.*, 2016). Ca^{2+} influx mediated by SOCE is helpful in restoring Ca^{2+} homeostasis and could prevent neuronal damage caused by ER-stress mechanisms after cerebral ischemia (Sirabella *et al.*, 2009; Parekh, 2010; Lang *et al.*, 2018). Conversely, it may also be the source of Ca^{2+} overload, thus triggering non-excitotoxic neuronal death (Zhang *et al.*, 2014).

Therefore, the role played by SOCE in neurons during cerebral ischemia is still controversial. Previous studies have also suggested that Ca^{2+} refilling in the ER, mediated by Orai1-STIM1 interaction, is an important neuroprotective mechanism

involved in ischemic tolerance provided by PC (Lehotský *et al.*, 2009; Secondo *et al.*, 2019). Thus, this project has the purpose to provide a deeper comprehension of the role played by SOCE components, Orai1, STIM1 and SARAF, in the development of ischemic brain damage and in the neuroprotection exerted by ischemic PC.

Considering that delayed ischemic tolerance requires a modulation of the transcriptional response to stroke and that activation of transcription is the main mechanism whereby immune cell functions are regulated (Garcia-Bonilla *et al.*, 2014), another important aim of the study is the investigation of the role played by the central and peripheral innate immune system in the establishment of PC-mediated ischemic tolerance. Indeed, the immune system is critically involved in the endogenous neuroprotective mechanisms elicited in the brain after preconditioning, as demonstrated by the evidence that activation of inflammatory pathways prior to ischemia, for example through administration of TLRs ligands or local/remote ischemic insults, confers robust neuroprotection (Stevens *et al.*, 2014; McDonough and Weinstein, 2018). As it occurs during an episode of ischemic stroke, the PC stimulus stimulates an inflammatory reaction, lower in magnitude, that is resolved during the early phase of ischemic tolerance, and simultaneously promotes a genomic reprogramming of the innate immune system, that will react differently to a future episode of severe ischemia, thus contributing to the resolution of inflammation (Garcia-Bonilla *et al.*, 2014). Recently, it has also been shown that peripheral immune cells contribute to the endogenous neuroprotection stimulated by the exposure to a sublethal harmful stimulus prior to a severe episode of stroke (Gesuete *et al.*, 2016).

Beside the important role played by SOCE in neuronal survival mechanisms, it has been demonstrated that this Ca^{2+} current represents the main source of Ca^{2+} in immune cells, where it regulates several functions such as differentiation, maturation, proliferation, chemotaxis, secretion of cytokines and ROS, and phagocytosis (Feske *et al.*, 2015; Demaurex and Nunes, 2016; Vaeth *et al.*, 2017). Although several studies have assessed the critical role played by SOCE components in innate

immunity, their potential involvement in the modulation of immune cell phenotypes during ischemic stroke and tolerance has not been previously investigated. Therefore, we have hypothesized that calcium signals generated by Orai1-STIM1 interaction may underlie the reprogramming and polarization of innate immune cells towards a beneficial state during ischemic PC, thus supporting the acquired ischemic tolerance.

Hence, this research project is focused on the validation of the experimental method of brain ischemic PC in a mouse model of transient focal cerebral ischemia induced by MCAo, by evaluating the effects of a sublethal ischemic insult, namely the PC stimulus, on cerebral ischemic damage and neurological deficits produced in mice by a more intense ischemic event, occurring after 3 days of reperfusion.

An important purpose of this study is the characterization of the expression of SOCE components, Orai1, STIM1 and SARAF, in mice brains after ischemia and ischemic PC. To this aim, we performed protein analysis to assess their cortical levels and colocalization studies to identify the specific cell type expressing these SOCE components in the brain.

By using this animal model, the project also aims at assessing whether modulation of central and peripheral innate immune system contributes to neuroprotection evoked by ischemic PC. The post-ischemic immune response was characterized in mouse brain, by assessing cortical levels of cytokines and specific immune markers, and in the periphery, through the evaluation of modifications in spleen weight and in leukocyte count after ischemia preceded or not by the preconditioning treatment.

Finally, to understand if a modulation in the expression of SOCE components occurs in immune cells during ischemia and ischemic PC, we also evaluated their expression in circulating leukocytes in mouse blood samples.

Chapter 3 - Materials and methods

3.1 - Animals

All the experiments of this research project were performed on adult C57Bl/6J male mice (10-20 weeks-old) weighing 26-28 g (Charles River Laboratories, Como, Italy), housed under controlled environmental conditions with ambient temperature of 22°C, relative humidity of 65%, 12 h light:12 h dark cycle, and free access to food and water.

Animal care and experimental procedures were carried out respecting the guidelines of the Italian Ministry of Health (DL 26/2014), in accordance with the European Directive 2010/63/UE. The protocol n. 975/2017 was approved by the Committee set by the Ministry of Health at the National Institute of Health (Rome).

All efforts were made to reduce the number of animals used and their suffering.

3.2 - Middle cerebral artery occlusion

Transient focal brain ischemia was induced in mice by MCAo, using the relatively non-invasive technique of the intraluminal filament (Longa *et al.*, 1989). This technique involves the use of a silicone-coated nylon filament (diameter: 0.23 mm, Doccol Corporation, Redlands, CA, USA), which adheres to the blood vessel of the Willis circle, in correspondence to the origin of the MCA, thus blocking blood flow.

Mice were anaesthetised with 5% isoflurane in air for the induction phase and were maintained with the lowest acceptable concentration of the anaesthetic (1.5-2%) during the surgical procedure. Body temperature was monitored with a rectal probe and maintained at 36.5-37.5°C with a heating pad.

Under a stereo dissecting microscope, a midline incision of about 1 cm was made on the neck, to identify and isolate the right common carotid artery (CCA) and its bifurcation into external carotid artery (ECA) and internal carotid artery (ICA), carefully separating them from surrounding vessels and nerves. The ECA was tied

with 6.0 silk suture and dissected to create a stump; the CCA and the ICA were temporary occluded with vascular clips.

A small incision was made on the ECA stump with Vannas scissors, about 1.5 mm from the bifurcation. Through this incision the nylon filament was inserted and then, after removing the vascular clip, gently advanced into the ICA for about 9-11 mm beyond the bifurcation, up to the origin of the MCA in the circle of Willis, where a mild resistance was felt. The filament was fixed in this position by a knot with silk suture to avoid displacements and subsequent bleeding (figure 3.1 A-B). After 60 min of MCAo, mice were anaesthetised and the filament was withdrawn to allow reperfusion.

Sham operated animals underwent to the same surgical procedure, except for filament insertion into the MCA.

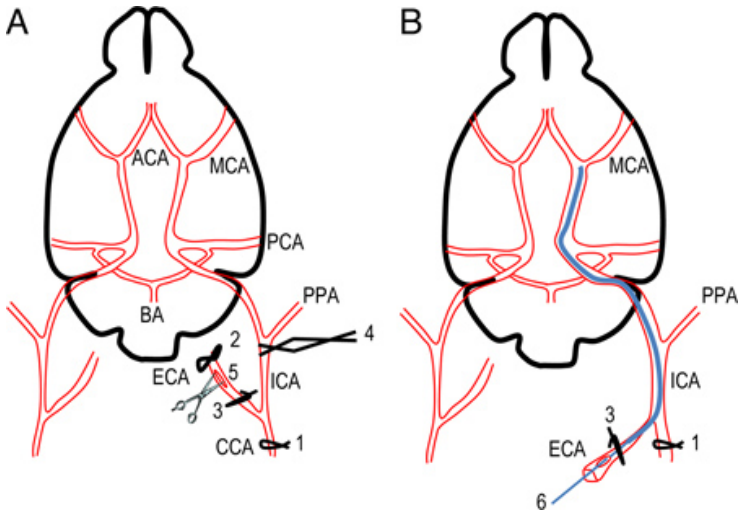

Figure 3.1 – Schematic diagram of the surgical procedure for MCAo by the intraluminal filament technique. A) Schematic representation of mouse principal cerebral arteries and surgical procedure to create the access for filament insertion. B) Introduction of the filament (blue) into the ICA up to the origin of the MCA in the Circle of Willis (Rousselet *et al.*, 2012).

During surgery, CBF was continuously monitored by Laser-Doppler flowmetry (Periflux System 5000, Perimed, Sweden). To this aim, a flexible fibre-optic probe was glued onto the parietal bone of the ischemic hemisphere, corresponding to the

supply region of the MCA. Successful occlusion was confirmed by a significant (>70%) reduction of cerebral blood flow (Amantea *et al.*, 2016) (figure 3.2).

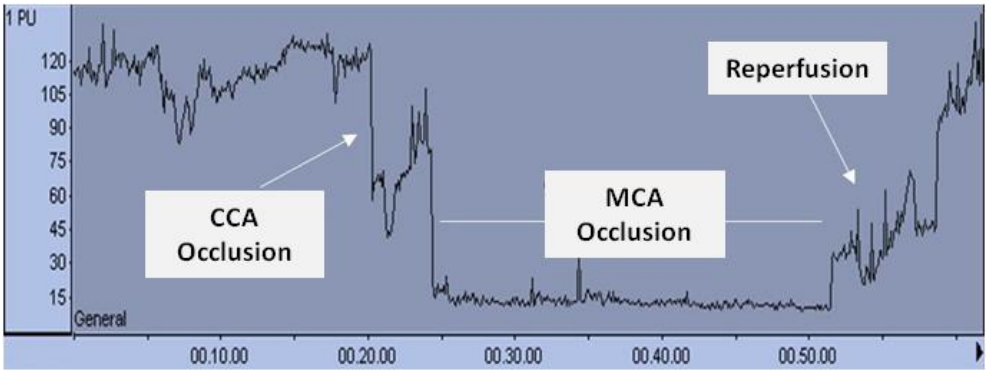

Figure 3.2 – Representative Laser-Doppler flowmetry trace of the CBF during MCAo in mice.

After the surgical procedure, mice were subcutaneously injected with 500 µl of saline solution and transferred into their cages in a warm environment, in order to facilitate recovery after the drop of body temperature caused by the anaesthetic, with free access to food and water.

3.3 - Brain ischemic preconditioning

The experimental procedure for the induction of brain ischemic PC used in this research project is based on a previously established paradigm, that has already been demonstrated to induce robust neuroprotection by eliciting delayed ischemic tolerance (Stenzel-Poore *et al.*, 2003; Zhang *et al.*, 2008; Lusardi *et al.*, 2010; McDonough *et al.*, 2020).

The preconditioning stimulus consists in a brief sublethal MCAo of 15 min, followed by 72 h of recovery and reperfusion, corresponding to the peak of PC-mediated neuroprotection. Then, mice were subjected to the more intense ischemic insult of 1 h, followed by 24 h of reperfusion (figure 3.3).

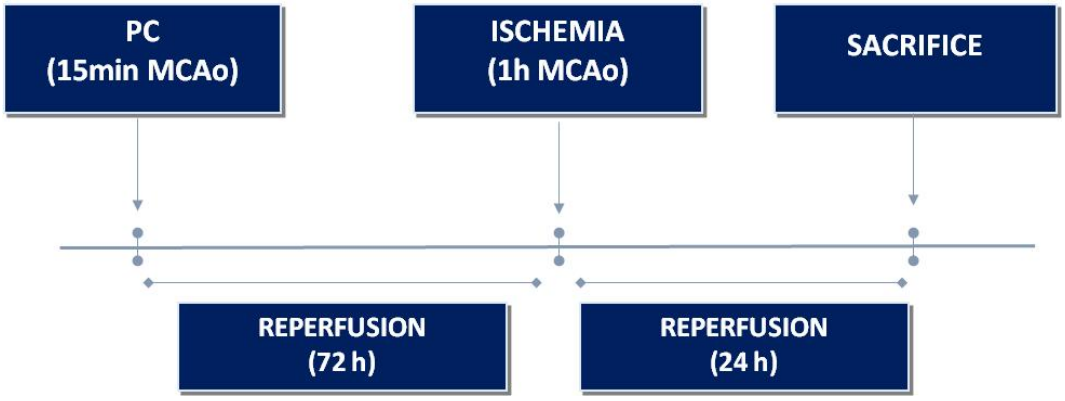

Figure 3.3 – Experimental protocol time-line for brain ischemic PC.

Sham surgery was performed to obtain control conditions for both PC (sham) and MCAo (SHAM) groups.

Mice were randomly allocated to the following experimental groups:

1) **SHAM**: sham surgery followed by 24 h of reperfusion;
2) **MCAo**: 1 h MCAo followed by 24 h of reperfusion;
3) **Sham + MCAo**: sham surgery followed, 72 h later, by 1 h MCAo and 24 h of reperfusion;
4) **PC**: 15 min MCAo followed by 72 h of reperfusion;
5) **PC + MCAo**: 15 min MCAo followed, 72 h later, by 1 h MCAo and 24 h of reperfusion.

3.4 - Quantification of ischemic brain damage

Cerebral infarct damage produced in mice by 1 h MCAo alone or preceded by ischemic PC, was assessed after 24 h of reperfusion. Animals were sacrificed and their brains were rapidly dissected and frozen. Each brain was then sectioned using a cryostat into 15 coronal slices (20 µm-thick) at 0.5 mm intervals from the frontal pole, and mounted on Superfrost-plus slides.

The sections were stained with cresyl violet, a basic dye that binds Nissl bodies only detectable in the cytoplasm of viable neurons. Nissl bodies were stained purple-blue, while the ischemic area was identified for the absence of staining (figure 3.4).

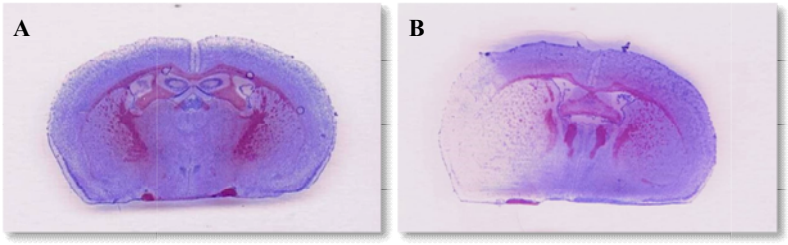

Figure 3.4 – Coronal brain slices stained with cresyl violet. Representative coronal brain slices of mice subjected to sham surgery (A) and 1h MCAo followed by 24h of reperfusion (B).

Nissl staining protocol included the following procedures to fix, rehydrate and stain tissue slices:

1) 15 min in absolute ethanol at -80°C, to fix the tissue;
2) 1 min in 100% ethanol at room temperature;
3) 1 min in 90% ethanol;
4) 1 min in 70% ethanol;
5) 1 min in 50% ethanol;
6) 1 min in distilled water;
7) 12 min in cresyl violet (Bio-Optica, Milan, Italy);
8) Quick rinse in distilled water to remove excess dye.

Stained sections were then scanned and blindly analysed using the image analysis software ImageJ (version 1.30). Infarct volume (mm^3) was determined by summing the ischemic areas of the coronal slices and multiplying the obtained value by the interval-thickness between sections (0.5 mm).

Moreover, to evaluate the role of the peripheral immune system, after the sacrifice, the spleen was removed and immediately weighted.

3.5 - Assessment of neurological deficits

Evaluation of the neurological state of mice subjected to 1 h MCAo, preceded or not by PC, was blindly performed after 24 h of reperfusion, using a modified scoring

system (Longa *et al.*, 1989), by which a score from 0 to 5 is assigned based on movement deficits (table 3.1).

SCORE	FUNCTIONAL DEFICIT
0	No deficit
1	Failure to fully extend the contralateral forepaw when held by the tail
2	Reduced resistance to a lateral push
3	Spontaneous circling toward the contralateral side
4	Falling to contralateral side
5	No spontaneous movements with depressed level of consciousness

Table 3.1 – Grading scale of functional deficits of mice subjected to MCAo.

To provide a deeper neurobehavioural analysis, we evaluated general and focal deficits produced by transient MCAo preceded or not by the preconditioning stimulus, by using the De Simoni Composite Neuroscore (Orsini *et al.*, 2012a; Llovera *et al.*, 2015). This composite neuroscore comprises 13 categories, divided into general and focal deficits, and scores range from 0 (healthy mouse) to 56 (the worst performance in each category).

GENERAL DEFICITS	
I. HAIR	0- Hair neat and clean. 1- Localized piloerection and dirty hair in 2 body parts (typically nose and eyes). 2- Piloerection and dirty hair in more than 2 body parts.
II. EARS	0- Normal. Ears are stretched laterally and behind, they react to noise. 1- Ears are stretched laterally but not behind (one or both), they react to noise. 2- Same as 1, but ears do not react to noise.
III. EYES	0- Open, clean and quickly following the surrounding environment. 1- Open and characterized by aqueous mucus. Slowly follow the surrounding environment. 2- Open and characterized by dark mucus. 3- Ellipsoidal shaped and characterized by dark mucus. 4- Closed.

IV. POSTURE	*Mouse on the palm and swing gently.* 0- The mouse stands in the upright position with the back parallel to the palm. 1- The mouse stands humpbacked. During the swing it flattens the body to gain stability. 2- The head or part of the trunk lies on the palm. 3- The mouse lies on one side, barely able to recover the upright position. 4- The mouse lies in a prone position, not able to recover the upright position.
V. SPONTANEOUS ACTIVITY	0- The mouse is alert and explores actively. 1- The mouse seems alert, but it is calm and sluggish. 2- The mouse explores intermittently and sluggishly. 3- The mouse is somnolent and numb, few movements on-the-spot. 4- No spontaneous movements.
VI. EPILEPTIC BEHAVIOR	0- None. 3- The mouse is reluctant to handling and shows hyperactivity. 6- The mouse is aggressive, stressed and stares. 9- The mouse shows hyperexcitability, chaotic movements and presence of convulsion following handling. 12- Generalized seizures associated with wheezing and unconsciousness.
	FOCAL DEFICITS
VII. BODY SIMMETRY	*Description of the virtual nose-tail line.* 0- Normal. a) Body: normal posture, trunk elevated, with fore and hindlimbs leaning beneath the body. b) Tail: straight. 1- Slight asymmetry. a) Body: leans on one side with fore and hindlimbs leaning beneath the body. b) Tail: slightly bent. 2- Moderate asymmetry. a) Body: leans on one side with fore and hindlimbs stretched out. b) Tail: slightly bent. 3- Prominent asymmetry. a) Body: bent, on one side lies on the bench. b) Tail: bent. 4- Extreme asymmetry. a) Body: highly bent, on one side constantly lies on the bench. b) Tail: highly bent.
VIII. GAIT	0- Normal. Gait is flexible, symmetric and quick. 1- Stiff, inflexible. The mouse walks humpbacked, slower than normal mice. 2- Limping with asymmetric movements. 3- Trembling, drifting, falling.

	4- The mouse does not walk spontaneously; when stimulated it walks no longer than three steps.
IX. CLIMBING	*Mouse on a gripping surface 45° to bench.* 0- Normal, the mouse climbs quickly. 1- Climbs with strain, limb weakness present. 2- Holds onto slope, does not slip or climb. 3- Slides down slope, unsuccessful effort to prevent fail. 4- Slides immediately, no effort to prevent fail.
X. CIRCLING BEHAVIOR	0- Absent; the mouse equally turns left or right. 1- Predominantly one-side turns. 2- Circles to one side, although not constantly. 3- Circles constantly to one side. 4- Pivoting, swaying, or no movement.
XI. FORELIMB SYMMETRY	*Mouse suspended by the tail.* 0- Normal. Both forelimbs are extended towards the bench and move actively. 1- Light asymmetry. Contralateral forelimb does not extend entirely. 2- Marked asymmetry. Contralateral forelimb bends towards the trunk. The body slightly bends on the ipsilateral side. 3- Prominent asymmetry. Contralateral forelimb adheres to the trunk. 4- Slight asymmetry, no body/limb movement.
XII. COMPULSORY CIRCLING	0- Absent. Normal extension of both forelimbs. 1- Tendency to turn to one side. The mouse extends both forelimbs but starts to turn preferably to one side. 2- Circles to one side. The mouse turns towards one side with a slower movement compared to healthy mice. 3- Pivots to one side sluggishly. The mouse turns towards one side failing to perform a complete circle. 4- Does not advance. The front part of the trunk lies on the bench.
XIII. WHISKER RESPONSE	*Touch gently the whiskers and the tip of the ears.* 0- Normal symmetrical response. The mouse turns the head towards the stimulated side. 1- Light asymmetry. a) The mouse withdraws slowly when stimulated on the ischemic side. b) Normal response on the contralateral side. 2- Prominent asymmetry. a) No response on the ischemic side. b) Normal response on the contralateral side. 3- Absent response ipsilaterally, slow response when stimulated on the contralateral side. 4- Absent response bilaterally.

Table 3.2 – De Simoni composite neuroscore. General and focal deficits scores for the assessment of functional outcomes in mice subjected to 1h MCAo.

3.6 - Enzyme-linked immunosorbent assay (ELISA)

Quantitative detection of cytokines IL-1β and IL-10 in mice brains was performed using ELISA kits pre-coated with monoclonal antibodies (IL-1β mouse ELISA kit, BMS6002, Invitrogen; mouse IL-10 ELISA kit PicoKine^{TM}, MBS175883, MyBioSource) according to the manufacturer's guidelines.

To this aim, mice were sacrificed and the ipsilateral (ischemic) and contralateral frontoparietal cortices (3.2 to -3.8 mm from Bregma) (Paxinos and Franklin, 2012) were rapidly dissected from the brains.

The samples of brain cortices were homogenized in ice-cold RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy) and centrifuged for 20 min at 20817 *g* at 4°C, to separate the soluble phase, containing proteins, from the other cellular components in the pellet. Supernatants were collected and protein concentration was quantified with the Bradford method (Bradford-Solution, PanReacAppliChem, ITW Reagents), by using serial (0 – 1 mg/ml) dilutions of a bovine serum albumin (BSA) solution as standard.

For each sample, 10 µL of lysate, diluted with sample buffer, were added to microwells and incubated for 2 h at room temperature, with proper biotinylated antibodies. The unbound antibodies were removed through three washing steps and the plate was incubated with streptavidin or avidin-biotin peroxidase complex (HRP), which binds with high affinity biotin-conjugated IL-1β and IL-10 antibodies adsorbed onto microwells. After washing, a solution of tetramethyl-benzidine (TMB) was added to the samples, that reacts with HRP to produce a blue coloured product in proportion to the amount of biotinylated antibodies present in the samples. After a further incubation of 10-30 min in the dark, the reaction was stopped by adding an acidic solution and the absorbance of each microwell was immediately read on a spectro-photometer at 450 nm.

Sample concentrations of IL-1β and IL-10 were determined by interpolating optical density values with standard curves created with serial dilution of mouse IL-1β and IL-10 standards, respectively. The obtained concentration was multiplied by the dilution factor and normalized with protein concentration values of each sample.

3.7 - Western blot analysis

In order to perform western blot analysis on brain tissue, mice were sacrificed and the ipsilateral (ischemic) and contralateral frontoparietal cortices (3.2 to -3.8 mm from Bregma) (Paxinos and Franklin, 2012) were rapidly dissected from the brains. The samples of cerebral cortices were processed as previously described for ELISA analysis, to obtain the lysates. Protein concentration in lysates was quantified with Bradford method, using BSA as standard.

For each sample, 40 μg of proteins were heated for 5 min at 95°C in Laemmli buffer (Sigma-Aldrich, Milan, Italy), to allow protein denaturation, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad Mini Pro-tean III and, then, electroblotted onto nitrocellulose membrane (NitroBind, Maine Manufacturing, Maine, USA) using a mini trans-blot (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with a blocking solution containing 5% non-fat milk in 0.05% Tween-20 Tris-buffered saline (TBS-T), for 1 h at room temperature, to reduce non specific binding and background.

For immunodetection, the blots were incubated overnight at 4°C with the specific primary antibodies. In particular, the expression of a specific marker of alternatively activated microglia/macrophages was assessed using the primary antibody rabbit anti-Ym1 (1:1000; 60130, StemCell Technologies). Moreover, modifications in cortical expression of SOCE components induced by ischemia and ischemic PC were evaluated with the following primary antibodies: rabbit anti-Orai1 (1:1000; ACC-062, Alomone Lab, Israel), rabbit anti-STIM1 (1:1000; ACC-063, Alomone Lab, Israel), rabbit anti-SARAF (1:1000; ACC-067, Alomone Lab, Israel).

The amount of proteins was normalized using β-actin (mouse anti-β-actin, 1:1000; sc-69879, Santa Cruz Biotechnology, Inc.) as internal control.

Then, the membranes were incubated with the proper species-specific peroxidase-linked secondary antibody (anti-rabbit 1:2000; Santa Cruz Biotechnology, Inc.; anti-mouse 1:2000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature.

Immunodetection of protein bands was performed with enhanced chemiluminescence kit (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc.) by exposing the membranes to X-ray films (Ultracruz Autoradiography Film, Santa Cruz

Biotechnology, Inc.). After development, the films were scanned and the densitometric analysis of the bands was performed using ImageJ software.

3.8 - Immunohistochemistry

For immunohistochemical analysis, mice were anaesthetized and perfused through the heart with a fixative. To this aim, an incision was made under the sternum; the skin was cut to expose the chest cavity that was opened on both lateral edges to allow the retraction of the sternum with clump scissors. Then, the diaphragm was carefully cut to expose the heart. A needle was inserted into the left ventricle up to the aorta and clumped in this position; the right atrium was immediately cut to allow blood to flow from the heart. By using a peristaltic pump, mice were transcardially perfused with 20-25 ml of heparinized (4000 U/ml) saline (0.9% NaCl), to remove the blood from the tissues (clearing of the liver is an indicator of a good perfusion), followed by approximately 20 ml of ice-cold 4% paraformaldehyde (PF) in phosphate buffered saline (PBS, 0.1 M; pH 7.4) to fix brain tissue.

The brains were rapidly dissected, post-fixed for 2 h in PF, cryoprotected in sucrose (30% in PBS) at 4°C for about 2 days and then frozen.

Using a cryostat, coronal brain slices (40 μm-thick) were sectioned at the level of the regions perfused by the middle cerebral artery (1.18 to -0.10 mm from Bregma) (Paxinos and Franklin, 2012).

To label neurons, myeloid cells and SOCE components, brain slices were incubated for 30 min at room temperature in blocking solution (5% normal donkey serum, 0.5% BSA, 0.3% Triton X-100 in PBS), and, then, overnight at 4°C with the following primary antibodies in PBS/0.3% Triton X-100/0.5% BSA: mouse anti-NeuN (anti-neuronal nuclei; 1:200; MAB377, Chemicon International, Temecula, CA) to label neurons, rat anti mouse Ly-6B.2 (clone 7/4; 1:200; MCA771GA, Bio-Rad Laboratories, UK) to label neutrophils and monocytes/macrophages (Rosas *et al.*, 2010), rat anti-Ly-6G (1:200; clone 1A8; BD Pharmin-gen, Italy) for neutrophils, rat anti-CD11b (1:200, MCA74GA, AbDSerotec) for microglia/macrophages, rabbit anti-Ym1 (1:200; 60130, StemCell Technologies) directed against alternatively activated microglia/macrophages, rabbit anti-Orai1 (1:100; ab59330, Abcam, UK),

rabbit anti-STIM1 (1:75; ACC-063, Alomone Lab, Israel), rabbit anti-SARAF (1:50; ACC-067, Alomone Lab, Israel).

After 3 washes in PBS, the sections were incubated for 2 h at room temperature with proper secondary antibodies conjugated with AlexaFluor488 or AlexaFluor568 (1:200; Invitrogen, Thermo Fisher Scientific, Italy). Then, nuclei were labelled by a 15-min exposure to 4',6-diamidino-2-phenylindole (DAPI, 1:500; Sigma-Aldrich, Milan, Italy). The sections were mounted on Superfrost-plus slides and coverslipped with aqueous mounting medium.

The immunostaining was examined with a confocal laser scanning microscope (Fluoview FV300, Olympus), equipped with a dedicated software module (cellSens) for image analysis.

3.9 - Flow cytometry

To perform flow cytometry analysis, mice were anaesthetised and blood samples were collected by heart puncture in tubes containing K_3EDTA as anticoagulant. The samples were gently inverted to ensure proper mixing with the anticoagulant and stored at 4°C before processing.

Blood samples were centrifuged at 300 *g* for 10 min to separate the pellet, containing leukocytes, from the plasma. The pellet was washed in PBS/0.5% BSA and centrifuged at 300 *g* for 5 min. To perform erythrocytes lysis, the pellet was resuspended in BD Pharm Lyse™ (BD Bioscience) for 4-5 min at room temperature on shaking, and then centrifuged at 300 *g* for 5 min; the supernatants were discarded, the pellets washed in PBS/0.5% BSA and eventually resuspended in lysis buffer until a clear supernatant was indicative of complete erythrocytes lysis.

After the last wash in PBS/0.5% BSA, the pellets were resuspended in 100 µl of PBS/1%BSA and incubated with anti-mouse CD16/32 antibody (1:50, TruStainFcX™, Biolegend, San Diego, CA, USA) for 5-10 min on ice, to block non-specific binding to the Fc receptor.

To characterize the expression of specific cell surface markers, leukocyte samples were then incubated for 45 min on ice in the dark with the following primary antibodies: ATTO-488-labelled rabbit anti-Orai1 (extracellular) (1:25; ACC-062-

AG, Alomone Lab, Israel) and/or PE/Dazzle594-labelled rat anti-Ly-6G (1.5:100; 127648, Biolegend, San Diego, CA, USA). After the incubation, the samples were washed twice, resuspended in 500 µl of PBS/0.5% BSA and analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

3.10 - Statistical analysis

Data were analysed using Graph-Pad Prism software for Windows (version 6.0, GraphPad Software, San Diego, CA). Normally distributed data are expressed as mean ± standard error of the mean (SEM), while non-normally distributed data are reported as median with interquartile range (IQR).

Comparisons between experimental groups were performed using one-way ANOVA followed by Tukey post-test or two-way repeated measures ANOVA followed by Bonferroni multiple comparisons post-test for normally distributed data, or the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test, for non-normally distributed data, as specified in each figure legend. Values of $p \leq 0.05$ were assumed to be statistically significant.

Experimental procedure	number of mice used
Assessment of cerebral infarct damage and neurological deficit	30
ELISA test	12
Western blot analysis	25
Immunohistochemistry	11
Flow cytometry	15

Table 3.3 – Table summarizing the number of animals used for each experimental procedure. 11 mice died during or early after surgery; 8 animals were excluded from the study for unsuccessful MCAo (less than 70% reduction of CBF).

Chapter 4 - Results

4.1 - Neuroprotective effects of brain ischemic preconditioning

The first aim of the present work was to assess whether the protocol chosen for ischemic PC provides neuroprotection against a severe insult produced,72 h later, by 1 h MCAo followed by 24 h of reperfusion in mice.

Nissl staining of coronal brain slices showed that the sublethal PC stimulus (i.e., 15 min MCAo, followed by 72 h of reperfusion) produces a small infarct limited to the striatum (pale areas in "PC" panel of figure 4.1). By contrast, 1 h MCAo resulted in a large infarct involving the entire region perfused by the middle cerebral artery, namely the striatum and the frontoparietal cortex (pale areas in "MCAo" panel of figure 4.1). The neuroprotective effect of ischemic PC was clearly visible in brain slices from preconditioned mice ("PC+MCAo" panel of figure 4.1), whereby penumbral areas, corresponding to medial striatum, motor cortex and rostral and caudal parts of the frontoparietal cortex, were preserved.

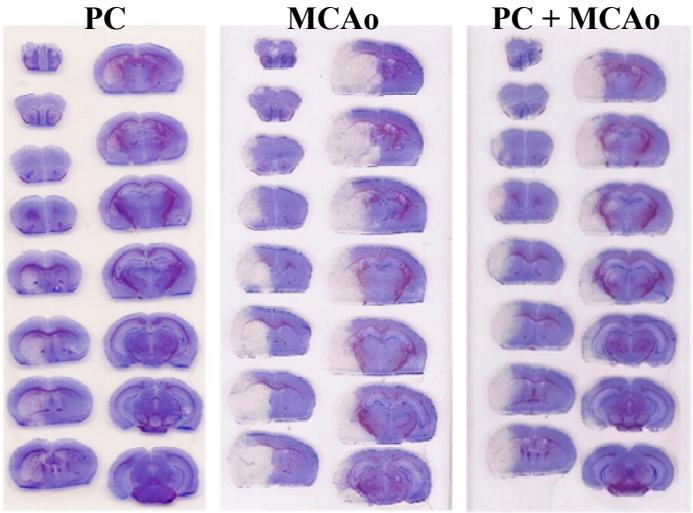

Figure 4.1 – Ischemic PC preserves penumbra regions from ischemic damage produced in mice by 1h MCAo. Representative Nissl-stained coronal brain slices showing cerebral infarct (pale areas) in mice subjected to 15 min MCAo followed by 72h of reperfusion (PC), 1h MCAo followed by 24h of reperfusion (MCAo) or both (PC + MCAo).

The quantification of cerebral infarct damage confirmed that ischemic PC, but not sham surgery, induces significant neuroprotection in mice against a subsequent more severe ischemic event. Indeed, mice exposed to 15 min MCAo, 72 h prior to the more severe ischemia of 1 h (PC+MCAo), displayed significantly reduced cerebral infarct area and volume as compared with 1 h MCAo alone (MCAo) or preceded by sham surgery (Sham+MCAo) (figure 4.2 A-B).

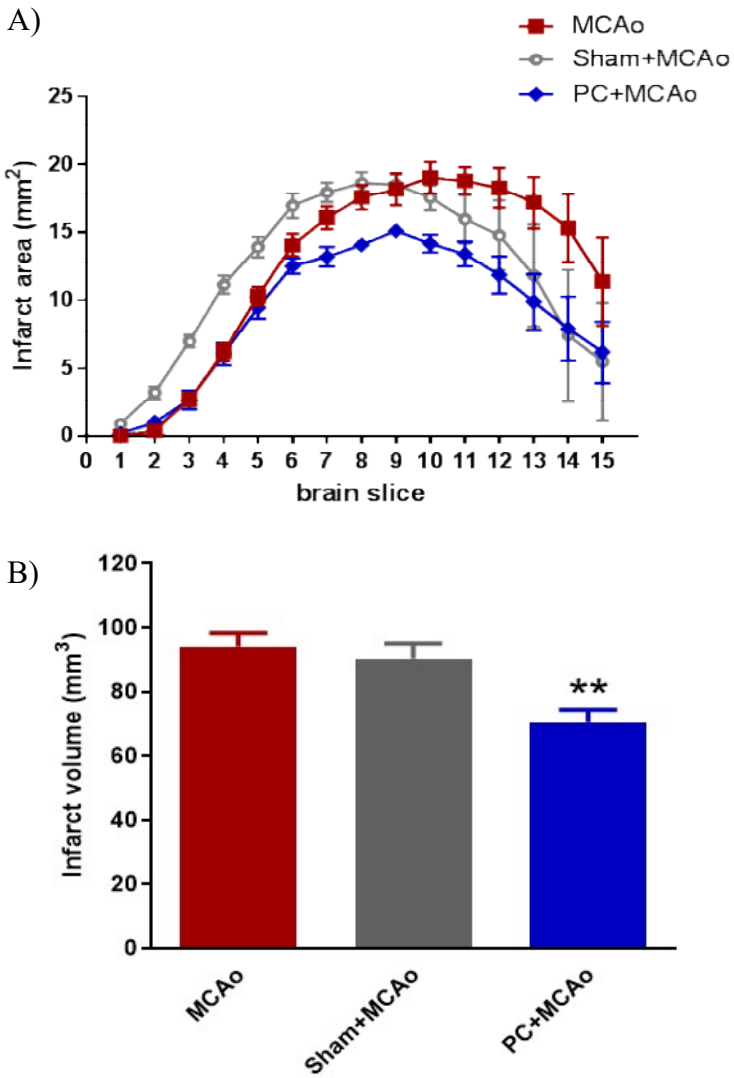

Figure 4.2 – Ischemic PC significantly reduces cerebral infarct area and volume in mice subjected to 1h MCAo. Quantification of infarct area (A) and volume (B), produced in mice by 1h MCAo followed by 24h of reperfusion (MCAo), alone or preceded, 72h before, by ischemic PC (PC+MCAo) or sham surgery (Sham+MCAo). **p ≤ 0.01 vs MCAo and vs Sham+MCAo (One-way ANOVA followed by Tukey post-test. Data are expressed as means ± SEM, n=8-12 animals per experimental group).

According to the histological data, the assessment of neurological deficits demonstrated that pre-exposure to ischemic PC (but not sham surgery) significantly prevents neurological deterioration produced by 1 h MCAo followed by 24 h of reperfusion in mice (figure 4.3).

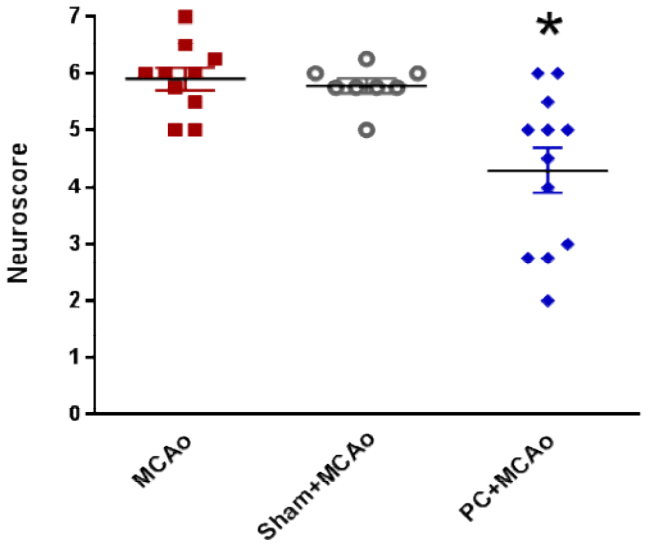

Figure 4.3 – Ischemic PC significantly improves neurological deficits of mice subjected to 1h MCAo. Assessment of neurological deficit in mice subjected to 1h MCAo alone (MCAo) or preceded, 72h before, by ischemic PC (PC+MCAo) or sham surgery (Sham+MCAo). *$p \leq 0.01$ vs MCAo and $p \leq 0.05$ vs Sham+MCAo (Kruskal-Wallis followed by Dunn's multiple comparison test. Data are expressed as medians with IQR, n=8-12 animals per experimental group).

A deeper analysis of neurobehaviour based on De Simoni composite neuroscore scale revealed that mice subjected to 1 h MCAo followed by 24 h of reperfusion displayed both focal and general deficits typically observed after focal brain lesions (Orsini *et al.*, 2012b; Llovera *et al.*, 2015). Moreover, in mice pre-exposed to ischemic PC we observed a tendency towards reduction (of 3.5 points) of total deficits (figure 4.4, table 4.1).

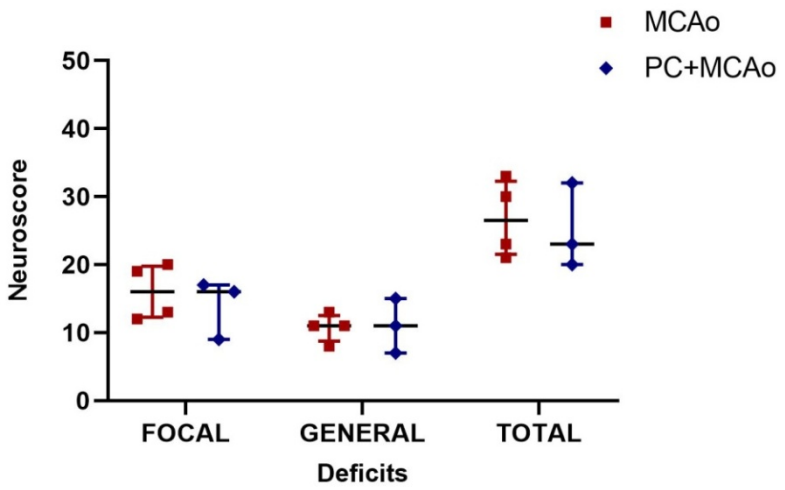

Figure 4.4 – Ischemic PC attenuates total deficits produced in mice by 1h MCAo. Assessment of neurological deficits using De Simoni Composite Neuroscore in mice subjected to 1h MCAo followed by 24h of reperfusion (MCAo) or preceded, 72h before, by ischemic PC (PC+MCAo). Data are expressed as medians with IQR, n=3-4 animals per experimental group.

	MCAo Medians with IQR	**PC+MCAo Medians with IQR**
FOCAL DEFICITS	16 (12.75-19.25)	16 (12.5-16.5)
GENERAL DEFICITS	11 (10.25-11.5)	11 (9-13)
TOTAL DEFICITS	26.5 (22.5-30.75)	23 (21.5-27.5)

Table 4.1 – De Simoni composite neuroscore. Data are expressed as medians with IQR, n=3-4 animals per experimental group.

Hence, our results demonstrated that the protocol selected for inducing ischemic PC (i.e., 15 min MCAo) represents an effective neuroprotective strategy by eliciting an ischemic tolerant state that protects against a subsequent episode of severe ischemia, as it significantly reduced histological and functional damage produced, 72 h later, by 1 h MCAo.

4.2 - Characterization of the expression of the molecular components of SOCE

Considering the importance of SOCE in maintaining intracellular Ca^{2+} homeostasis (Moccia *et al.*, 2015; Albarran *et al.*, 2016; Bollimuntha *et al.*, 2017) and its critical role in neuronal survival mechanisms after cerebral ischemia (Berna-Erro *et al.*, 2009; Zhang *et al.*, 2014; Secondo *et al.*, 2019), we aimed at assessing whether expression of its molecular components is affected by cerebral ischemia and, eventually, by the preconditioning neuroprotective stimulus. To this end, by western blotting and immunofluorescence analysis, we evaluated the cortical expression of the main components of SOCE, namely Orai1, STIM1 and SARAF, after 1 h MCAo alone or preceded by ischemic PC.

Regarding the calcium channel Orai1, western blot analysis revealed that its expression in ipsilateral (ischemic) and contralateral cortex is unaffected in mice subjected to PC alone, 1 h MCAo or both (PC+MCAo group) (figure 4.5 A). Immunofluorescence images showed that Orai1 is mainly expressed in $NeuN^+$ neurons in the ipsilateral cortex of mice subjected to 1 h MCAo. Moreover, $Orai1^+$ signal also colocalized with Ly-6B.2, a marker of myeloid cells, observed alongside the endothelium and in perivascular areas (figure 4.5 B).

This is an interesting finding, since the calcium selective channel responsible of I_{crac}, Orai1, plays a critical role in modulating innate immune cell functions (Demaurex and Nunes, 2016; Vaeth and Feske, 2018; Clemens and Lowell, 2019) and, more importantly, it is involved in the mechanisms underlying neutrophil activation and recruitment during an inflammatory response (Immler *et al.*, 2018; Grimes *et al.*, 2020). Thus, our immunofluorescence findings strongly suggest that Orai1 participates in the modulation of the immune response triggered by MCAo.

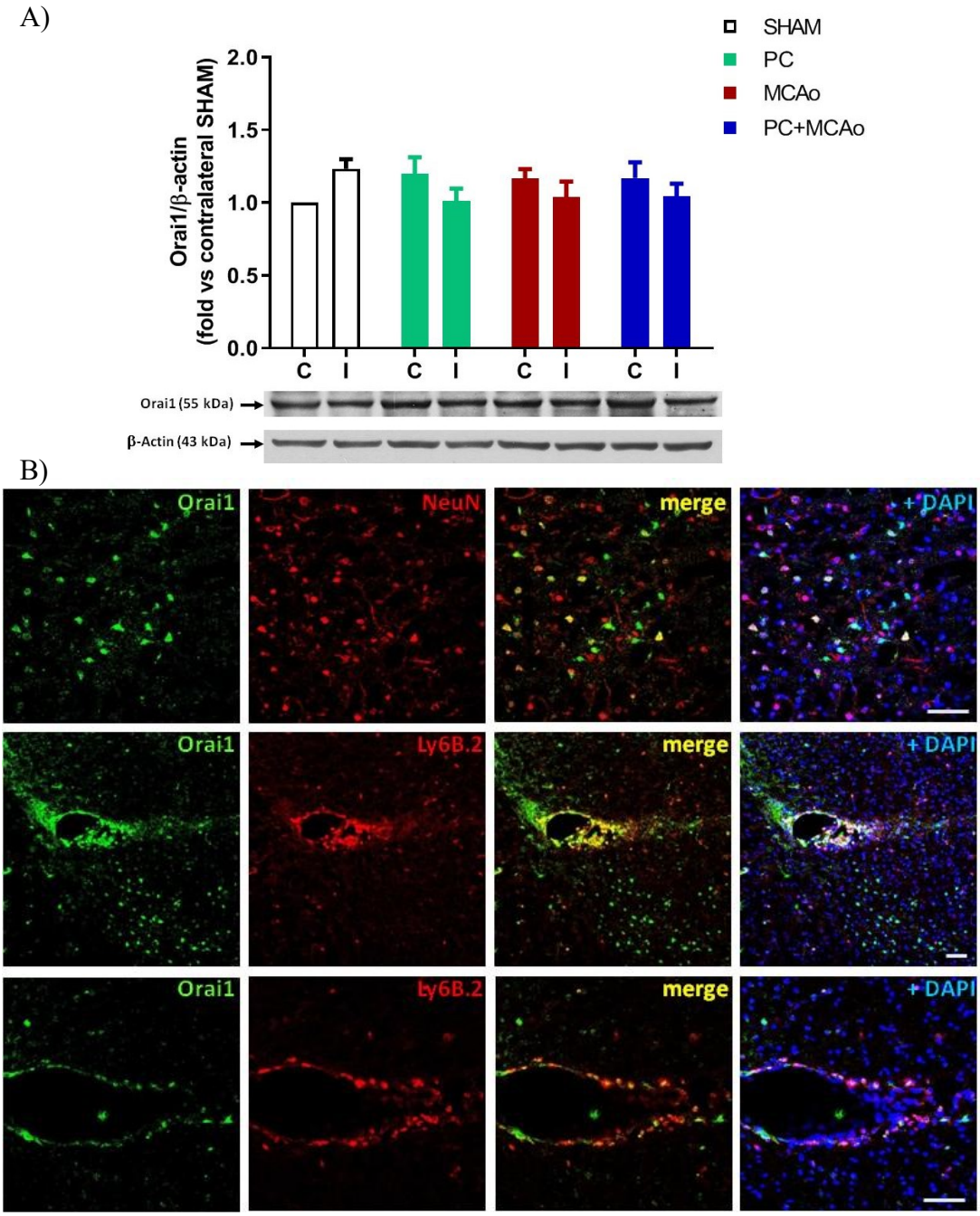

Figure 4.5 – Cortical expression of Orai1 is unaffected by MCAo and/or ischemic PC. A) Western blot analysis performed in ipsilateral (ischemic, I) and contralateral (C) cerebral cortical homogenates showing the expression of Orai1 in mice subjected to sham surgery (SHAM), 15 min MCAo followed by 72h of reperfusion (PC), 1h MCAo followed by 24h of reperfusion (MCAo) or both (PC+MCAo). (Data are expressed as means ± SEM, n=5 mice per experimental group). B) Representative immunofluorescence images from the ipsilateral parietal cortex of a mouse subjected to 1h MCAo followed by 24h of reperfusion, showing the expression of Orai1 (green fluorescence) in $NeuN^+$ neurons (red fluorescence) and in $Ly\text{-}6B.2^+$ myeloid cells (red fluorescence) infiltrating the brain from a blood vessel. Nuclei are counterstained with DAPI (blue signal). Scale bars= 175 μm.

Western blotting analysis of cortical levels of the ER Ca^{2+} sensor STIM1 demonstrated that its expression is significantly reduced in the ipsilateral (ischemic) cortex of mice subjected to 1 h MCAo as compared to contralateral or sham tissue (figure 4.6 A). By contrast, STIM1 expression in the ipsilateral and contralateral cortices of mice subjected to the sole PC stimulus was comparable to protein levels observed in sham group. However, the reduced expression of STIM1 observed in the ischemic cortex was preserved when MCAo was preceded by the preconditioning treatment (PC+MCAo), highlighting that neuroprotection by PC does not affect STIM1 reduction in the lesioned tissue (figure 4.6 A).

Immunofluorescence images performed on brain slices of mice subjected to 1 h MCAo revealed that STIM1 is only expressed in neurons. The pictures, showing the margin between infarcted and healthy tissue, confirm the result of protein analysis demonstrating the reduction of STIM1 immunosignal in $NeuN^{+}$ neurons (figure 4.6 B). Considering the neuronal expression of STIM1 in the brain, these results suggest that its reduced expression occurring in the ischemic cortex after 1 h MCAo/24 h reperfusion is probably due to neuronal loss, whereas, the sublethal preconditioning stimulus of 15 min of MCAo may not be sufficient to reduce its expression.

However, despite the reduction of cerebral damage observed in preconditioned mice (PC+MCAo), STIM1 expression in the ipsilateral cortex is not preserved (figure 4.6 A).

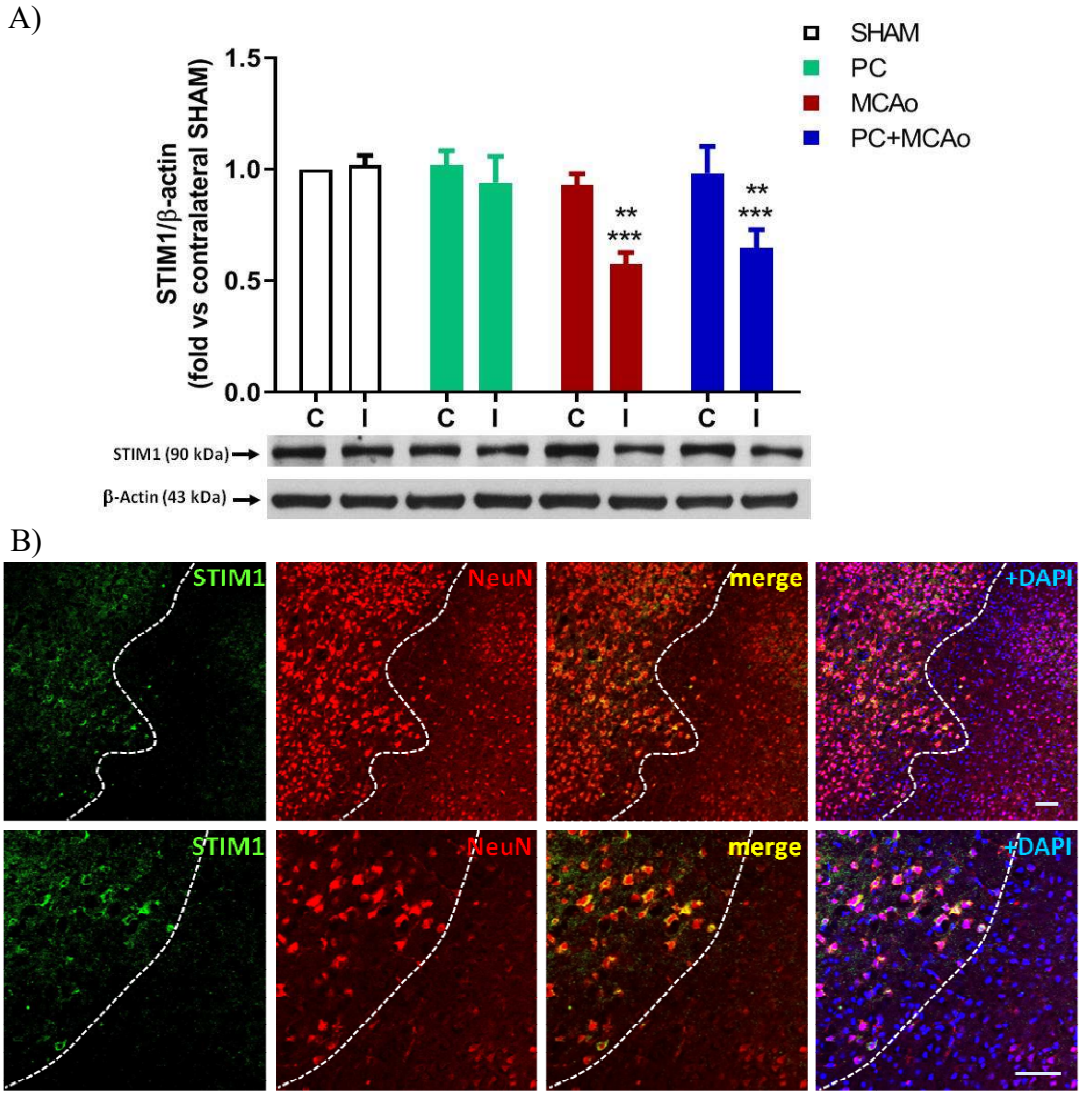

Figure 4.6 – Cortical expression of STIM1 is reduced following 1h MCAo/24h reperfusion regardless of previous ischemic PC. A) Western blot analysis performed in ipsilateral (ischemic, I) and contralateral (C) cerebral cortical homogenates showing the expression of STIM1 in mice subjected to sham surgery (SHAM), 15 min MCAo followed by 72h of reperfusion (PC), 1h MCAo followed by 24h of reperfusion (MCAo) or both (PC+MCAo). ***p ≤ 0.001 vs corresponding C, **p ≤ 0.01 vs SHAM I and vs PC I (two-way ANOVA followed by Bonferroni post-test. Data are expressed as means ± SEM, n=5-7 mice per experimental group). B) Representative immunofluorescence images acquired at the margin (dotted line) between the infarcted area (right side of the picture) and the healthy tissue, from the ipsilateral parietal cortex of a mouse subjected to 1h MCAo/24h reperfusion, showing the expression of STIM1 (green fluorescence) in $NeuN^+$ neurons (red fluorescence). Nuclei are counterstained with DAPI (blue signal). Scale bars= 175 µm.

Finally, we also evaluated whether ischemia and/or ischemic PC affect cortical expression of the negative regulatory factor of SOCE, SARAF.

Western blot analysis revealed that the sole PC stimulus (i.e., 15 min MCAo, followed by 72 h of reperfusion) did not affect the expression of SARAF; by contrast, the injurious ischemic insult of 1 h MCAo caused a significant reduction of SARAF expression in the ipsilateral cortex as compared to contralateral tissue or to the cortex of sham group (figure 4.7 A). Interestingly, this effect was abolished in mice exposed to neuroprotective PC prior to 1 h MCAo (PC+MCAo), whereby SARAF expression was comparable to that observed in sham group (figure 4.7 A).

Immunofluorescence experiments showed that, similar to STIM1, SARAF expression is exclusively observed in neurons (figure 4.7 B). Moreover, images acquired along the margin between the infarcted area and the healthy tissue, showed a clear reduction of SARAF immunosignal in $NeuN^+$ neurons in the lesioned tissue of mice subjected to 1 h MCAo, and the preservation of its neuronal expression in the preconditioned group (PC+MCAo) confirming the western blotting findings (figure 4.7 B).

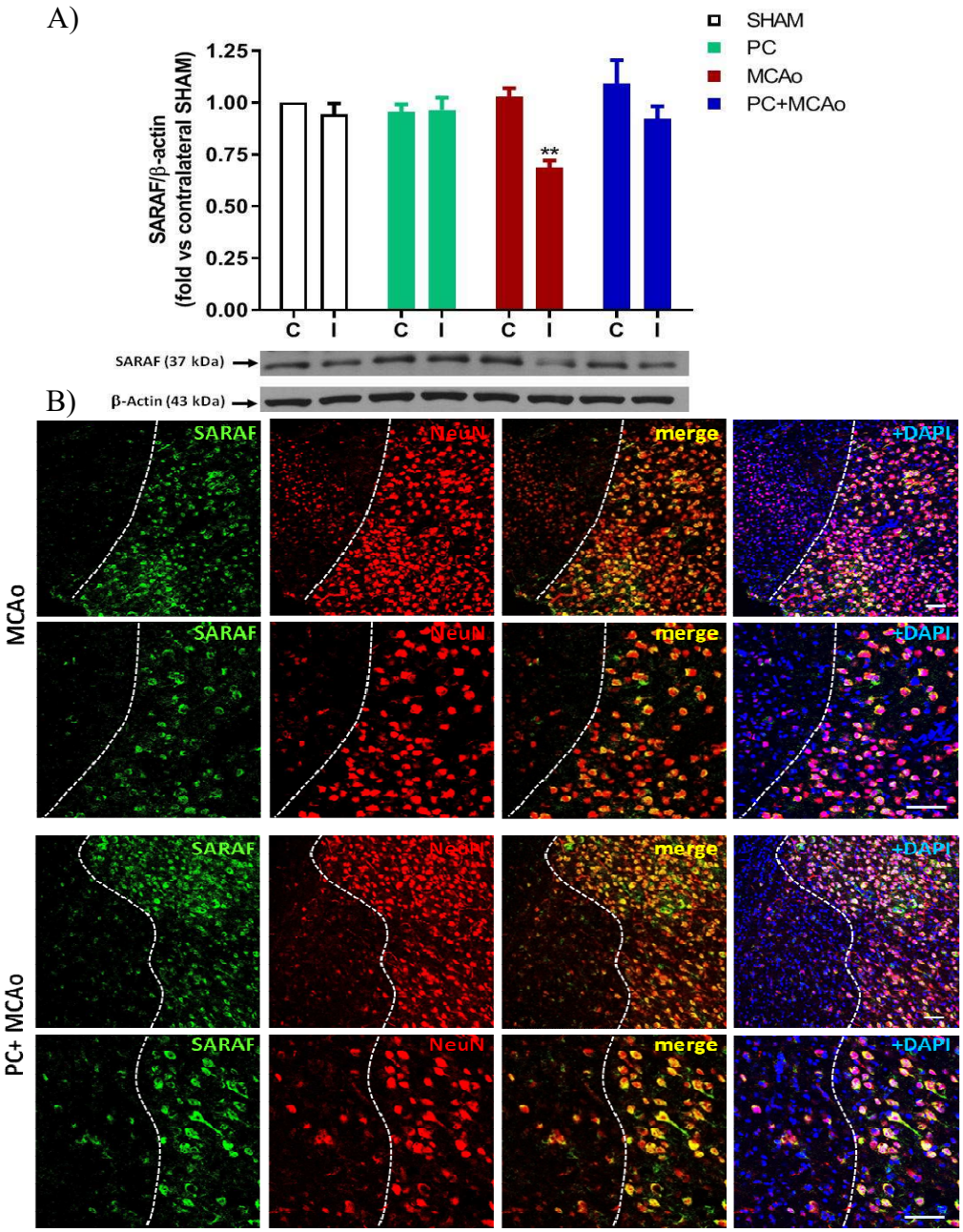

Figure 4.7 – Ischemic damage results in reduced cortical expression of SARAF, while ischemic PC abolishes this effect. A) Western blot analysis performed on ipsilateral (ischemic, I) and contralateral (C) cerebral cortical homogenates showing SARAF expression in mice subjected to sham surgery (SHAM), 15 min MCAo followed by 72h of reperfusion (PC), 1h MCAo followed by 24h of reperfusion (MCAo) or both (PC+MCAo). **p ≤ 0.01 vs corresponding C, vs SHAM I, vs PC I and vs PC+MCAo (Two-way ANOVA followed by Bonferroni post-test. Data are expressed as means ± SEM, n=5-6 mice per experimental group). B) Immunofluorescence images acquired at the margin (dotted line) between the infarcted area (left side of the picture) and the healthy tissue from the ipsilateral parietal cortex of a mouse subjected to 1h MCAo alone or preceded by PC (PC+MCAo) showing the expression of SARAF (green fluorescence) in $NeuN^+$ neurons (red fluorescence). Nuclei are counterstained with DAPI (blue signal). Scale bars= 175 μm.

4.3 - Characterization of the cerebral immune response

Another major aim of the present work was to characterize soluble and cellular mediators of the immune response after ischemia and following ischemic PC in mouse brain.

ELISA assay revealed that cerebral damage produced in mice by 1 h MCAo was associated with increased protein levels of the pro-inflammatory cytokine IL-1β in the ischemic (ipsilateral) cortex after 24 h of reperfusion, as compared to contralateral tissue or to ipsilateral cortex of sham operated animals. By contrast, protection provided by ischemic PC prevented this effect in the ipsilateral cortex of the preconditioned group (PC+MCAo), thus suggesting that attenuation of inflammation may underlie ischemic tolerance (figure 4.8).

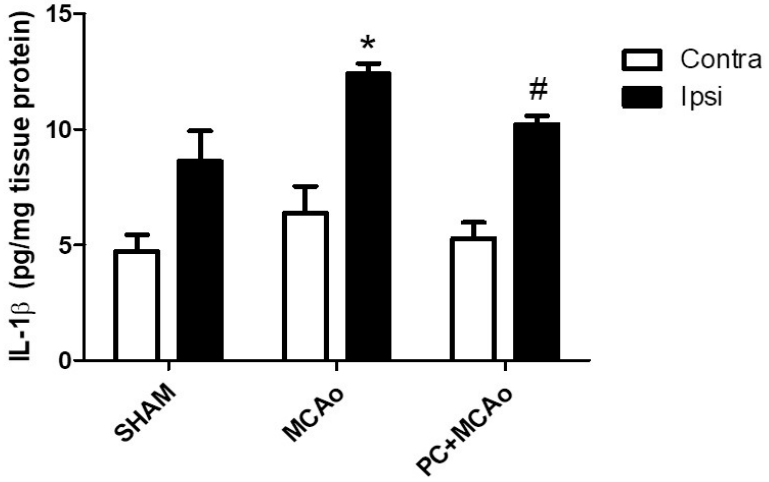

Figure 4.8 – Ischemic PC prevents the increase of IL-1β in the ischemic cortex of mice subjected to 1h MCAo. ELISA analysis of IL-1β protein levels in ipsilateral (ischemic) and contralateral cerebral cortical homogenates, assessed at 24h of reperfusion from sham surgery (SHAM), 1h MCAo alone (MCAo) or preceded by PC 72h before (PC+MCAo). *$p \leq 0.001$ vs contralateral and $p \leq 0.05$ vs sham ipsilateral, #$p \leq 0.01$ vs contralateral (Two-way ANOVA followed by Bonferroni post-test. Data are expressed as means ± SEM, n=4 mice per experimental group).

To assess whether ischemic PC also affects innate immune cell phenotypes in brain, we evaluated the expression of Ym1, marker of alternatively-activated M2

microglia/macrophages that are involved in the resolution of post-stroke inflammatory reaction (Kanazawa, Ninomiya, *et al.*, 2017).

Western blot analysis performed on cerebral cortical homogenates showed that the ischemic insult of 1 h MCAo induced a significant increase in the expression of Ym1 in the ischemic cortex as compared to contralateral tissue or sham group (figure 4.9). Interestingly, expression of Ym1 was not affected by exposure to the sole PC stimulus in the ipsilateral cortex, but a marked and significant elevation of the expression of Ym1 was observed in the ischemic cortex of preconditioned mice (PC+MCAo), as compared to contralateral tissue and to ischemic cortex of mice subjected only to 1h MCAo (figure 4.9).

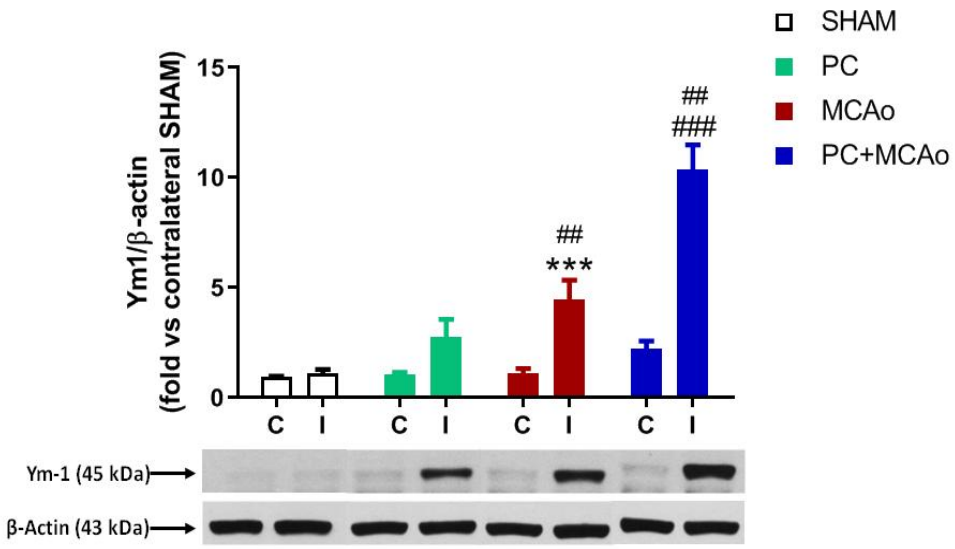

Figure 4.9 – Ischemic PC prior to 1h MCAo significantly increases the expression of Ym1 in the ischemic cortex. Western blot analysis of Ym1 expression in ipsilateral (ischemic, I) and contralateral (C) cerebral cortical homogenates of mice subjected to sham surgery (SHAM), 15 min MCAo followed by 72h of reperfusion (PC), 1h MCAo followed by 24h of reperfusion (MCAo) or both (PC+MCAo). ***p ≤ 0.001 vs SHAM I, ###p ≤ 0.001 vs SHAM I, PC I and MCAo I, vs PC C and vs MCAo C, ##p ≤ 0.01 vs corresponding C (Two-way ANOVA followed by Bonferroni post-test. Data are expressed as means ± SEM, n=4 mice per experimental group).

With the aim to identify the specific immune cell type expressing Ym1 in the brain after ischemia and ischemic PC, we performed an immunohistochemical analysis on coronal brain slices. Immunofluorescence images show that Ym1 expression is

evident in both striatum (St) and parietal cortex (P Cx) of the ipsilateral hemisphere (figures 4.10 and 4.11), whereas, signal is absent in the contralateral tissue (data not shown). Confirming the result obtained from western blotting analysis, Ym1 expression is dramatically elevated in the ischemic striatum and parietal cortex of mice pre-exposed to preconditioning before the injurious ischemia (PC+MCAo), as compared to non-preconditioned animals (MCAo) (figures 4.10 and 4.11). Colocalization analysis demonstrated that although some Ym1 immunopositive cells also express the neutrophilic marker Ly-6G, the majority of these alternatively activated immune cells are not neutrophils (figure 4.10).

Thus, we evaluated Ym1 expression in immune cells expressing CD11b, typical marker of microglia and macrophages. Immunofluorescence images demonstrate that $Ym1^{+}$ cells observed in the lesioned hemisphere at 24 h after ischemic injury are mainly amoeboid $CD11b^{+}$ myeloid cells, resembling monocytes/macrophages infiltrating from blood vessels, being distinguishable from ramified microglia that do not express Ym1 (figure 4.11).

These results suggest that elevation of Ym1 in the preconditioned brain stems from M2-polarized myeloid immune cells, mainly consisting in infiltrating monocytes that may provide a protective immunomodulatory effect.

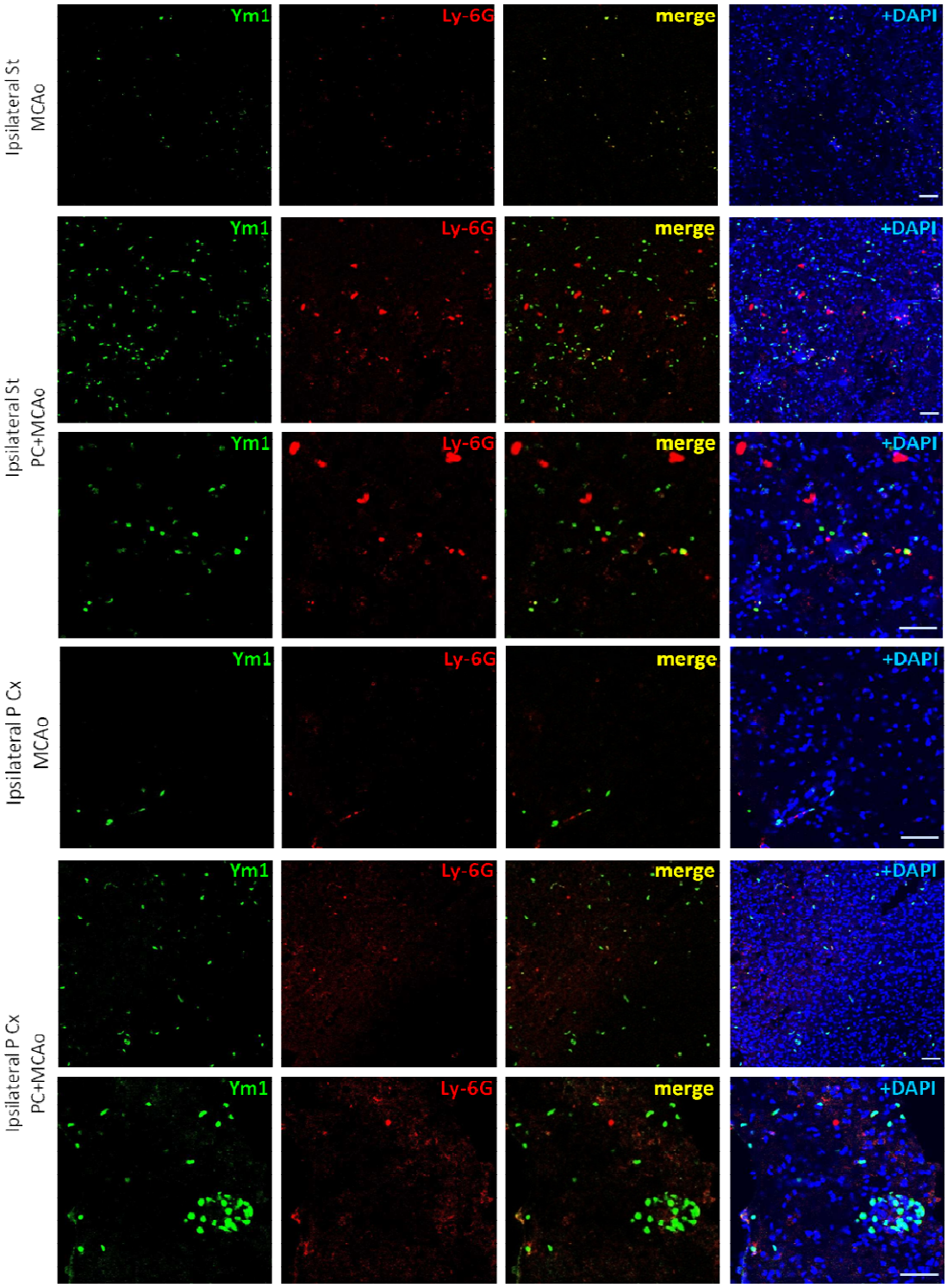

Figure 4.10 – The number of Ym1^{+} cells is elevated after ischemic PC in both the ipsilateral striatum and cortex. Immunofluorescence images from the ipsilateral striatum (St) and parietal cortex (P Cx) of mice subjected to 1h MCAo followed by 24h of reperfusion (MCAo), alone or preceded by ischemic PC (PC+MCAo), showing the expression of Ym1 (green fluorescence) and of the neutrophilic marker Ly-6G^{+} (red fluorescence). Nuclei are counterstained with DAPI (blue signal). Scale bars= 175 µm.

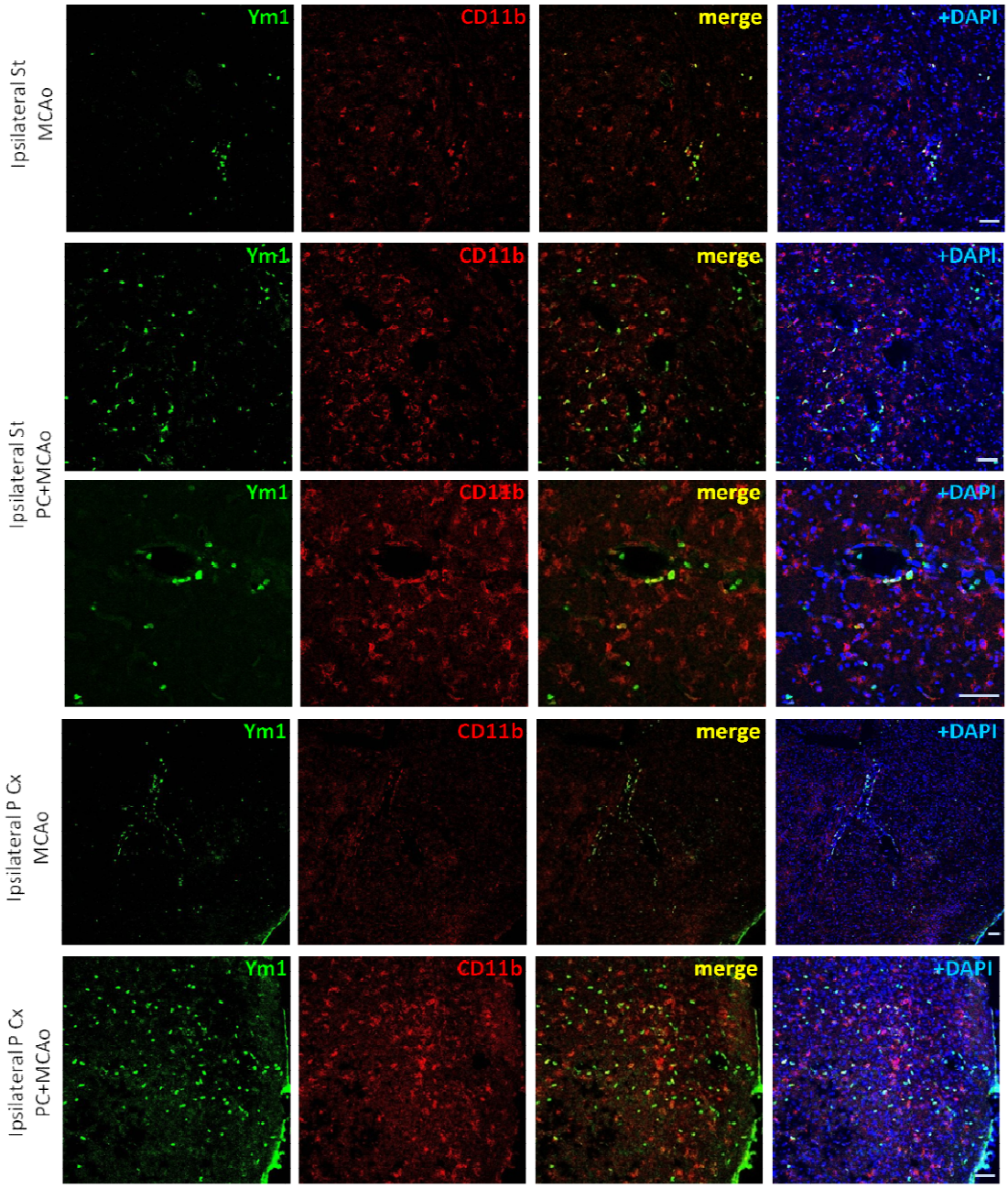

Figure 4.11 – Ym1 expression occurs in CD11b^{+} monocytes/macrophages, displaying an amoeboid shape and infiltrating from blood vessels. Immunofluorescence images from the ipsilateral striatum (St) and parietal cortex (P Cx) of mice subjected to 1h MCAo followed by 24h of reperfusion (MCAo), alone or preceded by ischemic PC (PC+MCAo), showing the expression of Ym1 (green fluorescence) in CD11b^{+} microglia and macrophages (red fluorescence). Nuclei are counterstained with DAPI (blue signal). Scale bars= 175 µm.

Previous work has already demonstrated that the antiinflammatory cytokine IL-10 can drive polarization of macrophages towards the M2 phenotype via the activation of the transcription factor STAT3 (Wang *et al.*, 2014; Porta *et al.*, 2015). In order to assess whether this cytokine may also be involved in M2 polarization triggered by ischemic PC, we performed ELISA assay on cerebral cortical homogenates.

The assessment of IL-10 cortical levels revealed no significant changes in its expression at 24 h of reperfusion from 1 h MCAo in both ipsilateral and contralateral hemispheres, as compared to sham group. Despite the reduced cerebral damage observed at this time of reperfusion in preconditioned mice (PC+MCAo) and the elevation of M2-polarized cells, ischemic PC did not affect IL-10 cortical levels, suggesting that its immunomodulatory and neuroprotective effects are not mediated by this antiinflammatory cytokine, at least at this time point (figure 4.12).

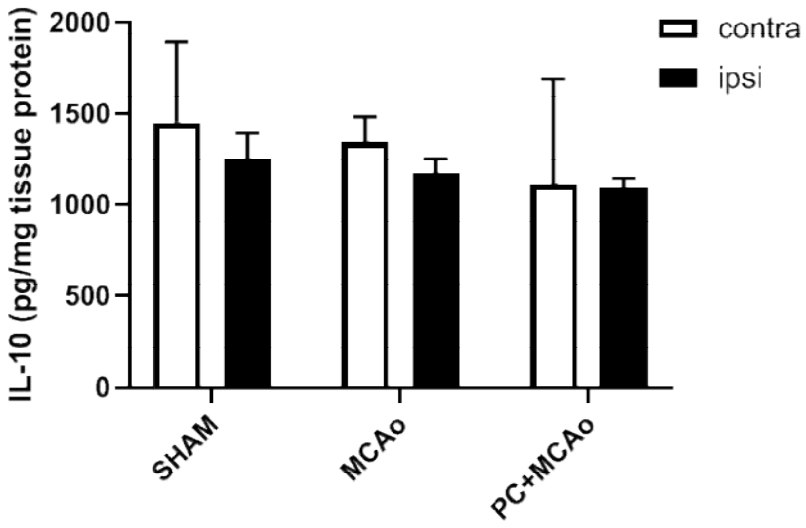

Figure 4.12 – Ischemic PC and MCAo do not affect cortical levels of IL-10. ELISA analysis of IL-10 protein levels in ipsilateral (ischemic) and contralateral cerebral cortical homogenates assessed at 24h of reperfusion from sham surgery (SHAM), 1h MCAo alone (MCAo) or preceded by PC 72h before (PC+MCAo). (Data are expressed as means ± SEM, n= 2 mice per experimental group).

4.4 - Characterization of the peripheral immune response

The above described data strongly suggest the involvement of the peripheral immune system in the neuroprotective mechanisms evoked by ischemic PC.

To confirm and extend these findings, we have analysed the responses evoked in the periphery. Firstly, we assessed the modification of spleen weight in mice subjected to focal cerebral ischemia, with or without preconditioning. Indeed, the spleen is the main reservoir of myeloid cells in the body that can be activated and mobilized during an inflammatory reaction. After cerebral ischemia, the spleen responds to injury by an acute transient reduction in its volume, followed by its expansion, in both animal models and stroke patients (Nous *et al.*, 2020). This biphasic modification in splenic volume associates with the release of splenocytes into the peripheral circulation, which then infiltrate the brain parenchyma contributing to neurodegeneration (Chiu *et al.*, 2016). Our data demonstrated that mice subjected to 1 h MCAo show a 70% increase in spleen weight after 24 h of reperfusion, as compared to sham group. Interestingly, this effect was abolished in mice pre-exposed to the neuroprotective PC stimulus (PC+MCAo), in which spleen weight returns to sham levels (figure 4.13), suggesting that the PC-induced tolerant state may involve the modulation of the peripheral immune response.

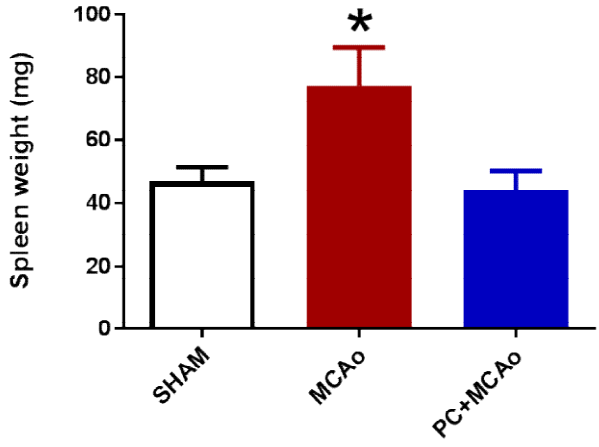

Figure 4.13 – Ischemic PC abolishes spleen weight increase observed in mice subjected to 1h MCAo. Assessment of spleen weight modifications in mice, at 24h of reperfusion after sham surgery (SHAM), 1h MCAo alone (MCAo) or preceded 72h before by PC (PC+MCAo).*p≤ 0.05 vs SHAM and vs PC+MCAo (One-way ANOVA followed by Tukey post-test. Data are expressed as means ± SEM, n=10-12 mice per experimental group).

Moreover, to characterize the involvement of peripheral immune cells in the ischemic-tolerant state, we evaluated the effects of ischemia and ischemic PC on neutrophil counts in mice blood samples, using the neutrophilic marker Ly-6G.

Flow cytometry analysis revealed that 1 h MCAo causes a consistent elevation of neutrophil counts (Ly-6G$^+$ events) in blood at 24 h of reperfusion as compared to sham-operated animals. By contrast, neutrophil counts in the blood of preconditioned mice is comparable to that observed in sham group (figure 4.14).
Therefore, it can be hypothesised that the preconditioning treatment, by avoiding the increase of circulating neutrophils induced by a severe ischemia, would eventually result in reduced brain infiltration of inflammatory neutrophils (likely detrimental given their negligible expression of Ym1 shown in figure 4.10).

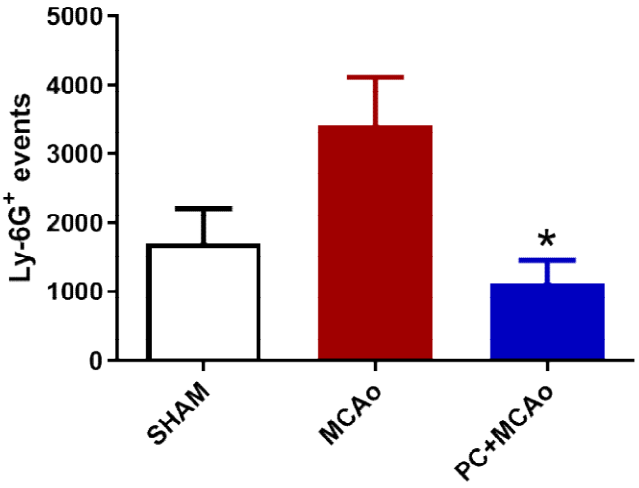

Figure 4.14 – Ischemic PC significantly attenuates elevation of neutrophil counts (Ly6G$^+$ events) produced in the blood of mice by 1h MCAo. Flow cytometry analysis of circulating neutrophils (Ly-6G$^+$ events) in mice blood samples collected after 24h of reperfusion from sham surgery (SHAM), 1h MCAo alone (MCAo) or preceded by PC 72h before (PC+MCAo). *p ≤ 0.05 vs MCAo (One-way ANOVA followed by Tukey post-test. Data are expressed as means ± SEM, n=5 mice per experimental group).

Given the evident modulation of central and peripheral immune response to stroke observed in this work, and considering that SOCE represents the main source of calcium in immune cells in response to their recruitment and modulates several of

their functions (Vaeth and Feske, 2018), we hypothesized an involvement of this Ca^{2+} current in the neuroprotection evoked by ischemic PC.

To clarify this issue, we analysed the expression of Orai1 (the only molecular component expressed in circulating leukocytes – see figure 4.5) in the whole population of circulating leukocytes and in neutrophils obtained from mice blood samples after cerebral ischemia and ischemic PC.

Flow cytometry analysis revealed that the ischemic insult of 1 h followed by 24 h of reperfusion dramatically increases the number of $Orai1^+$ leukocytes as compared to sham operated animals, regardless of whether the animals received or not the PC stimulus (figure 4.15 A). Interestingly, the percentage of $Orai1^+$/Ly-$6G^+$ events (63.2%) was significantly higher as compared to sham (1.1%) (figure 4.15 B), suggesting that in addition to the elevation of the absolute number of neutrophils (figure 4.14), the ischemic insult also elevates the relative Orai1 expression in these cells. Surprisingly, exposure to the PC stimulus prior to ischemia did not affect the percentage of $Orai1^+$/Ly-$6G^+$ events (72.7%) (figure 4.15 B).

Nevertheless, the evidence that in the PC+MCAo group we observed a reduced number of Ly-$6G^+$ cells as compared to MCAo group (figure 4.14) strongly suggests a significant modulation of the relative expression of Orai1 in these circulating myeloid cells.

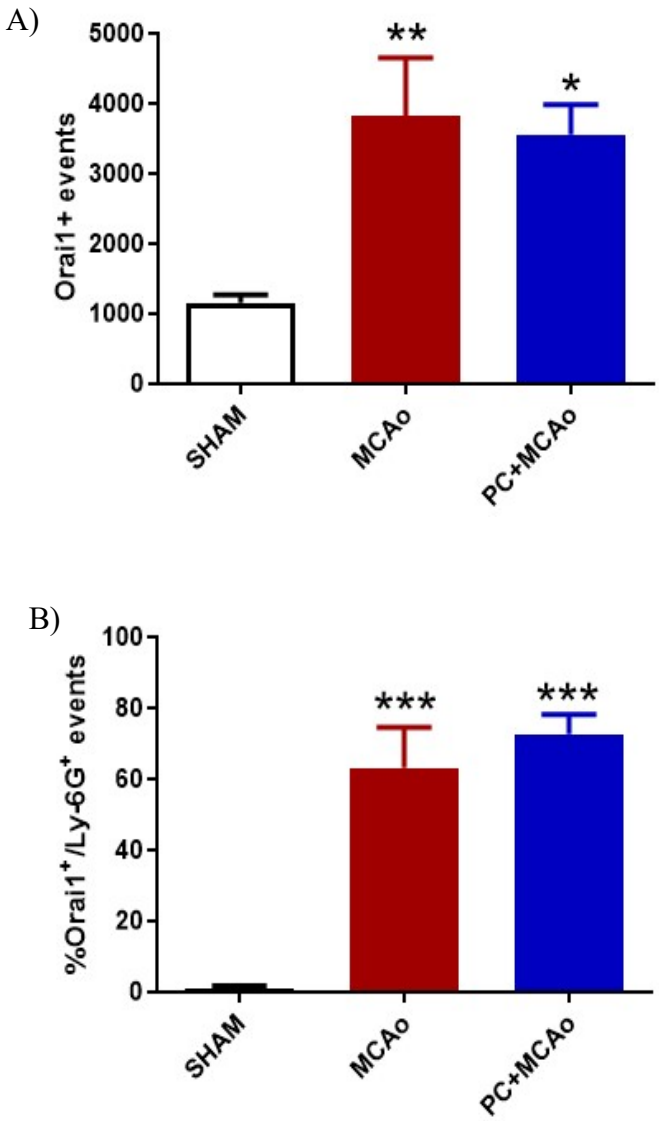

Figure 4.15 – Ischemic PC does not affect the increased expression of Orai1 in circulating leukocytes caused by ischemia and the majority of them also express the neutrophilic marker Ly6G. Flow cytometry analysis of Orai1^{+} leukocytes (A) and percentage of Orai1^{+}/Ly-6G^{+} neutrophils (B) in blood samples of mice subjected to sham surgery (SHAM), 1h MCAo alone (MCAo) or preceded by 15 min MCAo (PC) 72h before (PC+MCAo). *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 vs SHAM (One-way ANOVA followed by Tukey post-test. Data are expressed as means ± SEM, n= 4-5 mice per experimental group).

Chapter 5 - Discussion

In the present research project we have aimed at characterizing specific molecular and cellular mechanisms involved in the beneficial effects triggered by the stimulation of endogenous neuroprotection, particularly focussing on the immune system and SOCE. To this end, we used brain ischemic PC as a model to induce tolerance through a sublethal ischemic insult able to increase the resistance of the brain against a subsequent more intense ischemic event. Interestingly, clinical data supporting this evidence have shown the development of an ischemic-tolerant state in patients suffering from peripheral vascular diseases or sleep apnoea, that lead to chronic hypoperfusion and episodes of nocturnal hypoxemia (Koch *et al.*, 2007; Connolly *et al.*, 2013). Furthermore, a reduction in the harmful effects of stroke and myocardial infarction has also been demonstrated in patients when these insults are preceded respectively by a recent TIA or by angina episodes (Heusch and Gersh, 2017; Wang *et al.*, 2017).

The possibility to stimulate endogenous neuroprotection in stroke patients is an interesting strategy, that would allow to make neurons more resistant against a severe ischemic event, thus reducing the risk to develop severe functional deficits and improving the quality of life of patients surviving a stroke.

The experimental procedure of brain ischemic PC employed in this study is based on a well-defined protocol of transient focal preconditioning/transient focal harmful ischemia induced by exposing mice to 15 min of MCAo followed by 72 h of reperfusion, prior to the more severe ischemia of 1 h. This PC model has been demonstrated to elicit a robust and long-lasting delayed ischemic tolerance, which usually appears within 24 h and confers maximal neuroprotection after 72 h from the preconditioning treatment (Stenzel-Poore *et al.*, 2003; Zhang *et al.*, 2008; Lusardi *et al.*, 2010; McDonough *et al.*, 2020). The latency period of 72 h represents the time-window required to induce gene activation and *de novo* protein synthesis, that finally lead to a reprogramming of the transcriptional response to stroke resulting in brain developing an ischemic tolerant phenotype (Thompson *et al.*, 2013; Stevens *et al.*, 2014; McDonough and Weinstein, 2018).

Accordingly, the results of the present work showed that ischemic PC produced by 15 min MCAo, followed by 72 h of reperfusion, is effective in exerting significant neuroprotection in mice, by preserving cortical penumbral areas from the ischemic damage produced by 1 h MCAo and by significantly reducing neurological deficits.

Many *in vitro* and *in vivo* models of ischemic stroke have previously proven the neuroprotective potential of inhalational anaesthetics (pharmacological preconditioning); thus, we also evaluated cerebral infarct volume in mice subjected to sham surgery 72 h prior to the more severe ischemia of 1 h (Sham + MCAo experimental group). Indeed, it has been demonstrated that animals exposed to 1.2% or 2% isoflurane for 1 h for 5 consecutive days (Sun *et al.*, 2015; Tong *et al.*, 2015), 1.5% isoflurane for 30 min (Li *et al.*, 2013) or 1% isoflurane for 4 h (Zhu *et al.*, 2010), prior to MCAo, show reduced cerebral damage and lower neurological impairment.

To exclude the participation of isoflurane in PC-induced ischemic tolerance, mice were anaesthetized for about 30 min (5% isoflurane for the induction phase, then maintained at 1.5-2%) during sham surgery, resembling exposure occurring in animals subjected to the preconditioning MCAo. The analysis of cerebral ischemic damage revealed that isoflurane exposure does not affect infarct volume or neurological deficits produced in mice by an ischemia of 1 h, confirming the specificity of the brief sublethal ischemic event in stimulating endogenous neuroprotective mechanisms.

Therefore, we demonstrated that the neuroprotective effects of isoflurane are not involved in the established ischemic tolerance observed in our PC model, probably due to the prolonged time window (72 h) intercurring between the exposure to the anaesthetic and the injurious ischemia. Accordingly, most models of pharmacological preconditioning with anaesthetics show neuroprotection within the first 24 h from the exposure, but not at later stages (Kapinya *et al.*, 2002; Kitano *et al.*, 2007).

The Ca^{2+} current SOCE mediates calcium influx in a variety of cell types due to its ubiquitous expression (Vaeth and Feske, 2018) and it has been demonstrated that it is also involved in the mechanisms of hypoxic/ischemic neuronal injury (Berna-Erro *et*

al., 2009; Zhang *et al.*, 2014; Secondo *et al.*, 2019). During cerebral ischemia, the massive activation of Gq-linked receptors on neurons leads to release of Ca^{2+} from the ER; Ca^{2+} depletion in the ER causes an accumulation of unfolded proteins in ER lumen, that activates the unfolded protein response (UPR), which finally results in neuronal death through the mechanisms of ER stress (Rastogi and Srivastava, 2019). In order to re-establish calcium homeostasis in the ER, a consistent influx of Ca^{2+} from the extracellular space occurs, through the interaction between the ER Ca^{2+} sensor STIM1 and the high selective PM Ca^{2+} channel Orai1 (Secondo *et al.*, 2018). This calcium influx, mediating the refilling of ER, is helpful in restoring calcium homeostasis, and may prevent neuronal damage caused by the mechanisms of ER stress after cerebral ischemia (Sirabella *et al.*, 2009; Parekh, 2010; Lang *et al.*, 2018), however, it could also be the source of excessive calcium influx in neurons, thus causing a Ca^{2+}-dependent non-excitotoxic neuronal death (Berna-Erro *et al.*, 2009; Zhang *et al.*, 2014).

Interestingly, some studies have highlighted that one of the mechanisms whereby ischemic PC provides delayed ischemic tolerance is by inducing a genomic reprogramming leading to the attenuation of ER stress (Y. Q. Hu *et al.*, 2017), and it has also been demonstrated that the restoration of Ca^{2+} homeostasis through the interaction between Orai1 and STIM1 mediates ischemic tolerance in preconditioned rats (Ján Lehotský *et al.*, 2009; Secondo *et al.*, 2019).

Therefore, we hypothesised that neuroprotection evoked by ischemic PC in our mouse model of transient cerebral ischemia may be linked to the mitigation of ER stress response, through the modulation of the molecular components of SOCE.

The analysis of cortical expression of SOCE components demonstrated that Orai1 was not affected by cerebral ischemia and/or ischemic PC. This effect could be explained by the evidence that Orai1 in brain is expressed in neurons, but also in peripheral myeloid cells observed in perivascular regions and infiltrating the ischemic hemisphere. These $Orai1^{+}$ immune cells are immunopositive for Ly6B.2, an heavily glycosylated protein that is expressed in $Ly\text{-}6G^{+}$ neutrophils, $Ly\text{-}6C^{+}$ inflammatory monocytes and some activated macrophages (Rosas *et al.*, 2010), thus they may compensate for the loss of neuronal protein levels due to the ischemic damage in the tissue homogenate.

By contrast, STIM1 expression was significantly reduced in the frontoparietal ischemic cortex of mice subjected to 1 h MCAo, similarly to that observed in rats and hypoxic neurons (Secondo *et al.*, 2019).

Considering the result of immunofluorescence analysis, showing that STIM1 is exclusively expressed in neurons, this reduction of cortical protein levels could be attributed to neuronal loss caused by ischemic injury. However, despite the reduced cerebral damage observed in preconditioned mice, STIM1 cortical levels are not restored by the ischemic PC. This result may suggest that cerebral ischemic damage associates with a downregulation of the expression of STIM1 in neurons and this may impair SOCE functions thus leading to neuronal damage through ER stress.

The evidence that STIM1 and Orai1 expression is not affected by ischemic PC in our preconditioning model, is in contrast with a recent work demonstrating that both proteins are downregulated after ischemia in rats and hypoxic neurons, but ischemic PC prevents this effect thus restoring SOCE current, mitigating ER stress and providing neuroprotection (Secondo *et al.*, 2019). These contrasting results are probably due to the differences in preconditioning paradigms and animal species used (mouse vs rat).

Finally, we also analyzed cortical expression of SARAF, a negative regulatory factor of SOCE. As demonstrated for STIM1, also SARAF showed a neuronal distribution and its protein levels resulted significantly reduced by the ischemic insult of 1 h in the ipsilateral cortex. Interestingly, the reduction of SARAF expression was reverted in the preconditioned group, in which protein levels were comparable to those observed in ipsilateral sham cortex.

This effect was also observed in immunofluorescence images showing the neat reduction of SARAF immunosignal in the infarcted tissue after 1 h MCAo, and the preservation of its neuronal expression in the frontoparietal ischemic cortex of preconditioned mice.

SARAF is an ER-resident protein that associates with STIM1 and promotes a slow form of Ca^{2+}-dependent inactivation of SOCE, thus protecting neurons from excessive Ca^{2+} influx and maintaining Ca^{2+} homeostasis (Jardín *et al.*, 2018).

Recently, it has been shown that SARAF is also expressed in the PM, where it mediates a direct activation of Orai1 channel, independently from STIM1 expression,

thus allowing Ca^{2+} refilling also in the absence of STIM1 (Albarran *et al.*, 2016). Accordingly, the role of SARAF in the regulation of SOCE is strongly dependent on STIM1 expression. The functional role of SARAF-Orai1 interaction has been identified in STIM1-deficient cells, in which SARAF activates Orai1 thus allowing Ca^{2+} influx, whereas knockdown of SARAF expression dampens Orai1 activation (Albarran *et al.*, 2016). Thus, SARAF might play a crucial role in the activation of Orai1 in STIM1-deficient cells, while in presence of STIM1 this function is hidden by the more significant inactivating role (Albarran *et al.*, 2016).

This alternative function of SARAF could explain our findings demonstrating that, despite the downregulation of STIM1 observed in the ischemic cortex of preconditioned mice, SARAF may directly activate Orai1, whose expression is not altered by ischemia, thus mediating proper Ca^{2+} refilling in the ER, re-establishing Ca^{2+} homeostasis and protecting neurons from the mechanisms of ER stress.

Therefore, this interesting result further confirms the involvement of SOCE in neuronal damage caused by the ischemic injury and, in addition, originally demonstrates that the prevention of SARAF downregulation mediated by ischemic PC may be considered as an important neuroprotective mechanism aimed at preserving SOCE functions (figure 5.1), making SARAF a valuable target to protect neurons from ischemic damage.

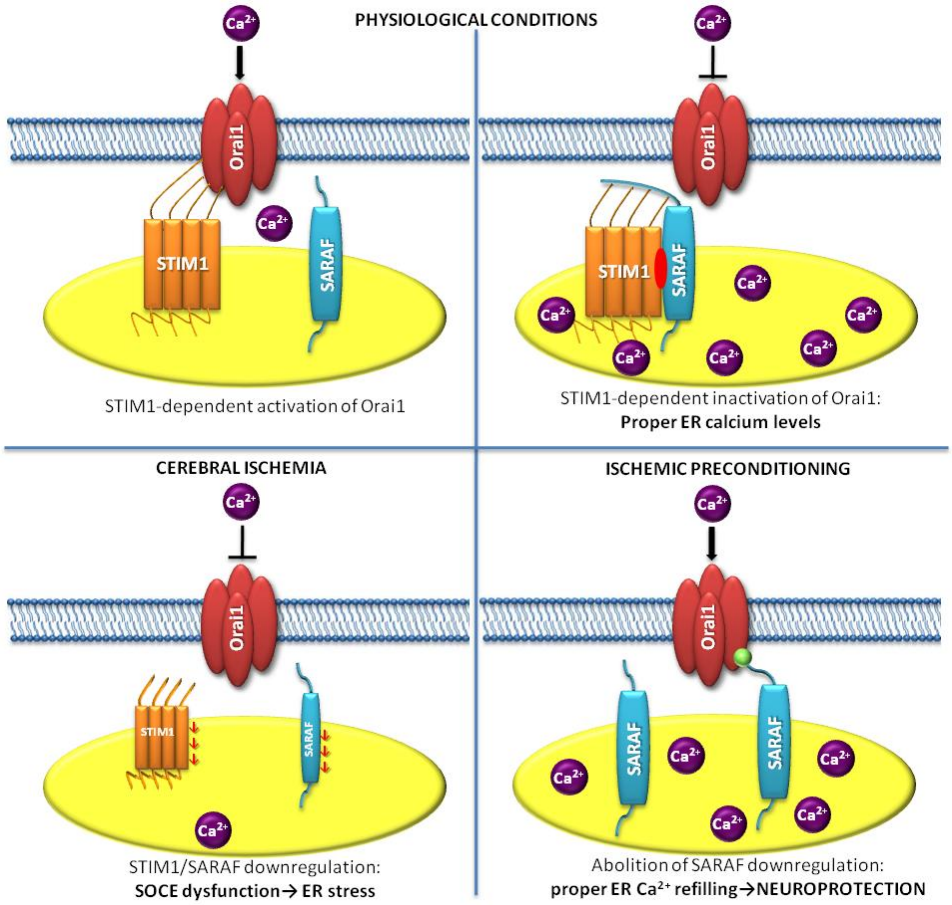

Figure 5.1 – Schematic representation of the role of SARAF under physiological conditions or following ischemia and ischemic PC.

Besides the investigation of the role of SOCE components during ischemia and in neuroprotection provided by ischemic PC, we aimed at assessing immune mechanisms involved in the establishment of cerebral ischemic tolerance.

Indeed, the evolution of cerebral damage following ischemic stroke is strongly affected by the innate and adaptive immune responses, involving soluble mediators and specialized cells resident in the brain or recruited from the periphery (Amantea *et al.*, 2015b; Qin *et al.*, 2020). The innate immune system is also an essential component of the delayed ischemic tolerance elicited in the brain by ischemic PC, as demonstrated by the evidence that stimulation of inflammatory pathways prior to ischemia (i.e. through administration of TLRs ligands) confers robust neuroprotection (Stevens *et al.*, 2014; McDonough and Weinstein, 2018).

During cerebral ischemia, DAMPs released by damaged neurons stimulate a strong inflammatory response through the activation of TLRs on immune cells, that sustains

neuronal damage by promoting the release of proinflammatory molecules (Gesuete *et al.*, 2014). Similarly, the sublethal PC stimulus triggers a mild inflammatory reaction, via TLR/NF-κB pathway, that is resolved during the early phase of ischemic tolerance, but simultaneously induces a reprogramming of innate immune reactions, which respond differently to a future episode of severe ischemia (Garcia-Bonilla *et al.*, 2014). In particular, previous work has demonstrated the crucial role played by TLR4 in the evolution of cerebral ischemic injury and in PC-mediated neuroprotection, since $TLR4^{-/-}$ mice show reduced cerebral damage after an episode of severe ischemia (Cao et al., 2007; Caso et al., 2007), but also attenuated neuroprotective effects after exposure to ischemic PC (Pradillo *et al.*, 2009).

In the ischemic brain, microglia is rapidly engaged in response to DAMPs released by damaged cells, mainly through the activation of TLRs, and trigger the recruitment of peripheral immune cells into the brain (McDonough and Weinstein, 2020). It has been already reported that mice subjected to ischemic PC, namely 15 min of transient MCAo, show an elevated number of activated microglia and infiltrating peripheral leukocytes in the ischemic cortex after 72 h of reperfusion (McDonough and Weinstein, 2016), further supporting that ischemic PC modulates the post-stroke immune reaction.

Therefore, the exposure to ischemic PC prior to a severe ischemia, protects the brain through a reprogramming of the post-stroke immune reaction, that leads to immune cells developing a protective phenotype, thus attenuating the expression of pro-inflammatory genes and promoting the production of anti-inflammatory cytokines (Hao *et al.*, 2020).

With the aim to further characterize the immunomodulatory effects of ischemic PC in mice, we evaluated cortical protein levels of the proinflammatory cytokine IL-1β.

Among the inflammatory mediators involved in the evolution of brain injury after stroke, IL-1β is considered a major contributor, acting through the stimulation of the expression of other cytokines and adhesion molecules (Pawluk *et al.*, 2020).

Previous evidence has already demonstrated that IL-1β is upregulated after ischemic stroke in rodent models and reaches its maximal levels after 12-24 h of reperfusion (Clausen *et al.*, 2008, 2016; Amantea *et al.*, 2010). In particular, the activation of IL-1 signalling pathway in neurons and brain endothelial cells after cerebral ischemia,

exacerbates infarct damage and neurological deficits in animal models by potentiating excitotoxicity, by promoting BBB disruption, enhanced expression of adhesion molecules and inflammatory mediators, neutrophil infiltration, platelet activation, and by reducing cerebral perfusion and post-stroke neurogenesis (Tsakiri *et al.*, 2008; Denes *et al.*, 2011; Allen *et al.*, 2012; Pradillo *et al.*, 2017; Wong *et al.*, 2019).

The clinical relevance of targeting these detrimental mechanisms is supported by the evidence that administration of IL-1 receptor antagonist (IL-1Ra) in stroke patients, that antagonizes both IL-1α and IL-1β actions, between 5-6 h from symptoms onset, reduces plasma inflammatory markers and is safe and well tolerated, thus improving clinical outcomes of patients; however, this treatment in combination with intra-arterial thrombolysis has demonstrated no improvement in functional recovery of patients at 3 months (Emsley *et al.*, 2005; Smith *et al.*, 2018).

Moreover, IL-1β is considered a key player of ischemic PC, since its systemic administration prior to ischemia triggers ischemic tolerance and reduces the subsequent post-ischemic inflammation (Ohtsuki *et al.*, 1996; Shin *et al.*, 2009).

Accordingly, our results showed that cortical expression of the proinflammatory cytokine IL-1β are increased in the ischemic hemisphere after 1 h of MCAo followed by 24 h of reperfusion, and interestingly this effect is prevented in preconditioned mice, suggesting that PC-mediated ischemic tolerance associates with the attenuation of the inflammatory reaction observed after ischemic stroke in the lesioned hemisphere.

Brain-resident and blood-borne immune cells play a dualistic role on the progression of ischemic cerebral damage, exerting both detrimental and beneficial functions depending on the specific phenotype acquired (Amantea *et al.*, 2018). In particular, local microglia and circulating leukocytes infiltrating the ischemic brain may polarize toward the classical proinflammatory M1 phenotype, or the alternatively-activated M2 state, which promotes the resolution of inflammation and tissue repair by exerting immunoregulatory functions (Kanazawa *et al.*, 2017). These regulatory immune cells may be implicated in the endogenous neuroprotective mechanisms elicited by ischemic PC (Gesuete *et al.*, 2016).

To evaluate whether ischemic PC affects immune cell polarization in the brain, we analysed cortical expression of Ym1, marker of M2-polarized microglia/macrophages.

Our results showed that the ischemic damage produced by 1 h of MCAo followed by 24 h of reperfusion, induces a discrete increase in the expression of Ym1 in the ischemic hemisphere, as demonstrated by previous studies (Perego *et al.*, 2011; Hu, X. *et al.*, 2012; Zhang *et al.*, 2018), while the preconditioning treatment prior to stroke further potentiates this effect, by producing a marked elevation of Ym1 immunopositive cells.

This interesting result suggests that one of the mechanisms whereby ischemic PC provides its neuroprotective effects is by promoting the polarization of innate immune cells toward the M2 phenotype, that exerts their reparative functions by releasing remodelling factors and anti-inflammatory cytokines leading to the resolution of post-stroke immune reaction (Kanazawa *et al.*, 2017).

By contrast, cortical expression of Ym1 is not affected in mice subjected to the sole PC stimulus at 72 h of reperfusion, corresponding to the time point in which maximal neuroprotection is observed, suggesting that the second injurious ischemia is required to elicit this neuroprotective mechanism.

According to our results, recent work demonstrated that hypoxic PC prior to transient MCAo exerts neuroprotection in rats by promoting microglia activation and enhancing their expression of markers of M2 polarization (i.e. Arg1, Ym1) (Huang *et al.*, 2019). Furthermore, it has been shown that preconditioning microglia by exposure to oxygen-glucose deprivation (OGD) optimal conditions (namely, 18 h of OGD) stimulates the acquisition of M2 antiinflammatory phenotype; while, the intra-arterial administration of preconditioned microglia stimulates the expression of remodelling factors involved in angiogenesis and axonal outgrowth, resulting in improved neurological outcome of rats subjected to focal ischemia (Kanazawa *et al.*, 2017b).

Colocalization studies, aimed at identifying the specific immune cell type expressing Ym1, demonstrated that some Ym1 immunopositive cells also express the neutrophilic marker Ly-6G, but the majority of them colocalize with CD11b, typical marker of microglia and macrophages. Immunofluorescence images showed that

Ym1/CD11b immunopositive myeloid cells are very likely infiltrating macrophages, as they are observed in perivascular regions and display an amoeboid shape, clearly distinguishable from ramified microglia that do not express Ym1.

In accordance with our results, recent studies have proven the involvement of peripheral immune cells in PC-mediated ischemic tolerance, showing that LPS-preconditioning, via the activation of TLR4 pathway, induces in mice the release of splenic Ly-6C^{hi} monocytes, that express typical markers of alternative polarization such as IL-10 and Arg-1 and, reaching the ischemic brain, contribute to the resolution of inflammation (Garcia-Bonilla *et al.*, 2018). In this study, it has also been shown that the elevation of peripheral monocytes in the ischemic brain is not accompanied by concomitant massive neutrophil accumulation (as we have instead observed) and that adoptive transfer of monocytes isolated from mice exposed to LPS are able to provide neuroprotection in naive mice, suggesting their critical role in the induction of cerebral ischemic tolerance (Garcia-Bonilla *et al.*, 2018). Moreover, an increase of peripheral myeloid cells has been detected in the ischemic hemisphere of mice subjected to ischemic PC (McDonough *et al.*, 2020).

Thus, these results strongly demonstrate that ischemic tolerance provided by ischemic PC involves the modulation of the peripheral immune response to stroke towards anti-inflammatory and beneficial/repairing mechanisms.

The anti-inflammatory cytokine IL-10 exerts several immunomodulatory functions and is critically involved in the mitigation of cerebral inflammatory reactions that follow ischemic stroke, by promoting vascular remodelling, reducing leukocytes extravasation and activation, and suppressing the production of proinflammatory cytokines (Garcia *et al.*, 2017). In particular, it has been demonstrated that this cytokine, by binding its receptor, is involved in the process of M2 macrophages polarization, through the activation of the transcription factor signal transducers and activators of transcription (STAT) 3 (Wang *et al.*, 2014; Porta *et al.*, 2015).

Moreover, a pivotal role of IL-10 in the establishment of ischemic tolerance in myocardium has been already demonstrated (Cai *et al.*, 2012). Regarding its role during cerebral ischemic tolerance, recent studies have shown an increased expression of IL-10 in LPS-preconditioned monocytes, that is required for their

neuroprotective functions (Garcia-Bonilla *et al.*, 2018), and that hypoxic PC promotes the expression of IL-10 in the ischemic cortex of rats subjected to transient focal ischemia, after 24 h of reperfusion (Huang *et al.*, 2019).
However, our data show that cortical levels of IL-10 are not affected by the ischemic damage after 24 h of reperfusion from 1 h MCAo, compared to sham group. Conversely, other studies demonstrated an up-regulation of IL-10 levels after 24 h from experimental stroke in rats (Fouda *et al.*, 2013). Moreover, also in the preconditioned group, no significant changes in IL-10 protein levels were detected, suggesting that the neuroprotective immunomodulatory effects of brain ischemic PC, namely the polarization of myeloid cells towards the reparative phenotype, are not mediated by this antiinflammatory cytokine, at least at this time of reperfusion.
Accordingly, a previous work demonstrated that IL-10 expression results upregulated in plasma after LPS-preconditioning, at 24 h of reperfusion from stroke, but not in brain (Vartanian *et al.*, 2011).

To further confirm the immunomodulatory properties of ischemic PC in the periphery, we initially examined its effects on spleen weight modifications induced by transient MCAo in mice.
The splenic response to stroke is a peripheral inflammatory reaction considered one of the main contributor of secondary neural injury, occurring after cerebral ischemia, caused by stored splenocytes released into the bloodstream and infiltrating the brain parenchyma enhancing neurodegeneration (Seifert and Offner, 2018). The spleen is the largest lymphoid organ in the body and is a reservoir of myeloid cells, that can be activated during an inflammatory reaction, by changing in number and location (Lewis *et al.*, 2019). After cerebral ischemia, the spleen responds to injury by an acute transient reduction in its volume, followed by its expansion, in both animal models and stroke patients (Nous *et al.*, 2020).
Our data showed that the 70% increase in spleen weight observed after 1 h of MCAo, followed by 24 h of reperfusion, is abolished in mice pre-exposed to ischemic PC, in which spleen weight is comparable to sham group. By contrast, previous evidence in different stroke models demonstrated that after cerebral ischemia the spleen transiently decreases in weight, reaching its smallest size at 48 h after MCAo; this

splenic atrophy is associated with the release of stored immune cells into the circulation, and as a consequence, with higher cerebral infarct volume (Ajmo *et al.*, 2008; Seifert *et al.*, 2012; Jin *et al.*, 2013; Seifert and Offner, 2018). Indeed, it has also been shown that splenectomy is neuroprotective in the context of ischemic stroke, by preventing the infiltration of immune cells into the brain and consequently the production of cytotoxic mediators (Ajmo *et al.*, 2008; Seifert *et al.*, 2012).

The apparent discordance with our findings may depend on the different time-point at which the measurement was made, being the increased spleen weight at 24 h of reperfusion an initial phase of activation, then followed by the release of immune cells in the periphery, detectable after 48 h. Therefore, this result may suggest that acute enlargement of the spleen observed at 24 h of reperfusion from transient MCAo in our stroke model, could be attributed to its intense activation in response to cerebral injury; interestingly, ischemic PC, by inhibiting this effect, may reduce the amount of activated immune cells released by the spleen that can infiltrate the ischemic brain and worsen the damage.

Confirming this hypothesis, flow cytometry analysis revealed that PC-induced neuroprototection results in a significant attenuation of neutrophil counts (Ly-6G$^+$ cells) elevation observed in the blood of mice at 24 h of reperfusion from 1 h MCAo, demonstrating that ischemic PC mitigates the acute response of the peripheral immune system to ischemic stroke thus protecting the brain.

Indeed, neutrophils are the first blood-borne immune cells to be recruited into the ischemic brain, where they accumulate causing further microvessels occlusion and exacerbate the damage by releasing proteases, cytokines, chemokines and ROS, resulting in BBB disruption, cerebral oedema and cell death (Lambertsen *et al.*, 2019). Consistent with our finding, it has been demonstrated that elevation of neutrophil counts is detected in the blood within 24 h from stroke onset and this correlates with worse functional outcomes, in both animal models and stroke patients (Perez-de-Puig *et al.*, 2015; Cai *et al.*, 2020).

As we observed in our PC model, previous studies have reported that LPS-preconditioning in mice is associated with a reduction of the amount of circulating neutrophils in the blood and, as a consequence, a reduced neutrophil infiltration in the ischemic hemisphere (Rosenzweig *et al.*, 2004). This is in line with the results of

immunohistochemical analysis, showing that the elevation of M2-polarized myeloid cells that we observed in the ischemic cortex of preconditioned mice is mainly sustained by infiltrating macrophages, and only some of these $Ym1^+$ cells coexpress the neutrophilic marker Ly-6G. However the clear involvement of neutrophils in PC-mediated ischemic tolerance has not yet been clarified (McDonough and Weinstein, 2018). Thus, the neuroprotection observed in preconditioned mice in this study may be also related to the reduction of the detrimental effects of neutrophils infiltration in the ischemic brain, confirming that the amount of circulating neutrophils in blood could be considered as an indicator of stroke outcome.

Taken together, these results clearly demonstrate that ischemic PC provides neuroprotection by modulating both central and peripheral immune responses to stroke, reducing the detrimental effects of inflammation on the progression of ischemic cerebral damage and priming the innate immune system to contribute to the resolution of post-ischemic inflammatory reaction. Thus, the identification of the molecular mechanisms involved in the beneficial immunomodulatory effects of ischemic PC would be of great interest for the discovery of potentially druggable targets aimed at potentiating endogenous neuroprotective responses.

Considering the critical role played by SOCE in the modulation of immune cell functions, we hypothesized a potential involvement of this Ca^{2+} current in the neuroprotective immunomodulatory mechanisms elicited by ischemic PC.
Indeed, SOCE represents the main source of calcium in immune cells in response to their recruitment and modulates several of their functions (Vaeth and Feske, 2018).
In particular, the engagement of immunoreceptors on immune cells results in a transient intracellular release of calcium from ER; therefore, the reduction of Ca^{2+} concentration in the ER lumen activates SOCE, thus providing a more robust and sustained calcium influx from the extracellular space (Feske, 2011). Ca^{2+} signals generated by Orai1-STIM1 interaction regulate several functions of immune cells, including differentiation, maturation, proliferation, chemotaxis, phagocytosis and secretion of cytokines and ROS (Demaurex and Nunes, 2016).

Interestingly, immunofluorescence images, performed on coronal brain slices, showed that among the molecular components of SOCE analysed in this work, only Orai1, in addition to its neuronal distribution, was found in myeloid cells expressing Ly-6B.2 marker, which comprises Ly-6G$^+$ neutrophils, Ly-6C$^+$ monocytes and some activated macrophages (Rosas *et al.*, 2010), accumulating in perivascular regions and infiltrating the ischemic hemisphere.

This observation in the brain is related to a dramatic increase of Orai1$^+$ leukocytes in the blood of mice at 24 h of reperfusion from the ischemia of 1 h, as compared to sham operated animals. Moreover, the majority of Orai1$^+$ events also express the neutrophilic marker Ly-6G, demonstrating that ischemia triggers the early activation and recruitment of circulating neutrophils, as previously demonstrated by other studies (Amantea *et al.*, 2015b; Planas, 2018), and enhances their relative expression of the Ca^{2+} channel Orai1.

These results demonstrate that the majority of myeloid cells expressing Orai1 observed in the ischemic brain are actually neutrophils and suggest the involvement of Orai1 in the recruitment of these cells after cerebral ischemia. This evidence is consistent with the critical role played by Orai1 in neutrophils. Indeed, Orai is the main CRAC channel expressed in these cells and drives important functions involved in their activation and recruitment during an inflammatory response (Immler *et al.*, 2018; Grimes *et al.*, 2020).

Surprisingly, ischemic preconditioning did not affect the elevation of Orai1 expression in circulating neutrophils produced by 1 h MCAo. Therefore, we demonstrated that ischemic PC significantly reduces the amount of circulating neutrophils released into the bloodstream after an episode of severe ischemia, but this effect is not mediated by the modulation of the calcium channel Orai1.

Unlike Orai1 (expressed both in neurons and in myeloid cells), the evaluation of cerebral localization of the SOCE-associated regulatory factor SARAF and the Ca^{2+} sensor STIM1, demonstrated that, they are only expressed in neurons. This is an unexpected observation, because it is well known that STIM1 regulates important functions in neutrophils including phagocytosis, degranulation and ROS production (Clemens *et al.*, 2017b), while STIM2 is critical for cytokines production via the NF-κB pathway (Demaurex and Saul, 2018). Also in macrophages both STIM isoforms

mediate effector functions, such as FcγR-dependent phagocytosis, chemotaxis and cytokines production via TLR-4 signalling pathway (Demaurex and Nunes, 2016). However, other findings indicate that neutrophils chemotaxis can also occurs in the absence of STIM (Demaurex and Saul, 2018), and macrophages lacking STIM proteins show no relevant impairment in inflammasome activation, phagocytosis and cytokine production (Vaeth *et al.*, 2015b).

In conclusion, the results of the present work confirm the neuroprotective effects of brain ischemic PC, that significantly reduced cerebral ischemic damage and functional deficits in mice subjected to transient MCAo.

The analysis of the expression of SOCE components in ischemic and preconditioned mice cortices demonstrated that dysfunctions of SOCE currents, depending on the reduced neuronal expression of STIM1 and SARAF, are involved in the mechanisms of ischemic damage. Interestingly, the prevention of SARAF downregulation induced by ischemic PC, that likely restores SOCE activity, is an important mechanism underlying the acquired ischemic tolerance in mice and could be exploited as a new target to induce neuroprotection in the context of stroke treatment.

Furthermore, this research project reaffirms that ischemic PC provides significant neuroprotection in mice, against a subsequent more severe ischemic event, through the modulation of the innate immune response following ischemia. In particular, the established ischemic tolerance is associated with a reduction in the expression of the proinflammatory cytokine IL-1β and with a marked elevation of M2-polarized macrophages in the ischemic hemisphere of preconditioned mice that exert beneficial functions by promoting the resolution of inflammation.

The immunomodulatory effects of ischemic PC were also observed in the periphery, where the preconditioning treatment results in the attenuation of the splenic response to stroke and, as a consequence, in a reduction of the amount of circulating neutrophils released into the bloodstream and recruited to the ischemic brain after an episode of severe ischemia, that contribute to exacerbate the damage.

However, our hypothesis of an involvement of SOCE components in the immunomodulatory effects of ischemic PC could not be validated. Indeed, we demonstrated that STIM1 and SARAF are only expressed in neurons, while Orai1,

that was found in myeloid cells infiltrating the brain, was not modulated by ischemic PC in the periphery. Thus, the effects of ischemic PC on the immune system may be ascribed to other molecular mechanisms.

The present work clearly demonstrates that the neuroprotective effects of ischemic PC may be attributed to its immunomodulatory effects in combination with the attenuation of ER stress response, through the modulation of SOCE-negative regulatory factor SARAF. This multi-target approach, that involves the modulation of both neurons and innate immune cells, makes PC-induced ischemic tolerance a promising strategy in the context of stroke treatment/prevention, that acting at various steps of the ischemic cascade, would allow to increase the efficacy of therapeutic interventions.

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