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CHARACTERIZATION OF A MOUSE MODEL OF HYPOXIC PRECONDITIONING IN NEONATAL HYPOXIA ISCHEMIA

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ABBREVIATIONS

- ADM Adrenomedullin
- AEDs Anticonvulsant Drugs
- aEEG Amplitude Integrated Electroencephalogram
- AMPA Alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid
- ANSCs Activated Neural Stem Cells
- ApoER2 Apolipoprotein E type 2 Receptor
- AS Aspgar Score
- ASCs Adult Stem Cells
- AS-ODNs Antisense Oligodeoxynucleotides
- ATA Atmosphere Absolute
- ATP Adenosin-triphosphat
- Bcl-2 B-cell lymphoma 2
- Bcl-xl B-cell lymphoma-extra large
- BDNF Brain Derived Neurotrophic Factor
- BMPs Bone Morphogenetic Proteins
- BP Blood Pressure
- BrdU [3H]-thymidine or 5-bromo-20-deoxy-uridine
- CA3 Cornu Amonis 3
- CAMKII Ca^{2+}Calmodulin-Dependent Protein Kinase II
- CB Calbindin
- CBD Ca^{2+}-binding domain
- CBF Cerebral Blood Flow
- CD133 Cluster of Differentiation 133
- cEEG Conventional Electroencephalogram
- CFU-E Colony Forming Unit-Erythroid
- CNS Central Nervous System
- CP Cerebral Palsy
- CR Calretinin
- CSF Cerebrospinal Fluid
- CT Computed Tomography
- DA Ductus Arteriosus
- DCX Doublecortin
- DFA Detrained fluctuation analysis
- DG Dentate Gyrus

- DRE Downstream Regulatory Element
- DREAM Downstream Regulatory Element Antagonist Modulator
- DV Ductus Venosus
- E1 Multi-ciliated ependymal cells
- E2 Bi-ciliated ependymal cells
- EAA Excitatory Amino Acids
- ECC Echocardiography
- EEG Electroencephalogram
- EGFR Epidermal Growth Factor Receptor
- Epo Erythropoietin
- EpoR Erythropoietin Receptor
- ERK Extracellular Signal Regulated Kinases
- ESCs Embryonic Stem Cells
- FADD Fas-Associated protein with Death Domain
- $FeCl_3$ Ferric chloride
- FO Foramen Ovale
- FR Free Radical
- GABA Gamma-aminobutyric acid transaminase
- GCL Granule Cell Layer
- GCSF Granulocyte Colony-Stimulating Factor
- GDNF Glial Cells line-Derived Neurotrophic Factor
- GFAP Glial Fibrillary Acidic Protein
- GLU-XIP Glutamate Exchange Inhibitory Peptide
- gp130 Glycoprotein 130
- GSH Glutathione reduced
- GSSG Glutathione oxidized
- HBO Hyperbaric Oxygen
- HIE Hypoxic-Ischemic Encephalopathy
- HIF-1α Hypoxia-inducible factor 1α
- HI Hypoxia Ischemia
- H_2O_2 Hydroperoxide
- HPC Hypoxia Preconditioning
- HRE Hypoxia Response Element
- ICE Interleukin Converting Enzymes
- IGF-1 Insulin like Growth Factor 1

- IL Interlukin
- IFN Intermediate Filament Protein
- IPC Ischemic Preconditioning
- IVC Inferior Vein Cava
- LA Left Atrium
- LPS Lipopolysaccharide
- LV Lateral Ventricles
- MAPK Mitogen-Activeted Protein Kinase
- MCAO Middle Cerebral Artery Occlusion
- MBP Myelin Binding Protein
- mGluR Metabotropic receptors
- ML Molecular Layer
- mmo/l Millimoli/Litro
- MMPs Matrix Metalloproteinases
- mitoKATP mitochondrial ATP-sensitive K^+
- MPTP Mitochondrial Permeability Transition Pore
- MRI Magnetic Resonance Imaging
- MSCs Mesenchymal Stem Cells
- mTOR Mammalian Target of Rapamycin
- NCAM Neural Cell Adhesion Molecule
- NCX Na^+/Ca^{2+} Exchanger
- NeuN Neuronal Nuclei Marker
- NFKβ Nuclear Factor Kβ
- NHE Na^+/H^+ Exchanger
- NICD Notch Intracellular Domain
- NMDA N-methyl-D-aspartate
- nNOS Nitric Oxide Synthase
- NSCs Neural Stem Cells
- NSE Neuron-Specific Enolase
- NSPs Neural Stem Progenitors
- O_2^- Superoxide Radical
- OGD Oxygen Glucose Deprivation
- OH Hydroxyl Radical
- $ONOO^-$ Peroxynitrite
- PA Perintal Asphyxia

- PC Preconditioning
- PDGFs Platelet-Derived Growth Factor
- PFI Prolonged Focal Ischemia
- pHi Intracellular pH
- PI3K/Akt Phosphonidite 3-Kinase and Protein Kinase B
- P7 Postnatal day 7
- PIP2 Phosphatidylinositol 4,5 bisphosphate
- pMCAO Permanent Middle Cerebral Artery Occlusion
- PSA-NCAM Poly-sialylated Neural Adhesion Molecule
- PSCs Pluripotent Stem Cells
- PT Prothrombin Time
- PTT Partial Thromboplastin Time
- PV Parvalbumin
- qNSCs Quiescent Neural Stem Cells
- RA Right Atrium
- RER Rough Endoplasmic Reticulum
- RNS Reactive Nitrogen Species
- ROS Reactive Oxygen Species
- RV Right Ventricle
- SGZ Subgranular Zone
- SHH Sonic Hedgeghog Homolog
- SOD Superoxide Dismutase
- SOM Somatostatin
- STAT Signal Transducer and Activator of Transcription
- SUR1 Sulfonylurea Receptor 1
- SVC Superior Vena Cava
- TGFα Transforming Growth Factors α
- TGFβ Transforming Growth Factor β
- TGFs Transforming Growth Factors
- tMCAO Transient Middle Cerebral Artery Occlusion
- TNFα Tumor Necrosis Factor α
- TPM Topiramate
- VEGF Vascular Endothelial Growth Factor
- VLDLR Very-Low-Density Lipoprotein Receptor
- V/SVZ Ventricular–Subventricular Zone

- XIP Exchange Inhibitory Peptide
- WMI White Matter Injury
- Wnts Wingless protein

SUMMARY

BACKGROUND: Hypoxic-ischemic (HI) damage in neonatal brain is a major risk factor for different human disorders. So far, no drug is presently available to manage this clinical emergency, therefore, the attention of researchers working in the field has been given to endogenous neuroprotective strategies, in order to identify new druggable targets. In particular, one of the best endogenous neuroprotective strategies is Preconditioning (PC), which is a subliminal stimulus able to protect the brain from a subsequent harmful stimulus.

AIM: The first aim of the present work was to identify a new model of hypoxic preconditioning. Then, we investigated whether hypoxic preconditioning (HPC) is able to stimulate the differentiation of neural stem cells (NSCs) in a mouse model of neonatal hypoxia ischemia. In addition, we investigated the relationship between the alteration of ionic homeostasis and the activation of endogenous neurogenesis mediated by NCX. The plasma membrane protein Na^+/Ca^{2+} exchanger (NCX), whose activity has been linked to brain ischemic pathophysiology in adult animals.

METHODS: Seven-day-old C57BL/6 mice were divided into different experimental groups. Hypoxia ischemia was induced by using Rice-Vannucci model. Briefly, they were subjected to ligation and cutting of the right common carotid artery followed by an exposure to hypoxia (92% N_2 and 8% O_2) at different time intervals and sacrificed at different times of reperfusion. Histopathological damage in the hippocampus was determined by measuring the expression level of Propidium Iodide (PI), whereas the neurogenesis was evaluated by using immunohistochemistry with different markers. The development of sensorimotor reflexes was examined by behavioral tests. The increase of NCX expression was evaluated by using immunohistochemical analyses in hippocampus dentate gyrus.

RESULTS: As expected, the greatest damage was found in mice subjected to ischemia plus 60' hypoxia (HI 60') and sacrificed 7 days after ischemia induction. A significant reduction in the hippocampal damage was observed in mice subjected to hypoxia for 20 minutes at postnatal day seven (P7) followed, 3 days later, by HI 60' (P10) and sacrificed at postnatal day eleven (P11). Indeed, this result suggests that 20' hypoxia functions as a preconditioning stimulus. In animals subjected to hypoxic-ischemic insult the damage was mainly localized in CA1 and CA3 regions, whereas the dentate gyrus was spared. Concerning the mechanism by which preconditioning may exert its effects, we found that preconditioning stimulus was able to trigger an increased expression of Nestin, a marker of neurogenesis, in seven-day-old mice. In addition, hypoxic preconditioning was able to determine the increase of proliferating cells ($BrdU^+$/$PSANCAM^+$) and neuroblast cells ($PSANCAM^+$) in the dentate gyrus of HI 60' (P10) mice, sacrificed at P11. In subventricular zone hypoxic preconditioning determined the increase of neuroblast cells ($PSANCAM^+$). Moreover, we observed an increase of NCX1 and NCX3 positive cells in dentate gyrus of preconditioned hypoxic-ischemic animals. Interestingly, the preconditioned hypoxic-ischemic animals also showed a better performance in the cliff avoidance and geotaxis reflex test than HI 60' mice.

CONCLUSIONS: Our results indicate that hypoxic preconditioning is associated to endogenous neurogenesis in immature brain after insult. The increase of neurogenic process is probably correlated to ionic homeostasis maintenance, regulated by NCX proteins. Therefore, NCX proteins can represent potential pharmacological targets for the treatment of brain damage associated to neonatal hypoxic-ischemic encephalopathy (HIE).

1 INTRODUCTION

1.1 NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY (HIE)

1.1.1 DEFINITION AND EPIDEMIOLOGY

The word hypoxia indicates a state of focal oxygen deficit in brain or in other tissues. This condition triggers a mild or severe metabolic acidosis according to the duration of noxious stimulus. In case of prolonged hypoxia or global oxygen deficit, defined “anoxia”, there is a rapid increase of anaerobic glycolysis. Hypoxia is usually followed by ischemia, which consists of a reduction or block of blood flow to a tissue.

It is well known that perinatal asphyxia (PA) and perinatal stroke are correlated, both events belonging to the same pathogenic process. The PA is caused by an alteration of respiratory gas exchange, both placental or pulmonary, and triggers the development of metabolic acidosis (MacLennan A., 1999). The PA can occur in three different moments during gestational age: before partum, during partum and after birth (van Handel M. et al., 2007; American College of Obstetrics and Gynecology, 2003). Similarly to PA, the perinatal stroke occurs from the 20th week of the gestational age, including in utero, to the postnatal 28th day (Nelson KB., 2007). Perinatal stroke includes different types of insult, such as neonatal hypoxia ischemia, perinatal arterial ischemic stroke, neonatal cerebral sinovenous ischemic stroke, and presumed perinatal stroke (Fernàndez-Loòpez D. et al., 2014). In particular, perinatal and neonatal hypoxia ischemia represents an insult that causes acute encephalopathy, seizures, and severe neurologic and cognitive deficits. HIE is a major cause of acute mortality and chronic neurologic deficit in infants and children. Currently, the World Health Organization has estimated that HIE is the fifth largest

cause of children death, till 5 years of age (8%). Statistics suggest an occurrence of systemic asphyxia in 2–4 per 1000 full-term births and an incidence approaching of 60% in premature and low weight infants (Volpe JJ. et al., 1992; Vannucci RC. et al., 2000). Between 20–50% of asphyxiated newborns with hypoxic–ischemic encephalopathy die within the newborn period, and up to 25% of the survivors will show permanent neuropsychological handicaps, including cerebral palsy, mental retardation, epilepsy or learning disability (Vannucci SJ. and Hagberg H., 2004).

1.1.2 ETIOLOGY

HIE is characterized by a multifactorial etiology; maternal, fetal and uteroplacental factors are involved in its development. During the life in utero, the lungs do not work yet, indeed they are not inflated, so the placenta sustains the fetus oxygen and carbondioxide gas exchange, in particular thanks to the chorionic villi which take oxygen from the mother's blood and removes carbon dioxide. The oxygenated blood goes through the umbilical vessels, from the maternal blood to the fetus; on the contrary, the two umbilical arteries carry the carbondioxide from the fetus blood to the mother's blood. The blood, coming from umbilical vein, passes through the *ductus venosus* (DV) or *ductus Aranzio*, avoiding the liver immature, and enters the inferior vein cava (IVC) which carries blood to the right *atrium* (RA) of the heart. Here, *" Eustacchio valve*", a wide membrane, directs the blood flow from RA to the left *atrium* (LA) through the foramen ovale (FO), which has an opening similar to a septal valve, excluding the pulmonary circulation (Hines MH., 2013). The blood through the right ventricle (RV) is pumped in the pulmonary trunk where it meets a second shunt, the *ductus arteriosus* (DA) or *ductus Botallo*, a short vessel which connects the pulmonary trunk to the aorta. As the lungs are collapsed, at high pressure, the blood tends to enter the systemic circulation through the DA (Hines MH., 2013). The aorta conveys blood to the fetal tissues. At the same time, from the superior vena cava (SVC) the deoxygenated blood tries to go directly through the tricuspid valve into the RV; this is the reason whereby it is spread in the pulmonary artery. However, due to the lungs are not yet inflated, the pulmonary vascular resistance remains strong. This fetal process allows the oxygen transport where it is most necessary, the heart and the brain. At birth, the placenta ceases its functions: the three shunts (foramen ovale, *ductus venosus* and *ductus arteriosus*) connect the

right side with the left side of the heart, originating a close vascular circulation (Hines MH., 2013). At this step, the umbilical cord is cut and the venous return from the IVC to the RA decreases, closing the DV. Simultaneously, the lungs start working increasing the venous blood flow towards the right internal. Thanks to the respiratory functions, the blood is oxygenated in the lungs and reach the left atrium through the pulmonary veins. The increasing venous blood in the lungs determines the foramen ovale closure interrupting the communication between the atria. Usually, within 24-48 hours from birth, the pulmonary respiration together with the hormonal changes, the placenta elimination and the umbilical cord (disappearance of prostaglandins, of which the placenta is the biggest producer), induce the DA closure (Hines MH., 2013).

During the gestational age, and in the specific maternal–fetal dyad, these different risk factors can be connected among them, trigging pathologic changes which may predispose to neonatal stroke. It is well known that serious maternal factors as cardiac arrest, asphyxiation, severe anaphylaxis, status epilepticus, hypovolemic shock, severe diabetes, hypertension, and preeclampsia (Wu YW. et al., 2005) may cause HIE. Preeclampsia reduces placental blood flow, determining fetal cerebral hypoperfusion and potential emboli or ischemic injury, global or focal (Fernàndez-Loòpez D. et al., 2014). In addition, it is known that a significant increase of the coagulation factors (V- VII- VIII- IX- X- XII), of the von Willebrand factor and of the plasma fibrinogen concentrations occurs during the pregnancy (Heier LA. et al., 1991; Bremme KA., 2003). Concomitantly, fibrinolytic activity decreases in pregnancy and returns to normal values within 1 hour from the placental ejection. Consequently, tissue plasminogen activator decreases and PAI-1 activity increases. All these physiological alterations of coagulation may cause a pathological process in the

mother, such as thromboembolism, that in turn triggers a major risk of thromboembolism in the fetus (Heier LA. et al., 1991; Bremme KA., 2003). Currently, it is known that other factors can induce neonatal stroke, for example: the increase of lipoprotein (A), the presence of genetic polymorphisms of MTHFR (C677T), Factor V Leiden (G1691A) and prothrombin (G20210A) (Gunther G. et al., 2000; Lynch JK. et al., 2005). Similarly, prothrombotic states connected to neonatal stroke may be induced by some deficiencies such as anticardiolipin antibodies, activated protein C or protein S, antithrombin (Silver RK. et al., 1992; Lynch JK. et al., 2005). In addition, the consumption of drug abuse substances such as cocaine during pregnancy may cause arterial vasoconstriction and vasospasm in the fetus, especially in the first and third trimester (Heier LA. et al., 1991). Finally, childbirth difficulties like vacuum delivery and emergency Cesarean section can be associated with a major risk of neonatal stroke (Fernàndez-Loòpez D. et al., 2014). According to this complex background, it is evident that the placental abnormalities as chorioamnionitis and chronic poor perfusion of the placenta, with acute changes during the partum time, can cause the perinatal arterial ischemic stroke. In retrospective studies, examining the placenta of infants and children affected from cerebral palsy, including hemiplegic cerebral palsy, later in life, it has been observed a high incidence of chronic infarctions, vascular pathology and anomalies of umbilical cord (Kraus FT. et al., 1997; Kraus FT. et al., 1999). Others risk factors are associated with fetal abnormalities like intracerebral hemorrhage, congenital cardiac arrhythmia, cardioembolic events and severe isoimmune hemolytic.

1.1.3 DIAGNOSIS

Since HIE is characterized by a complex physiopathology and multifactorial etiology, researchers use many different criteria for studying it. In such a complicated clinical picture it is really important to get an early diagnosis in order to operate a rapid therapeutic intervention. Therefore, an appropriate neurological examination in the first life days is the more useful predictor for determining potential brain injury occurred in the perinatal period. Currently, the guidelines to diagnose a moderate or severe HIE, in full-term newborn and premature infants born after 34 weeks of gestation, is based on four essential criteria: the analysis of encephalopathy early signals; the analysis of metabolic acidosis; the analysis of multi-systematic organ dysfunction and of the brain image. In fact, to diagnose a case of perinatal asphyxia must be reported all of the following indicators: abnormal heart rate or meconium-stained amniotic fluid, delay in onset of spontaneous respiration; metabolic acidosis (cord pH<7.0 or 7.1 and base deficit >12 mmol/l) in an umbilical artery blood sample; persistence of an Apgar score of <6 or 7 for longer than 5 minutes; neonatal neurologic *sequelae* (cerebral palsy, hypotonia, seizures, mental retardation, auditory, visual and language difficulties); multiple organ involvement (kidney, lungs, liver, heart, intestines); and exclusion of other etiology such as trauma, coagulation disorders, metabolic disorders, infections conditions and genetic disorders (American College of Obstetricians and Gynecologist and American Academy of Pediatrics, 2003; Shankaran S., 2003). Therefore, the diagnosis is based on several steps coming in succession and according to the individual data obtained. The first aspect to monitor in the earliest minutes of life, in full-term infants, is the spontaneous respiration and the Apgar score. The Apgar score (AS) consists of a rating assigned at birth, expressing the vitality of the newborn and considering five vital signs which

are indicative of the infant's ability to survive on his own. A score from 0 to 2 is given to each of all these aspects. The evaluated aspects are following: the skin color, breathing, heart rate, muscle tone, reflexes. The Apgar score, which is the sum of the scores obtained on the five vital signs, therefore, can have a maximum of 10 points (newborn in excellent conditions) to a minimum of 0 points (index of serious difficulties at delivery). This score must be repeated twice: the first one is made about a minute after birth and the second time five minutes later. The identification of spontaneous respiration alterations and the low Apgar score must be associated with laboratory studies such as the analysis of serum electrolyte levels, prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen levels. The blood gas is monitored to assess acid-base status and to avoid hyperoxia and hypoxia, as well as hypercapnia and hypocapnia. Later, the renal parameters and cardiac and liver enzymes are analyzed; anomalous values can indicate the hypoxic-ischemic injury degree to these organs. The following step is the analysis of other molecules into the body fluids and, if increasing, they are, of course, disease indicators. It is well known that in the hypoxic brain there is an increasing expression of specific molecules, such as S100β, Neuron-specific enolase (NSE), Activin A, Adrenomedullin, IL-1b, and IL-6 (Bennet L. et al., 2010). The S100β is a glial protein at low PM (10 KDa), so-called because of its 100% of solubility in ammonium sulfate. It is part of a multigenic proteins family which acts as calcium mediator (S100) (Moore BW. et al., 1965). Mainly, dimers localized in the cytosol, are activated by binding ionized calcium and regulated by cellular activities transcription, differentiation, growth and motility. It is expressed in the astroglial cells of CNS, in the Schwann cells, in the peripheral nervous system, in adipocytes, chondrocytes and melanocytes. Brain proteins are constituted of S100β for 0.05%. It has a neurotrophic role in physiological

concentration and neurotoxic effect at high concentration. NSE is a glycolytic isoenzyme, it is expressed only in the neurons of central and dorsal root ganglia, autonomic ganglia, and some peripheral neuroendocrine cells (Pickel VM. et al., 1976; Kirino T. et al., 1983). Activin A belongs to the superfamily of Transforming growth factor β (TGFβ) and is composed of homodimers of subunits β, mainly synthesized in different areas of the central nervous system (hypothalamus, pituitary, olfactory bulbs). Although it has a neuromodulator role, there is evidence of activity as mitogen for different neuronal populations, survival nerve factor and neuronal differentiation inhibitor. The adrenomedullin (ADM) is a peptide hormone (52 aa). ADM has vasodilator and hypotensive effects, inotropic and natriuretic effects and is stimulated by the increase of cardiac pressure and volume overload. It is expressed in the cardiovascular, pulmonary, renal, gastrointestinal system, and has endocrine effects in brain tissue. Recent studies suggest that the plasma concentration ADM has increased in several diseases such as hypertension, congestive heart failure, chronic renal failure and septic shock (Jougasaki M. et al., 1996; Nishio K. et al., 1997; Bennet L. et al., 2010). Its determination in plasma is limited because of its biological instability and its short half-life. The interleukins are cytokines secreted by various cells of the immune system and are the major effector molecules of inflammation, as they respond to different stimuli and are also present in inflammatory process (Bennet L. et al., 2010). The family of interleukins 1 counts 11 active ligands on different specific receptors. IL-1β is synthesized as inactive pro-peptide and stored in vesicles (Bennet L. et al., 2010). Inflammatory stimuli, such as lipopolysaccharide (LPS) and ATP, determine the maturity of the pro-peptide by the enzyme ICE (interleukin converting enzymes, also called caspase-1) and the secretion of IL-1β, that plays a crucial role in the fast response (Bennet L. et al.,

2010). IL-6 is a cytokine produced by different cells such as macrophages, microglia, astrocytes etc. It has involved in the proinflammatory and anti-inflammatory processes. As proinflammatory molecule IL-6 is released in the peripheral region of the ischemic zone, in the brain of patients it can be measured in the serum where it can be found at high concentrations (Bennet L. et al., 2010). While, as anti-inflammatory, it inhibits the synthesis of Tumor necrosis factor α (TNF-α) and induces the synthesis of the soluble receptors for IL-1 and TNF-α. In ischemia condition, the injection of IL-6 can induce a reduction of damage. Therefore, IL-6 has a protective role in cerebral ischemia (Clark WM. et al. 2000; Suzuki S. et al., 2009). All these markers are not characterized by high specificity because of their high concentrations either in physiological or in pathological conditions. Indeed, in the case of S100β it has been reported that an increase in several clinical conditions such as traumatic delivery (Schulpis KH. et al., 2006; Bennet L. et al., 2010), preterm gestational age (Gazzolo D. et al., 2000) as well as being affected by the sex of the infant (Gazzolo D. et al., 2003; Bruschettini M. et al., 2005; Vicente E. et al., 2007), pre-existing neural injury, intrauterine growth restriction or chronic hypoxia (Loukovaara M. et al., 2009; Bennet L. et al., 2010), central and peripheral hypoperfusion (Routsi C. et al., 2006), treatments such as prenatal maternal glucocorticoids and anesthetics infant (Bruschettini M. et al., 2005; Vicente E. et al., 2007), perinatal infection/inflammation (Bokesch PM. et al., 2002; Friel LA. et al., 2007; Garnier Y. et al., 2009). Furthermore, S100β and NSE may be released from the umbilical cord and placenta, not only in the brain (Wijnberger LD. et al., 2002). Up until now, it has not been defined the precise time window in which S100β reaches a pick, in the disease case. The severity levels of the disease are not always correlated with the different S100β concentration. The analysis of these parameters can predict the evolving injury in a

timely manner. To have a complete and quick diagnosis it is necessary to examine instrumental analysis and biomarker data at the same time. Potential affect subjects to echocardiography (ECC), to highlight anomalies heart like congenital cardiac arrhythmia. Electroencephalography (EEG), standard and amplitude-integrated EEG, are fundamental in the fetuses who developed severe brain injury, the EEG remains highly abnormal (Bennet L. et al., 2010). An epileptiform EEG transients can be found in preterm infants (Scher MS. et al., 1994; Vecchierini-Blineau MF. et al., 1996; Bennet L. et al., 2010) and high levels of these transients are associated with adverse neurodevelopmental outcomes (Scher MS. et al., 1994; Okumura A. et al., 2003; Bennet L. et al., 2010). In addition, the EEG features may also have prognostic value, for example, the variation of the power spectrum (Wong FY. et al., 2007; Bennet L. et al., 2010), evaluation of nonlinear quantitative EEG measures of frequency (Doyle OM. et al., 2007), and long-range correlations in sub-band EEG signals by detrended fluctuation analysis (DFA) may be predictive of outcome and the phase of injury (Jiang D. et al., 2009; Bennet L. et al., 2010). Conventional EEG (cEEG) and amplitude-integrated EEG (aEEG) are performed in the early neonatal period and are highly predictive regarding the long-term outcomes (Obrecht R. et al., 1982; Murray DM. et al., 2009; Bennet L. et al., 2010). Finally, the diagnostic and prognostic capacity of the EEG may be improved when combined with other markers, such as the cerebral oxidative metabolism measure (Tichauer KM. et al., 2009). Other investigations consist of Magnetic resonance imaging (MRI) of the brain, Cranial ultrasonography, Computed tomography (CT) scanning of the head. During an MRI, pictures are taken from the top of the skull to the base and from the front to the rear. Scanning resumes, therefore, all the areas of the brain allowing the analysis of each layer. When the brain is damaged, the lesioned area will form a scar; parts of

the brain that have suffered injuries/scars give off a high signal (hyperintense signal) which differentiate them from normal tissue. Generally, hypoxic-ischemic lesions are detected on magnetic resonance through 4 signals: signal strength increased in the basal ganglia; signal strength increased in the thalamus; signal strength absent or reduced in the back of the internal capsule (white matter structure that carries information over the basal ganglia); dissemination of liquid reduced the diffusion weighted images (Bennet L. et al., 2010). Cranial ultrasonography (ultrasound) is not able to capture all anomalies associated to hypoxic-ischemic encephalopathy. However, it can detect bleeding and the size of the ventricle, the white matter and parasagittal prominent cystic lesions. Ultrasound can be used to detect cerebral edema within 24 hours of hypoxic-ischemic insult, and the results include an overall increase of echogenicity (Bennet L. et al., 2010). These analyses allow the determination of injured areas, providing indicative information about the entity of hypoxic- ischemic event occurred. It is well known that the pathologic lesions and the resulting remote outcomes are different in term newborns compared to preterms. In the first case the lesions are localized in the gray matter; in the preterms the white matter is damaged. Finally, it is necessary to perform retinal and ophthalmic examination, and the pediatric Hearing test. It is an audiometric test that allows to evaluate of person's hearing ability by measuring the sound capacity to reach the brain. This test is performed considering that an increased deafness incidence has been found among infants affected by hypoxic-ischemic encephalopathy who require assisted ventilation.

In clinical practice and for scientific purposes, thanks to an encephalopathy score, it is possible to classify potential HIE affected people. Several classification systems elaborated by Leviton and Nelson provide an overview of qualitative and quantitative

grading schemes. In particular, in 1976, Sarnat and Sarnat developed the most common classification system of disease severity levels, on which is based the modern examination practice. According to the Sarnat system, children assignment is a score of 1, 2 or 3 (1=mild HIE; 2= moderate HIE; 3= severe HIE). In the mild HIE, during less than 24 hours, it is observed hyperalertness, stretch reflexes, sympathetic effect, a normal EEG. In the moderate HIE, it is observed an obtundation, hypotonia, strong distal flexion and multifocal seizures. Infants show a severe HIE present flaccidity and the suppression of brain stem and autonomic functions. In according to these symptoms the EEG is characterized by abnormal patterns of brain activity. To obtain an early diagnosis we have to start from the clinical symptoms to identify cases of cerebral palsy, learning disabilities, and complex behavioral disorders developing later in childhood. However, Sarnat grading is a valid predictive classification system in infants with mild and severe encephalopathy (van de Riet JE. et al., 1999; Haataja L. et al., 2001; Van Schie PEm. et al., 2010). In these cases neonatal MRI has demonstrated a high sensitivity but a lower specificity for predicting good motor outcomes (Keeney SE. et al., 1991; Kuenzle C. et al., 1994; Van Schie PEm. et al., 2010), therefore, children with normal MRI usually have a favorable motor outcome (Haataja L. et al., 2001; Belet N.et al., 2004; Miller SP. et.al., 2005; Van Schie PEm. et al., 2010). Thus, Sarnat grade I and normal neonatal MRI accurately predicts a good motor outcome after neonatal hypoxic-ischemic insult, whereas Sarnat grade II and abnormal MRI predicts a poor outcome with less accuracy. So children with abnormal MRI and altered motor abilities need routinely control concerning their neurological and psychomotorial conditions. Many of these periodical clinical assessments are most sensitive to injury severity beyond the

narrow time window for the treatment beginning (i.e. later than 6 h) (Sarnat HB. and Sarnat MS., 1976).

	Stage 1	Stage 2	Stage 3
Level of Consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular Control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex Reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong; low threshold	Weak; incomplete	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic Function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; poor light reflex
Heart Rate	Tachycardia	Bradycardia	Variable
Bronchial and Salivary Secretions	Sparse	Profuse	Variable
GI Motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common; focal or multifocal	Uncommon
EEG Findings	Normal (awake)	Early: low-voltage continuous delta and theta Later: periodic pattern Seizures: focal 1-to 1-Hz spike-and-wave	Early: periodic pattern with Isopotential phases Later: totally isopotential
Duration	1-3 days	2-14	Hours to weeks

Table 1. Sarnat scoring scale (Sarnat HB. and Sarnat MS., 1976).

1.1.4 PATHOPHYSIOLOGY AND MECHANISMS OF CELL DEATH

Hypoxic-ischemic encephalopathy is caused by the reduction of cerebral blood flow (CBF) to the brain (Ferriero DM., 2004; Perlman JM., 2004), that in turn is due to the alteration of respiratory gas exchange, both placental or pulmonary. In the first phase of insult the organism induces different responses to attenuate the damage in brain, heart, and adrenal glands. At first CBF increases, then there is a lowering of brain temperature and release of gamma-aminobutyric acid (GABA) neurotransmitter. In the cells an increase of anaerobic glycolysis occurs, this triggers the accumulation of lactate, which determines the typical acidosis.

1.1.4.1 EXCITOTOXICITY

Cell death process triggered by HIE can be divided into two main phases. In the early phase there is a progressive cell depolarization (Gunn AJ. et al., 1997; Davidson JO. et al., 2015), and the accumulation of excitatory amino acids, which causes necrotic cell death (Tan WK. et al., 1996; Davidson JO. et al., 2015). In the second phase there is a great release of free radicals (FR), the activation of excitotoxic pathways, and nitric oxide production induced by reperfusion (Juul SE. and Ferriero DM., 2014). These events determine the necrotic and apoptotic death of a large number of cells. In the second phase of prolonged hypoxia or HI injury, the alteration of cellular energy (ATP) induces the depolarization of neurons and glia and the release of excitatory amino acids (EAA) into the extracellular space (Vannucci SJ. and Hagberg H., 2004). In the central nervous system the excitatory neurotransmission is mainly related to glutamate activity. Glutamate interacts with two classes of receptors: ion channels and metabotropic receptors (mGluR) (Palmada M., et al. 1998; Kleman NW. et al., 2010). It is well known that glutamate is

an important trophic factor for the immature brain, besides it plays a key role in development, differentiation, migration and survival affecting progenitor cell proliferation (Kleman NW. et al., 2010). In particular, during hypoxic-ischemic events there is a strong activation of N-methyl-D-aspartate (NMDA), alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors (Gucuyener K. et al., 1999; Juul SE. and Ferriero DM., 2014). The great activation of NMDA receptors causes the increase of intracellular levels of calcium, and consequently it triggers the pro-apoptotic pathways mediated by caspase-3. Finally, the alteration of nuclear structure and DNA damage occur (Juul SE. and Ferriero DM., 2014). All these events cause a profound lesion in the developing brain, and most of the cells undergo death for necrosis or apoptosis. In 1969, Onley and Sharpe were the first to introduced the concept of “excitotoxicity”, demonstrating that neurons exposed to their own neurotransmitter glutamate could die (Olney JW. and Sharpe LG., 1969). Later on, it has been demonstrated that during anoxia, glutamate is released in extracellular compartments and determines neurotoxic effects, triggering a great influx of Ca^{2+} and Na^{+} ions into neurons through its receptors (Choi DW., 1998). Recently, this theory has become the object of different criticisms, because it can only explain some events occurring in the acute phase of anoxic insult (Di Renzo Gf. et al., 2009). In addition, it has been demonstrated that the new generations of glutamate receptor antagonists were not able to protect the brain in animal models of brain ischemia and in human stroke (Di Renzo Gf. et al., 2009).

The limits of this theory can be summarized in the “Hossmann postulates”:

1. Glutamate exposition and ischemic injury induce different metabolic and biochemical response. In fact, after ischemia there is a suppression of energy metabolism and occurs changes in protein synthesis (Djuricic B. et al., 1994).

In contrast, the exposure to a high dose of glutamate does not change energy metabolism and protein synthesis (Hossmann KA., 1993). This aspect indicates that the exposition of high dose of glutamate is able to preserve the proteins from stroke, so it indusces a different damage compared to brain ischemia (Di Renzo Gf. et al., 2009).

2. In experimental models of brain ischemia, the glutamate levels have been measured after insult, demonstrating that the increase of glutamate is not necessarily required for induction of pathological process (Di Renzo Gf. et al., 2009).
3. During ischemia in core region the neuronal death is mainly due to energy depletion, in contrast, in penumbra area the damage can be mediated by the exposition of glutamate toxicity (Kaku DA. et al., 1993).
4. The structural integrity of ischemic penumbra is fully preserved, therefore, excitotoxicity induced by glutamate is not possible in this area (Di Renzo Gf. et al., 2009).
5. Phenomenon of glutamate toxicity has a complete evolution 24 hours after insult *in vitro* experimental studies (Choi DW. et al., 1987), in contrast, *in vivo* it has a complete evolution after 6 hours. Therefore, at same time point, the penumbra area becomes the ischemic core (Back T. et al., 1994).
6. During ischemic event occurs a greater release of inhibitory neurotransmitters (GABA) than glutamate in blood flow, therefore, there is a strong reduce of excitotoxicity (Matusumoto K. et al., 1993).

In conclusions, preclinical studies have suggested that NMDA antagonists are protective in focal ischemia only if administered immediately after insult (Di Renzo Gf. et al., 2009).

1.1.4.2 NON-NMDA DEPENDENT PATHWAYS

Beside glutamate receptors, other membrane proteins involved in ionic homeostasis maintenance have been linked to stroke pathophysiology. Among these are listed: NHE, NCX, $NC_{ca\text{-}ATP.}$ The plasma membrane Na^+/H^+ exchanger (NHE) and H^+ pump are activated to remove the excess of H^+ ions. NHE regulates intracellular pH (pHi) homeostasis and cell volume. Nine isoforms have been cloned, which have different subcellular distribution, kinetic properties, and physiological functions (Xue J. and Haddad GG., 2009). NHE isoform 1 is expressed in all central nervous system (CNS) and is associated with several pathophysiological conditions (Ma E. and Haddad GG., 1997; Luo J. and Sun D., 2007; Kleman NW. et al., 2010). It is activated by ischemia and induces an increase of intracellular Na^+ ions, which promotes Ca^{2+} influx by reverse mode operation of the Na^+/Ca^{2+} exchanger (NCX) (Lee C. et al., 2005; Kintner DB. et al., 2007; Kleman NW. et al., 2010). The great increase of intracellular Ca^{2+} levels and the consequent cell death may involve in the activation of apoptotic mitochondrial pathway, changes of pHi, brain edema; activation of Mitogen-activated protein kinases (MAPKs); and release of excitatory amino acids and ROS. During neonatal HI, the non-selective $NC_{Ca\text{-}ATP}$ cation channel contributes to edema formation (Pedersen SF. et al., 2006). The $NC_{Ca\text{-}ATP}$ channel conducts monovalent cation currents and requires nanomolar concentrations of Ca^+ to open (Chen M. et al., 2001; Kleman NW. et al., 2010). Sulfonylurea receptor 1 (SUR1) regulates this channel, that is activated by depletion of intracellular ATP. In adult animal models, the blockade of SUR1 by glibenclamide reduced cerebral edema, infarct volume and mortality by 50% (Simard JM. et al., 2006; Kleman NW. et al., 2010). In contrast, in a neonatal rat model of HI, glibenclamide is able to protect the brain from moderate insult only (Luo J. and Sun D., 2007; Kleman NW. et al., 2010).

1.1.4.3 OXIDATIVE STRESS

The excitotoxic pathways are directly involved in the activation of neuronal nitric oxide synthase (nNOS) and the generation of nitric oxide. The increase of intracellular levels of NO induces mitochondrial dysfunction and formation of reactive oxygen species (ROS) (Vannucci SJ. and Hagberg H., 2004). The ROS include superoxide anion (O^{2-}), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). All these substances have as target, lipids, proteins and DNA. The damage of these cellular components causes cell death. During oxidative stress, cytoplasmic superoxide dismutase (SOD1) and mitochondrial superoxide dismutase (SOD2) are activated in brain (Vannucci SJ. and Hagberg H., 2004). SOD enzymes convert the oxygen free radicals in H_2O_2, which can be detoxified by catalase or glutathione peroxidase and eliminated as H_2O (Vannucci SJ. and Hagberg H., 2004). Therefore, the alteration of antioxidant system contributes to the increase of brain damage induced by hypoxic-ischemic injury.

1.1.4.4 INFLAMMATION

During cerebral ischemia, in both the parenchyma and the systemic circulation the inflammatory response is activated. In neonatal hypoxia ischemia, even minutes after the insult intervenes an immediate innate immune response (Algra SO. et al. 2013; Liu F. et al., 2013). In particular, the cytokines, important inflammatory mediators, are release in situ of inflammatory reaction (Saliba E. et al., 2001; Liu F. et al., 2013) and maintain brain tissue homeostasis (Hopkins SJ., 2003; Liu F. et al., 2013). In general, the roles of cytokines are pleiotropic, and they can have both pro- or anti-inflammatory effect in the context of ischemic insult. The cytokines related to the inflammatory responses to stroke are interleukins (IL-1, IL-6, IL-10), tumor

necrosis factor-α, and transforming growth factor-β (Han HS. et al., 2003; Liu F. et al., 2013). In the CNS, IL-1β and TNF-α are produced by microglia, astrocytes, and neurons, these cytokines are characterized by potent pro-inflammatory effects. In peripheral blood samples of human newborns affect by HIE (at P1, P3, and P7) were found higher levels of IL-1β and TNF-α than controls (Liu J. et al., 2010; Liu F., et al., 2013).

1.1.4.5 NECROSIS AND APOPTOSIS

Hypoxic-ischemic injury induces necrotic and apoptotic cell death. Both can be classified on the basis of biochemical and morphological criteria (MacManus JP. and Linnik MD., 1997; Vannucci SJ. and Hagberg H., 2004). Necrotic death is induced by severe hypoxic-ischemic insult and is characterized by the disruption of membrane integrity, the release of cytoplasmic molecules into the extracellular space and a secondary inflammatory response (Vannucci SJ. and Hagberg H., 2004). In contrast, programmed cell death or apoptosis is a regulated cell commit suicide. The process is regulated by a family of cysteine proteases, called caspases. Caspase-3 is involved in the cleavage of different substrates that are vital for cell survival (Cohen GM., 1997; Vannucci SJ. and Hagberg H., 2004). Caspase-3 is activated by intrinsic or extrinsic pathways. The activation of intrinsic pathway induces the release of cytochrome *c* and formation of the apoptosome and, subsequently, caspase-9 activation. The activation of extrinsic pathway mediates the binding between specific ligands and their receptor, known as death receptors. In particular, the FAS-ligand binds the FADD-receptor, which leads to caspase-8 cleavage and activation of caspase-3 (Vannucci SJ. and Hagberg H., 2004). In the neonatal hypoxic-ischemic

brain the apoptosis is the main cell death mechanism (McDonald JW. et al., 1997; Kleman NW. et al., 2010). Northington and his colleagues demonstrated that in the early hours after HI induction there was a great number of necrotic cells in the ipsilateral forebrain, in the striatum and cortex of P7 rats (Northington FJ. et al., 2001; Kleman NW. et al., 2010). While the number of apoptotic cells increased in ipsilateral ventral basal thalamus and in the ipsilateral cortex respectively at 24 and 48 hours after induction of insult (Northington FJ. et al., 2001; Kleman NW. et al., 2010).

1.1.5 THERAPY IN HIE

Early diagnosis of hypoxic ischemic encephalopathy is extremely important because timely treatment may reduce the devastating effects of the insult. Currently, in the neonatal intensive departments in case of suspicion of HIE insult, vital signs together with of biochemical and blood gas values are monitored, resulting in correction of metabolic changes. In particular, clinical procedures provide resuscitation and stabilization, the possibility to proceed with artificial ventilation, monitoring of blood pressure (BP), trying to maintain above 35-40 mmHg, checking of fluid and levels of glycemia. Nowadays, the therapies used have been suggested by international protocols, and their effectiveness depends on the fact that they must be administered in a given time frame from the insult.

1.1.5.1 ANTI-EXCITOTOXIC AGENTS

In the early phase of hypoxic-ischemic insult the infants are treated with anti excitotoxicity agents. In 1990, Grigg and Anderson reported that the use of competitive and noncompetitive NMDA receptor antagonists, in rat hippocampal slices, was able to ameliorate the hypoxia-induced functional failure (Grigg JJ. and Anderson EG., 1990; Villa RF. and Gorini A, 1997). Recent clinical trials conducted in Australia and in the USA supported the use of antenatal magnesium. In particular, in New Zealand, woman at 30 gestation weeks were subjected to infusion of 16 mmol of $MgSO_4$ followed by 8 mmol for up to 24 hours, this study demonstrated that $MgSO_4$ reduced death or determined substantial gross motor dysfunction, but there were not any significant differences in mortality rate or cerebral palsy in survivors. In the USA, it has been demonstrated that the use of $MgSO_4$, in the perinatal period,

reduced the negative effects of severe cerebral palsy (CP) (Costantine MM. et al., 2011; Juul SE. and Ferriero DM., 2014).

It is well known that the noble gas Xenon is an antagonist of the NMDA receptors. During hypoxic condition, Xenon induces the increase of Hypoxia-inducible factor 1α (HIF-1α) expression (Ma D. et al., 2009; Juul SE. and Ferriero DM., 2014); and so the upregulation of erythropoietin (Epo), vascular endothelial growth factor (VEGF) and glucose transporter 1 protein (Ma D. et al., 2009; Juul SE. and Ferriero DM., 2014).

1.1.5.2 ANTI-OXIDANT AGENTS

In the early phase of hypoxic-ischemic insult the infants can be also treated with anti-oxidant agents. Allopurinol is a xanthine-oxidase inhibitor, at high concentrations it inhibits the formation of free radical. In preclinical studies in postnatal seven-day rats after HI, administration of allopurinol (135 mg/kg subcutaneously) 15 min after cerebral hypoxia ischemia, reduced brain edema and attenuated long-term brain damage (Palmer C. et al., 1993; Juul SE. and Ferriero DM., 2014). The use of allopurinol in humans have been reported in several clinical trials. In some of them allopurinol was assumed by mothers of fetuses with a suspected intrauterine hypoxia (Kaandorp JJ. et al., 2010; Torrance HL. et al., 2009). In each trial a specific marker was monitored during the gestational age, the health of newborns was monitored up to two years. These studies showed that there was an increase of marker levels in cord blood of newborns affected by hypoxia ischemia at N36 weeks gestation (Torrance HL. et al, 2009). Recently, it has been demonstrated that a new class of oxygen free radical scavenging agents, non-glucocorticoid 21-

aminosteroids, has a protective role in cerebral ischemia models (Perkins WJ. et al., 1991; Villa RF. and Gorini A, 1997).

1.1.5.3 ANTI-INFLAMMATORY AGENTS

Recent studies showed the neuroprotective role of melatonin in white matter and learning disabilities. *In vivo* model, it has been demonstrated that blocking of melatonin release produces a substantial white matter injury (Welin AK. et al., 2007; Juul SE. and Ferriero DM., 2014). It has been observed that a significant reduction of melatonin induced the activation of microglia in the neonatal model of hypoxia ischemia (Villapol S. et al., 2011; Juul SE. and Ferriero DM., 2014). In piglet model it has been demonstrated that melatonin, in combination with hypothermia, preserves brain function measured by aEEG, and reduced cell death in the thalamus, the region most affected by the asphyxia insult (Robertson NJ. et al., 2013, Juul SE. and Ferriero DM., 2014) Moreover, it has been reported that Omega-3 polyunsaturated fatty reduces brain damage and improve neurological functions in HI mice after insult. The Omega-3 compound works as inhibitor and prevents the release of inflammatory mediators (Zhang W. et al., 2010; Juul SE. and Ferriero DM., 2014). In rodent model of HI, it has been demonstrated that the administration of ibuprofen daily for 1 week determined the reduction of brain injury, because the drug contrasted the effects of inflammatory mediators (Wixey JA. et al., 2012).

1.1.5.4 ANTI-CONVULSANT AGENTS

Up until now, it is well known that moderate and severe hypoxic-ischemic insult are always accompanied by seizures, resulting in a deterioration of the cerebral

lesions, therefore, the effective prevention of neonatal seizures could attenuate brain injury. Anticonvulsant drugs (AEDs) are a heterogeneous class of drugs useful to suppress repetitive neuronal depolarization and high frequency that evoke seizures. Many of these drugs act by affecting the conductance of K^+, Na^+, Ca^{2+} and thus interfere with neurons membrane potentials. In addition, they prevent the spread of neuronal depolarization within the brain and offer protection against brain damage caused by seizures. Currently, topiramate and levetiracetam are indicated for the treatment of perinatal asphyxia affections (Kim JM. et al, 2009). Topiramate (TPM) is a monosaccharide substituted, characterized by good absorption, high bioavailability, and good tolerability. TPM possess different mechanisms of action: (I) inhibits AMPA/kainate (Follett PL. et al., 2004; Kaminski RM. Et al., 2004; Koh S. et al., 2004; Angehagen M. et al., 2005; Sfaello I. et al., 2005; Filippi L. et al., 2012), (II) blocks the pre-synaptic voltage-sensitive Na^+ channels of excitatory synapses (Zona C. et al., 1997; Filippi L. et al., 2012), (III) blocks the high voltage-activated calcium currents (Costa C. et al., 2006; Filippi L. et al., 2012), (IV) impairs mitochondrial permeability transition pore (MPTP) (Kudin AP. Et al., 2004; Filippi L. et al., 2012), (V) inhibits carbonic anhydrase isoenzymes (Dodgson SJ. et al., 2000; Filippi L. et al., 2012), and enhances GABA-mediated chloride flux and GABA-evoked chloride currents in murine brain neurons, thus increase seizure threshold (White HS. et al., 1997). In animal models of HI (postnatal day 7), it has been demonstrated that the immediate administration of TPM after injury induces neuroprotection, mainly because of the AMPA-kainate activity reduction (Follett PL. et al, 2004). In neonatal stroke model, the effects of TPM treatment were observed in multi-strategy therapies. The combined treatment of TPM and hypothermia conferred protection, improving functional performance better than hypothermia alone (Liu Y. et al., 2004; Cilio MR.

et al., 2010). Notably, the combined treatment of TPM, levetiracetam and phenytoin induced the increase of cell death, in contrast, TPM and levetiracetam treatment reduced the brain injury (Kim J. et al., 2007a). It has been reported that levetiracetam has a good efficacy in seizure treatment (Mazarati AM. et al, 2004) and reduced the infarct volume in focal ischemia (Hanon E. and Klitgaard H., 2001).

1.1.5.5 ERYTHROPOIETIN

Erythropoietin (Epo) is a glycoprotein hormone, produced in humans mainly by peritubular interstitial cells of kidneys, that perceive the alterations of oxygen levels in organism. Therefore, the Epo production is regulated by a sensitive feedback system. During hypoxic condition, HIF-1α translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β, generating the heterodimeric complex (HIF-1α and HIF-1β), able to bind to hypoxia response element (HRE) in the 3' enhancer of the gene for erythropoietin, finally inducing the increase of expression of Epo gene (Wang GL. et al., 1995; Ke Q. and Costa M, 2006). The increase of Epo expression stimulates the rapid expansion of erythroid progenitors. Conversely, during normoxic conditions, HIF-1α is rapidly degraded by prolyl-hydroxylase. The Epo acts in bone marrow hematopoietic framework by stimulating the colony forming unit-erythroid (CFU-E), reducing apoptosis and increasing the frequency of mitosis (faster differentiation in proerythroblast). It is well known that in humans and murine brain, during both fetal development and adult life, a local production of Epo occurs (Juul SE. et al., 1998; Juul SE. et al., 1999). Epo binds to its specific receptor, Erythropoietin receptor (EpoR), expressed on the surface of the membranes. EpoR is a transmembrane glycoprotein, that is a component of cytokine type I receptor superfamily; it is expressed on erythroid cells but also on the different brain cells,

such as glial cells (Nagai A. et al., 2001), neurons (Morishita E. et al., 1997), endothelial cells (Yamaji R. et al., 1996), of different brain regions, such as hippocampus, cortex, internal capsule and midbrain (Digicaylioglu M. et al., 1995). Binding of Epo on its receptor determines the homodimerization and activation of the same receptor. This, in turn, leads to the activation through phosphorylation of receptor associated to tyrosine kinase (Janus Kinase 2); phosphorylation and activation of MAPK, extracellular signal regulated kinases (ERK1/2), as well as the phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) pathway and signal transducer and activator of transcription (STAT5). All these pathways are involved in the activation of anti-apoptotic pathways, that induces the expression of anti-apoptotic Bcl-xl (Bcl2 family member), which mediates protective effects on neurons and oligodendrocytes (Digicaylioglu M.et al., 2001; Juul SE. and Ferriero DM., 2014). In particular, Epo signaling inhibits early mechanisms of brain injury by its anti-excitotoxic (Zacharias R. et al., 2010), anti-oxidant (Kumral A. et al., 2005) effects. In neonatal hypoxic-ischemic model, it has been demonstrated that the administration of high doses of Epo decreased the brain damage, reducing neuronal loss, improved the functional activities, and promoted neurogenesis and oligodendrogenesis (Gonzalez FF. et al., 2007; Iwai M. et al., 2007). Epo also protected the vascular integrity of capillaries following injury through VEGF (Bocker-Meffert S. et al., 2002; Ke Q. and Costa M, 2006; Wang L. et al., 2008) and participated in preservation of the blood-brain barrier (Marti HH. et al., 2000; Ribatti D. et al., 2003; Ke Q. and Costa M, 2006). In addition, it has been demonstrated that Epo also mediated the anti-inflammatory effects, in fact, it reduced the activation of astrocytes reactive cells, microglia, immune cells and relation of inflammatory cytokines in the damaged area (Sun Y. et al., 2005; Juul SE. et al., 2009). Recently, it has been demonstrated that

recombinant Epo and the mimetic drugs reduction brain damage in neonatal hypoxia ischemia (Sola A. et al., 2005; Kellert BA. et al., 2007; Traudt CM. et al., 2013) and in intraventricular hemorrhage or periventricular leukomalacia (Kumral A. et al., 2007). The drugs do not cause problem also in high dose (McPherson RJ. et al., 2007) and are able to pass the blood-brain barrier. In particular, the administration of Epo doses of 1000-5000 U/kg/dose resulted in sustained neuroprotection, improving both short and long-term structure and function. Encouraging data on animal models induced to the translation on clinic trials; this trials should not be used in prenatal time because the Epo doesn't cross placenta. Nowadays, there are Phase I/II trials, which tend to establish Epo safety and translational pharmacokinetics profiles in preterms (Fauchere JC. et al., 2008; Juul SE. et al., 2008) and term neonates (Wu YW. et al., 2012). In addition, there are trials that analyze the effect of combination of Epo or darbepoetin with hypothermia in term HIE, however, data are not also clear.

1.1.5.6 HYPOTHERMIA

Therapeutic hypothermia represents the first accepted treatment able to attenuate brain injury in neonatal HIE (Walsh BH. et al., 2011). Nevertheless, the mechanisms involved in hypothermic neuroprotective effect are not fully known. The neuroprotective effect of hypothermic treatment has been demonstrated in neonatal animals that were subjected to a protocol of controlled cooling to 33–34 C°, within 6 hours at HIE insult, and were maintained at this temperature for 72 hours (Azzopardi DV. et al., 2009). This treatment inhibits many of the processes involved in primary and secondary energy failure (Laptook AR., 2009b). Moreover, it has been observed in rats, that hypothermia induces neuroprotection in hippocampus, through an increased expression of glial cells line-derived neurotrophic factor (GDNF), and with

the decrease of expression of TNFα and IL-6 and their mRNA levels (Xiong M. et al., 2009; Kleman NW. et al., 2010). Therefore, in neonatal model of HI the hypothermic treatment determines the inhibition of inflammatory response astrocyte, due to the reduction of glial fibrillary acidic protein (GFAP) levels (Xiong M. et al., 2009; Kleman NW. et al., 2010). Thus, a rapid and accurate diagnosis allows only the cooling of infants with moderate to severe HIE (Azzopardi DV. et al., 2009).

Currently, in HIE affected seven clinical trials on the therapeutic efficacy of hypothermia have been analyzed (Gluckman PD. et al, 2005; Azzopardi DV et al, 2009; Simbruner G. et al, 2010; Zhou WH. et al, 2010). These trials are characterized by stringent inclusion criteria. The infants must have been born at least after 36 weeks of gestation. They must have a weight of 1800-2000 g at birth. The newborn must show evident clinical signs, such as Apgar score of 5 or less at 10 minutes after birth, severe acidosis (pH<7), lethargy, coma, abnormal tone or posture, abnormal reflexes (stretch reflexes, grasp, Moro). In addition, the affected must have bradycardia, abnormal pupils, apneas and an alteration of aEEG.

In particular, Azzopardi et al. performed a randomized trial on asphyxiated newborns at 36 weeks of gestation. The affected were divided into two groups: the first group was subjected, within 6 hours from the birth, at cooling of the body to 33.5°C for 72 hours plus intensive care, while the second group was subjected to intensive care only. They demonstrated that the hypothermic treatment improved only neurologic functions in the first group compared with the second one (Azzopardi DV et al, 2009). The infants were monitored until 18 months of age.

In contrast, Zhou et al. demonstrated that the HIE newborns subjected to cooling at temperature of 34.5°± 0.2°C for 72 hours, 6 hours after birth, showed a decrease of

the combined death and severe disability (Zhou WH. et al, 2010). The infants were monitored until 18 months of age.

1.2 ANIMAL MODELS OF NEONATAL WHITE MATTER INJURY (WMI)

Currently, the mechanisms involved in HIE pathophysiology, are not completely known as the experimental models are not able to reproduce the complex background of this disease. The majority of the available information has been obtained thanks to the use of hypoxic-ischemic animal models. In the most part of these experimental studies a model of unilateral hypoxic–ischemic brain damage has been utilized in immature rat (Rice JE. et al., 1981; Vannucci RC. et al., 1996; Vannucci RC. et al., 1997). Recently, this model has been extended to immature mouse (Sheldon RA. et al., 1998). In the latest years, from this model other animal models have been developed in order to mimic the different pathophysiological features of the perinatal brain injury.

1.2.1 RICE AND VANNUCCI MODEL

The first model of neonatal HI was developed by Vannucci and Rice in rats (Rice JE. et al., 1981). The Vannucci/Rice method consists of an unilateral carotid artery ligation carried out in postnatal day seven rats followed by exposure to 8% oxygen/balanced nitrogen at 37°C. The unilateral carotid artery ligation alone does not induce ischemic damage because the circle of Willis maintains the reoxygenation of brain tissues. In contrast, the unilateral common carotid ligation and the exposition to 8% oxygen induce to hypoxemia and hypocapnia produced by hyperventilation (Vannucci RC. et al., 2005). These events cause the activation of anaerobic glycolysis and the lactic acid accumulation. It is well known that this model reproduces the damage to ipsilateral hippocampus, striatum, thalamus and cortex (Welsh FA. et al., 1982). In rats, the model is reproduced at P7, which correlates

histologically to a 32 to 34 week human neonate (Vannucci RC. et al., 2005). In mice, the model is reproduced at P7 to P10 (Barks JD. et al., 2008; Doverhag C. et al., 2008; Stone BS. et al., 2008). In this period, brain mice have morphological similarities to the third-trimester neonatal brains, in particular for what concerns cellular proliferation, cortical organization, synapse number, neurotransmitter synthetic enzymes and electrophysiology (Liu XH. et al., 1999). According to age and species, animals are subjected to different time intervals of hypoxia. It is well known that 150 minutes of hypoxia induces a severe damage in rats (Northington FJ. et al., 2001; Jiang X. et al., 2008). In P7 mice 30 minutes of hypoxia may cause moderate injury while 1 hour causes a severe injury (Kendall GS. et al., 2006). As concern the regional distribution of brain damage, in rats, 48 hours after insult, necrotic and apoptotic cells are mainly localized in the ipsilateral cortex and striatum (Northington FJ. et al., 2001), while in mice the damage is restricted to ipsilateral hippocampus (Kendall GS. et al., 2006).

1.2.2 CHRONIC AND INTERMITTENT HYPOXIA MODEL

Cerebral palsy may develop in infants with chronic lung disease, correlated to premature birth or multiple hypoxic episodes. Some pathological features of developmental WMI can be reproduce through continuous or intermittent hypoxia in neonatal rodent models. In the model of chronic hypoxia, from postnatal days 3 to P13 or to P33, the rodents are placed with their dam in a hypoxic chamber in which the levels concentration of O_2 are continuously monitored. Hypoxia was maintained at 9.5 ± 1.0% O_2, the air continuously diffused within the chamber, and excess carbon dioxide and humidity are filtered. Twice weekly the chamber was opened for less than 5 minutes, then the water and food are changed (Ment LR. et al., 1998; Back

SA. et al., 2006; Chahboune H. et al., 2009; Scafidi J. et al., 2009). It has been demonstrated that mice subjected to chronic perinatal hypoxia showed long-term neurobehavioral deficits (Chahboune H. et al., 2009), ventriculomegaly and hypomyelination. The ventriculomegaly is a consequence of the loss of cerebral tissue, while the hypomyelination is correlated to reduction of early OL progenitors (Back SA. et al., 2006).

In the model of intermittent hypoxia, from postnatal days 2 or 3 (P2 or P3) for 28 days, the mice are exposed to alternating cycles of 2 min of 6.0–7.5% O_2 followed by 3 min of normoxia, which were generated by balances of 100% N_2 and 100% O_2 gases (Douglas RM. et al., 2003). CO_2 levels and humidity were maintained near physiological levels. This cycle is replicated during daylight hours for all experimental period (Douglas RM. et al., 2003). It has been demonstrated that the exposition of intermittent hypoxia caused a significant neuronal injury in the hippocampus and thalamus (Douglas RM. et al., 2007). Thus, chronic and intermittent hypoxia determine a different altered genes responsible for cell signaling, development, metabolism and cell death (Zhou D. et al., 2008).

1.2.3 INTRAUTERINE HYPOXIA ISCHEMIA MODEL

Intrauterine models of transient global ischemia have been described in the rat and rabbit by housing pregnant dams in hypoxic conditions or by the abruption of placental blood flow by inflation of a balloon catheter inserted into the uterine artery, respectively (Cai Z. et al., 1995; Hersey K. et al., 1995; Cai Z. et al., 1998). This model reproduces many histopathological aspects of developmental WMI (Derrick M. et al., 2007). In rabbits, the lesions involve the cerebral gray and white matter, in some cases, there is an intraventricular hemorrhage with ventriculomegaly and

periventricular white matter loss. In addition, the surviving rabbit kits show a wide range of hypertonic motor deficits that resemble CP. This model allows to analyze the clinical-pathological correlations with neurobehavioral outcomes and neuroradiological assessments (Drobyshevsky A. et al., 2005). Moreover, intrauterine hypoxia ischemia model has also been extensively used to study the cellular mechanisms of WMI (Buser JR. et al., 2010).

1.3 PRECONDITIONING

The brain is constantly subjected to influence of intrinsic and extrinsic stimuli. During stressful and pathological conditions brain is able to activate endogenous neuroprotective strategies able to preserve its integrity. Among these neuroprotective strategies, preconditioning has been widely studied in the last decades for its potential therapeutic implications. Preconditioning (PC) represents a subliminal injurious stimulus applied before a longer harmful ischemia (Kirino T., 2002; Dirnagl U. et al., 2003; Gidday JM., 2006) is able to exert a remarkable neuroprotection, thus establishing a state of tolerance to anoxic conditions (Pignataro G. et al., 2012). The toxicologist Paracelsus studied for the first time this phenomenon during the 16th century. He observed that low levels of cellular stress induced the activation of a protective response, as is summarized from one of his important mentions: “The dose makes the poison” (Stetler RA. et al., 2014). PC as phenomenon protective strategy applicable to different organs. In fact, in 1986 Murry et al., observed that intermittent brief ischemia followed by reperfusion protects the myocardium from acute ischemic episodes (Murry CE. et al., 1986). Currently, PC is being largely studied in brain ischemia, however, several researchers are examining this phenomenon also in other neurodegenerative diseases, such as Parkinson’s (Stetler RA. et al., 2014). It is well-known that PC protection can be activated into two different phases: “rapid or acute preconditioning” (Perez-Pinzon MA. et al., 1997). While “rapid preconditioning” starts 3–5 minutes after the preconditioning stimulus and ends 1 hour later; “delayed preconditioning” (Perez-Pinzon MA. et al., 1997), begins 2–3 days after preconditioning and ends 1 week later (Dirnagl U. et al., 2003). Rapid preconditioning induces a transient and less strong neuroprotection than delayed preconditioning, in addition, it is not associated with de novo protein synthesis (Barone FC., et al., 1998;

Dirnagl U. et al., 2003). Conversely, delayed preconditioning is associated with longer molecular changes (Durukan A. and Tatlisumak T, 2010), as the neosynthesis of heat shock protein 72, the induction of transcription factors, and the activation of anti-apoptotic proteins (B-cell lymphoma 2) (Brambrink AM. et al., 2000) and antioxidant enzymes (Toyoda T. et al., 1997). The effects of preconditioning stimuli have been studied through *in vitro* and *in vivo* models of stroke, demonstrating that they induced anti-apoptotic mechanisms with consequent survival of tissue after acute ischemia, mainly through the activation of PI3K/AKT signaling pathway (Hausenloy DJ. et al., 2006). Ischemic preconditioning (IPC) triggers the synthesis of antioxidant molecules, such as MnSOD, through activation of a specific pathway. At first time, it induces the reduction of ATP synthesis and promotes the activation of mitochondrial ion channels, such as the mitochondrial ATP-sensitive K^+ (mitoKATP) channel (Otani H. 2004; Sart S. et al., 2014). This event determines a mild ROS release, which triggers the activation of protein kinase C, the activation of nuclear factor kappa beta and finally the synthesis of MnSOD (Otani H. 2004; Sart S. et al., 2014). Therefore, IPC treatment protects neurons and non-neuronal tissues, furthermore, it reduces infarct volume at least 33% in IPC if compared with non-IPC treated controls (Schaller B. and Graf R., 2002).

1.3.1 HYPERBARIC OXYGEN PRECONDITIONING AND NEONATAL HYPOXIA ISCHEMIA

Hyperbaric oxygen (HBO) is a condition obtained through infusion of 100% O_2 flow in a pressurized chamber. This state may protect the brain from following focal and global ischemia. In fact, it has been demonstrated, in gerbil and in rat, that the

exposition to 2-2.5 pressures of atmosphere absolute (ATA) every day for 3 or 5 sessions induced tolerance against global ischemia (Wada K. et al., 1996, Cheng O. et al., 2011). In adult rats, subjected to middle cerebral artery occlusion (MCAO), it has been demonstrated that the exposition to 2.5 ATA for 1 hour per days for 5 days until 24 hours before induction of insult, protected brain against global or focal cerebral ischemia (Ostrowski RP. et al., 2008). In contrast, in the same *vivo* model, it has been demonstrated that the exposition to 2.5 ATA for 1 hour at 24, 12, 6 hours before induction of insult, the preconditioning induce less protective effects (Ostrowski RP. et al., 2008). In HI model it has been proven that the exposition to 2.5 ATA for 2.5 h, followed to HI insult 24 h later, protects immature brain (Freiberger JJ. et al., 2006; Li Z. et al., 2008b).

1.3.2 HYPOTHERMIA AND HYPERTHERMIA PRECONDITIONING IN NEONATAL HYPOXIA ISCHEMIA

Currently, the therapeutic hypothermia is considered the first accepted treatment in neonatal HIE disease (Walsh BH. et al., 2011). In fact, in some clinical trials, HI full-term newborns are subjected to hypothermia, that is considered the gold standard method to protect the immature brain. In addition, it has been demonstrated that the hypothermia can represent a preconditioning stimulus. In particular, in adult rats subjected to twenty minutes of hypothermia (31–32°C), and following subjected to focal ischemia it has been proved that the hypothermia induced a delayed preconditioning, with the effect beginning 6 h after preconditioning and persisting for 2 days after (Nishio S. et al., 1999; Nishio S. et al., 2000). *In vitro* model of ischemia have been observed that 20’ of hypothermia (33°C), before OGD reduces Purkinje cell death in rat cerebellar slices (Yuan HB. et al., 2004; Yuan HB. et al., 2006).

Recently, it has been also reported that hyperthermia is able to act as a preconditioning stimulus *in vitro* and *in vivo* models. In particular, it has been proved that hyperthermic preconditioning prevents disruption of blood–brain barrier, in this way it has been analyzed the reduction of damage in newborn rat subjected to HIE insult (Tomoaki I. et al., 1999). In adult rats it has been well established that the exposition of animals for 15 min in a water bath set at 42°C, before MCAO, reduced infarct volume 18 or 24 h later injury (Xu H. et al., 2002) and improved learning abilities and memory following diffuse axonal injury (Su Z. et al., 2009). About HI model, there are few evidences on the protective role of hyperthermic preconditioning.

1.3.3 PHARMACOLOGICAL PRECONDITIONING IN NEONATAL HYPOXIA ISCHEMIA

The neuroprotective effect of different drugs used as preconditioning agents has been evaluated in several studies. In particular, it has been characterized the neuroprotective effect of the inhalation of anesthetics like sevoflurane and isoflurane, in adult and neonatal cerebral ischemia (Kitano H. et al., 2007; Li L. and Zuo Z., 2009; McAuliffe JJ. et al., 2007; Zhao P. et al., 2007; Zhao P. and Zuo Z., 2004; Zheng S. and Zuo Z., 2004; Zhu W. et al., 2010). In 2012, Yang et al. reported that the effects of sevoflurane preconditioning in mice subjected, 5 days before MCAO, to 2.5% sevoflurane for 1 hours everyday and sacrificed 72 hours after ischemia induction. The results showed that there was a reduction of brain damage and improved of neurobehavioral performance in preconditioned mice compared to control mice. In this study, they suggested that the effects of sevoflurane preconditioning can be mediated by activation of canonical Notch signaling pathway.

In particular, their results demonstrated that the sevoflurane preconditioning increased of Notch intracellular domain (NICD) protein that then travels to the nucleus to associate with the DNA-binding protein (RBP-J) (Yang Z. et al., 2012). These events determined that activation of proteins involved in different physiological pathways, including proliferation, differentiation processes (Kopan R et al., 2009; Andersson ER. et al., 2011) and proteins involved in neuroprotective mechanisms (Yang Z. et al., 2012). *In vivo* model of HI, it has been demonstrated that the exposition to 1% or 2% of isoflurane 15 minutes and 24 hours before the HI insult protects the brain of P7 pups (Sasaoka N. et al., 2009), and improves later functional outcomes in adult mice (McAuliffe JJ. et al., 2007; Zhao P. et al., 2007).

1.3.4 HYPOXIC PRECONDITIONING IN NEONATAL HYPOXIA ISCHEMIA

Hypoxic preconditioning (HPC) stimulus triggers a rapid state of tolerance and has a limited time frame, of approximately 72 hours (Bernaudin M. et al., 2002; Prass K. et al., 2003; Zhan L. et al., 2010; Stetler RA. et al., 2014). Hypoxic preconditioning has been studied *in vitro* and in the neonatal and adult in *vivo* model. The stimulus is reproduced in hypoxia chamber, where oxygen is replaced by nitrogen, typically to normobaric hypoxic conditions (8% oxygen). In the neonatal rats (P6–7) subjected to hypoxia (8% O_2; 92% N_2) in a humidified chamber held at 37°C, then followed by normoxia for 24 hours, and then pathological HI, it has been demonstrated that hypoxia triggers preconditioned state (Gidday JM. et al., 1994; Ota A. et al., 1998). Neural and behavioral outcomes are improved in preconditioning group compared to non-preconditioned groups, for an extended time period (Gustavsson M. et al., 2005). In 2013, Ara et al. evaluated the neural stem progenitors (NSPs) proliferation,

differentiation and neurogenesis in SVZ of newborn piglets subjected to 3 hours of hypoxic preconditioning, 24 hours before severe hypoxia ischemia. These researchers demonstrated that PC induced proliferation of endogenous NSPs up to 7 days after the hypoxic–ischemic injury. NSPs derived from SVZ of newborn preconditioned piglets formed a greater number of neurospheres, generated twice of neurons and astrocytes in *vitro* (Ara J. et al., 2013). In 2014, Suryana et al. analyzed in rat pups (P6) subjected to 3 hours of hypoxia and subsequent to HI, 24 hours later, the effect of maturation of the oligodendrocyte lineage cells. They demonstrated that hypoxic preconditioning prevented the loss of myelin binding protein (MBP) staining caused by HI, and they suggested that potentially mild hypoxia could be used to protect or reverse the maturational arrest of pre-OLs in conditions of myelination process alteration (Suryana E. et al. 2014).

Hypoxic preconditioning might have an age-dependent evolution, in fact, this protection was not observed in slices, derived from aged animals (>2 years of age) and subjected to severe OGD (Bickler PE. et al., 2010; Stetler RA. et al., 2014). These results suggest that the mature brain is more susceptible to oxidative injury (Xu K. et al., 2007; Stetler RA. et al., 2014), and has less capacity to repair itself after sublethal or lethal insult (Mattson MP. et al., 2002; Stetler RA. et al., 2014). Interestingly, in human pluripotent stem cells (PSCs) it has been demonstrated that hypoxic preconditioning and oxidative stress induces anti-apoptotic mechanisms through the stabilization of HIF-2α, which inhibits p53 and increased Bcl-2 expression (Das B. et al., 2012; Sart S. et al., 2014). While, in mesenchymal stem cells (MSCs) cultured hypoxic preconditioning supports HIF-1α stabilization, which is normally degraded by HIF prolyl-4-hydroxylases in normoxia conditions (Muscari C. et al. 2013, Sart S. et al., 2014) (Fig. 1). HIF-1α regulates the activity of glycolytic enzymes

and inhibits the pyruvate dehydrogenase, these events induce the shift of metabolic from oxidative phosphorylation to glycolysis, producing ROS decrease (Sart S. et al., 2014). So HIF-1α is a necessary component of the protective mechanisms involved in HPC. In fact, in HIF-1α knockout mice, subjected to hypoxic-ischemic injury, the damage is more severe than wild-type HI mice and HPC is not able to protect HIF-1α from severe hypoxia ischemia (Sheldon RA. et al., 2014).

Also *in vivo* model of IPC it has been demonstrated that this stimulus enhanced to neurogenesis in the dentate gyrus. In fact, LEE et al. studied the neuroprotective mechanisms of IPC and examined whether neurogenesis and angiogenesis increased following severe ischemia in rats subjected to IPC pretreatment. Their results demonstrated that 10 minutes of IPC treatment induced to cell proliferation in SVZ, with a peak of cell proliferation 7 days after IPC treatment. The proliferated cells differentiated into mature neurons within 14 days to IPC stimulus (Lee SH. et al., 2007). In according with previous studies (Stagliano NE. et al., 1999; Dirnagl U. et al., 2003) LEE et al. observed about 30% reduction of infarct volume and an improvement of functional outcome, which persisted for 28 days, after subsequent prolonged focal ischemia (PFI). Therefore, authors identified the increase of angiogenesis in the ischemic penumbra after PFI suggesting that repair mechanisms of endogenous neurogenesis involved the replacement of lost neurons; the release of trophic substances, and self-regeneration (Picard-Riera N. et al., 2004).

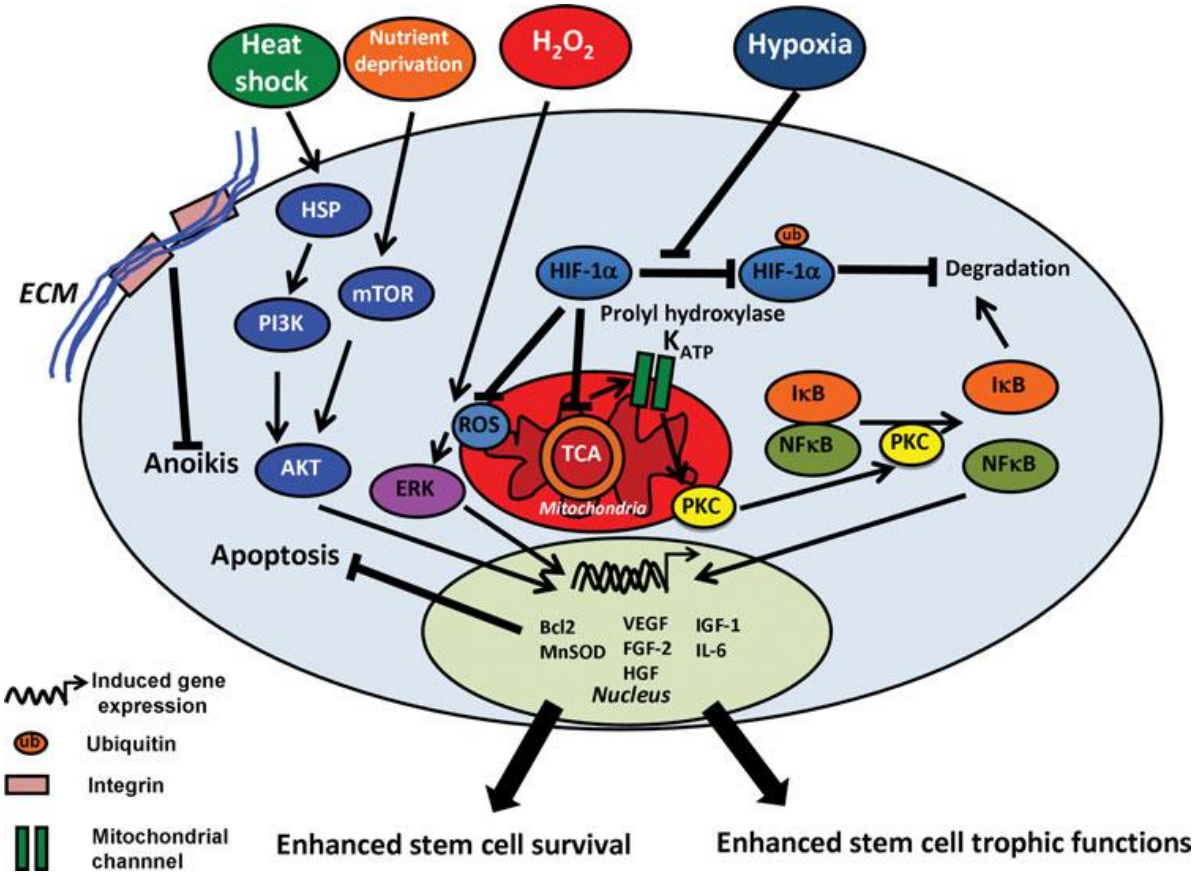

Figure 1. Molecular mechanisms involved in the neuroprotection mediated by preconditioning in stem cells (Sart S. et al., 2014).

Different stimuli are able to induce the expression of anti-apoptotic molecules and trophic factors (Heat shock treatment, chronic oxidative stress exposition; chronic hypoxia exposition). Chronic exposition to hypoxia inhibits the degradation of hypoxia-inducible factor (HIF)-1a, mediated by prolyl-hydroxylase through ubiquitination. The stabilization of HIF-1a reduces oxidative phosphorylation, determining the opening of mitoKATP channels and the activation of protein kinase C (PKC) (Sart S. et al., 2014). PKC activates nuclear factor kappa beta (NFkβ) signaling, that induces the expression of antioxidant proteins (MnSOD) and anti-apoptotic proteins (Bcl-2) (Sart S. et al., 2014). In addition, chronic hypoxia induces the transcription of neurotrophic factors (VEGF, FGF, BDNF) (Sart S. et al., 2014).

1.4 NEUROGENESIS

The CNS is composed of different cell lines: neurons, oligodendrocytes, astrocytes, and non-neural cell types. Neurons are the main effector cells of the CNS, they are involved in the mechanisms of elaboration and transport of information entering and leaving the CNS. Oligodendrocytes determine the myelination of related axons, which induces to the rapid propagation of action potentials through neurons (Maldonado-Soto AR. et al., 2014). The astrocytes have a role of support of the CNS, as they regulate the traffic of molecules, which pass from blood to the brain. In addition, they are involved in the cellular phagocytosis and maintaining of brain homeostasis (Barres BA. et al., 2008; Molofsky AV. et al., 2012; Maldonado-Soto AR. et al., 2014). During embryonic life, until birth, the cerebral cells are formed by the same pool of stem cells: radial glial cells (Kriegstein A. et al., 2009; Maldonado-Soto AR. et al., 2014). The stem cells are unspecialized cells, characterized by high proliferative potential and are able to renew themselves through cellular division, for indefinite periods, generating specialized cell types which constitute the various tissue and organs. Stem cells can be distinguished into two different types, in according to the stage of development and differentiate potential: Embryonic stem cells (ESCs) and Adult stem cells (ASCs). ESCs are pluripotent cells, they are able to renew themselves and generate all the different cell types of organism, in appropriate conditions. ESCs come from an embryo at the blastocyst stage, then 4-5 days after fertilization. Adult stem cells are multipotent undifferentiated cells, they maintain the homeostasis of tissue in which they are found and are involved in repairing the tissue after a damage. In the mid-1960s Altman and Das identified proliferating cells in the mature rat brain (Altman J. and Das GD.,1965; Altman J. and Das GD.,1966; Maldonado-Soto AR. et al., 2014). Subsequently, the development of proliferation

markers, such as [3H]-thymidine or 5-bromo-20-deoxy-uridine (BrdU), which labels DNA during the S-phase, confirmed the presence of proliferating cells in the mature mammalian brain (Maldonado-Soto AR. et al., 2014). The neural stem cells (NSCs) have self-renewal and pluripotent capacity, they generate the three neural cell phenotypes. Therefore, neurogenesis is a multi-step process characterized by different phases: proliferation; differentiation; migration; and synaptic integration of new neurons in the local neural circuit. During specific time-frames of adult neurogenesis, these cells can be distinguished morphologically by using specific markers. In the adult mammalian brain, NSCs are localized into two regions, called niches, characterized by specialized microenvironments that support their self-renewal and differentiation throughout their life. In the ventricular–subventricular zone (V/SVZ) of the lateral ventricles (LV) the new olfactory bulb neurons are continuously generated, they are functionally integrated into neural circuits. In the same way, in the subgranular zone (SGZ) of the dentate gyrus (DG) the new granule cells are generated (Vargas VS. et al., 2013). In both niches, the NSCs have features of astrocytes, with radial-like morphology and exhibit hallmark ultra-structural (Pastrana E. et al., 2011; Vargas VS. et al., 2013).

1.4.1 NEUROGENESIS IN VENTRICULAR–SUBVENTRICULAR ZONE

The V/SVZ is a major germinal area in the adult mammalian brain, it is characterized by a thin layer of dividing cells adjacent to the lateral walls of the LV. In 1997, Doetsch F. and her colleagues established the ultrastructural and immunocytochemical features of cell types in the adult rodent SVZ. The V/SVZ is composed of three main layers (ependymal layer, neuroblast network and perivascular layer) (Vargas VS. et al., 2013). Ependymal layer is composed of multi-

ciliated ependymal cells (E1) and bi-ciliated ependymal cells (E2), which are arranged as a series of pinwheels (Mirzadeh Z. et al., 2008; Vargas VS. et al., 2013). Their nuclei are spherical, the chromatin is not clumpy, and in their apical cytoplasm there are many mitochondria and basal bodies (Doetsch F. et al., 1997). The neuroblasts are neuron precursors, they have an elongated cell body with one or two processes, a cytoplasm containing many free ribosomes, a small Golgi apparatus, a few short cisternae of rough endoplasmic reticulum (RER), and many microtubules oriented along the long axis of the cells. Their nuclei have an abundant lax chromatin with two to four small nucleoli, the nuclei may be invaginated (Doetsch F. et al., 1997). The neuroblasts are connected with the other cells through small junctional complexes, which are distributed over the cell surface and contain endocytic vesicles. These compartments may be exchange sites of signals. The neuroblasts are organized as a network of chains throughout the SVZ (Doetsch F. et al., 1996; Vargas VS. et al., 2013). Along the entire length of SVZ extends a planar venous plexus (Shen Q. et al., 2008; Tavazoie M. et al., 2008; Vargas VS. et al., 2013). In addition, the V-SVZ niche is characterized by many populations of astrocytes, that have different morphologies and locations (Pastrana E. et al., 2011; Vargas VS. et al., 2013). The stem cells radial glial-like (Type B) express astroglial markers, such as glial fibrillary acidic protein (GFAP) (Donega V. et al., 2013). There are two subtypes of these cells, type B1 and B2, which have a different cytoplasm, chromatin and distribution compared with ependymal cells. These cells are quiescent *in vivo* and can be activated by specific signals in physiological conditions, such as pregnancy, and pathological states, as seizures (Scharfman HE. et al., 2009) stroke (Yoo SW. et al., 2008) or hypoxia ischemia (Kadam SD. et al., 2008). They divide themselves and give rise to active proliferating cells (Type C). These cells are large,

more spherical with a few processes, their nuclei contained deep invaginations and mostly lax chromatin (Doetsch F. et al., 1996). They are characterized by a large Golgi apparatus, a few ribosomes, and some small junctional complexes (Doetsch F. et al., 1996). Type C cells, through the intermediate transit amplification, generate neuroblasts (Type A), then the immature neurons migrate far from the olfactory bulb (Pastrana E. et al., 2011; Vargas VS. et al., 2013). Finally, these neurons differentiate into olfactory GABAergic granule interneurons, glutamatergic juxtaglomerular neurons or dopaminergic periglomerular interneurons, and integrate into the local neuronal circuits (Carleton A. et al., 2003; Brill MS. et al., 2009; Braun SM. and Jessberger S., 2014). The B1 stem cells are highly polarized cells, which interact with the cerebrospinal fluid (CSF) and the center of pinwheels through a small apical process. Furthermore, they interact through their soma with the intermediate SVZ and through long basal process with vasculature via (Vargas VS. et al., 2013) (Fig.2). SVZ is connected with CSF, which fills the ventricles and is primarily produced by a mini-organ floating in the ventricles, choroid plexus. In the CSF/ependymal compartment the cell–cell interactions occur and CSF composition influences V/SVZ stem cells and their progeny. CSF, during the embryo life, promotes the proliferation of neural progenitors, through the release of IGF2. While in adult CSF, there are many growing factors and ligands involved in V-SVZ stem cell life such as SHH, Wnts, TGFs, IGFs, PDGFs, BMPs, Slits and retinoic acid (Lehtinen MK. et al., 2011; Marques F. et al., 2011; Lee C. et al., 2012; Vargas VS. et al., 2013). In addition, there are extracellular matrix fragments and exosomes, that may contain factors regulating the proliferation and the differentiation of stem cells (Marzesco AM. et al., 2005; Street JM. et al., 2012; Vargas VS. et al., 2013). Furthermore, it is well known that the rostral flow of the CSF establishes the vectorial

gradients, that may contribute to regulate the directional migration of neuroblasts (Sawamoto K. et al., 2006; Vargas VS. et al., 2013).

Then, B1 stem cells receive signals from three niche compartments: the CSF/ependymal cell niche (apical), the intermediate SVZ niche and the perivascular niche (Fuentealba LC. et al., 2012; Vargas VS. et al., 2013) (Fig. 2).

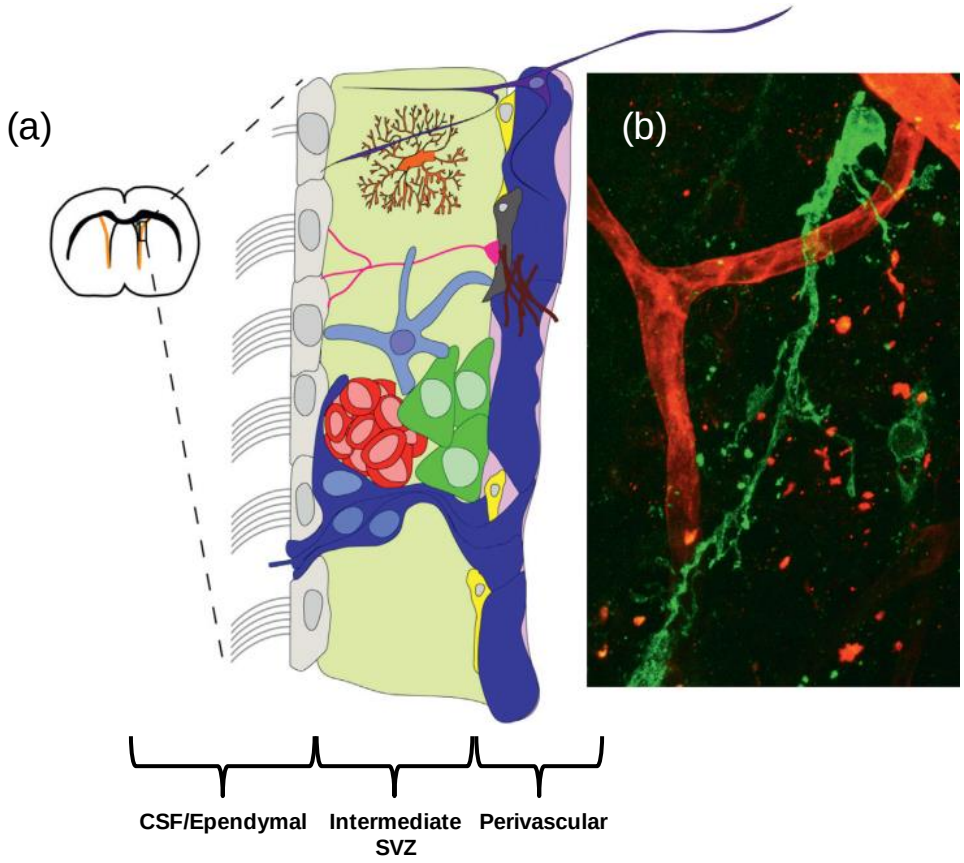

Figure 2. Cell types in the V/SVZ niche (Vargas VS. et al., 2013).
(a) The image shows the cells and compartments of adjacent area to the lateral ventricles (orange). The CSF/ependymal compartment is composed of the CSF, ependymal cells (multi-ciliated E1 cells and bi-ciliated E2 cells, gray) and B1 stem cells (dark blue) that contact the ventricle, as well as axons (Vargas VS. et al., 2013). The intermediate SVZ niche is formed by B1 stem cells (dark blue), transit amplifying Type C cells (green), and neuroblasts (Type A cells, red) (Vargas VS. et al., 2013). There are other cells: astrocytes (light blue), microglia (orange) and long distance axon terminals (purple). The perivascular niche is composed of blood vessels (pink), surrounded by pericytes (yellow) and astrocyte end feet (dark blue) (Vargas VS. et al., 2013). In addition, in this area there are macrophages and fibroblasts (dark gray). (b) B1 stem cells (green) are highly polarized cells, which interact through a long basal process with the blood vessels (red) (Vargas VS. et al., 2013).

1.4.2 NEUROGENESIS IN DENTATE GYRUS

The subgranular zone is a tight layer of cells located between the granule cell layer (GCL) and hilus of the dentate gyrus. Also in the SGZ, the NSCs are in contact with blood vessel, differently from V-SVZ cells, they are not related with ventricular lumen. NSCs extend their radial processes into the molecular layer (ML), through the GCL (Bonaguidi MA. et al., 2011; Encinas JM. et al., 2011; Braun SM. and Jessberger S., 2014), and generate, through a short-lived intermediate progenitor, the new neurons (Fig. 3). These cells migrate far from the GCL, within 3 weeks, then, the new granule cells project out a large dendritic arbour into the ML and an axon into the hilus (van Praag H. et al., 2002; Zhao C. et al., 2006; Toni N. et al., 2007; Toni N. et al., 2008; Braun SM. and Jessberger S., 2014). In this way, they integrate into the local circuit (Vargas V.S. et al., 2013). In the adult SGZ there are two types of stem cells (Type I and Type II) (Fukuda S. et al., 2003; Steiner B. et al., 2006). The Type I progenitors are radial glial-like cells, they express both marker GFAP and Nestin compared to the other astrocytes of SGZ. Indeed, the astrocytes express GFAP marker only (Seki T. et al., 1993; Donega V. et al., 2013). The Nestin is an intermediate filament protein Type VI (IFN), it is expressed by astrocytes and radial glial cells during the brain development. It starts to disappear from postnatal day eleven in the rat cortex (Kalman M. and Ajtai BM., 2001; von Bohlen O. und Halbach, 2007), therefore, it is downregulated and is replaced by intermediate filament proteins, as neurofilaments. In the early stages of neurogenesis there is a balance between quiescence and proliferation of NSPCs. Many intrinsic and extrinsic cellular factors, regulating this balance, have been identified, including Notch signaling (Ables JL. et al., 2010; Ehm O. et al., 2010; Lugert S. et al., 2010), Wnt signalling (Lie DC. et al., 2005, von Bohlen O. und Halbach, 2007), Sox2 transcriptional activity (Favaro R. et al., 2009; Suh HK. et al., 2007; von Bohlen O. und Halbach, 2007), and

lipid metabolic processes (von Bohlen O. und Halbach, 2007; Knobloch M. et al., 2013). From Type I cells derive the Type II progenitors (Type D cells) (Fukuda S. et al., 2003; Steiner B. et al., 2006), that divide themselves and give rise the immature neurons. These cells are characterized by the expression of two specific proteins: doublecortin (DCX) and poly-sialylated neural adhesion molecule (PSA-NCAM) (Donega V. et al., 2013). Doublecortin is a protein involved in the microtubule polymerization, it is expressed in neuroblasts and newborns neurons (Gleeson JG. et al. 1999; Francis F. et al. 1999; von Bohlen O. und Halbach, 2007). PSANCAM is a membrane-bound glycoprotein, which belongs to the class of neural cell adhesion molecule (NCAM) (Nacher J. et al., 2002). It is characterized by a long chain of polysialic acid, which gives anti-adhesive properties to the molecules (Rutishauser U., 1996). PSANCAM is involved in cell migration (Ono K. et al., 1994; Rutishauser U., 1996), axonal growth and fasciculation (Doherty P. et al., 1990 ; Zhang H. et al., 1992; Nacher J. et al., 2002), and synaptic reorganization (Miller PD. et al., 1994; Seki T. et al., 1996; Nacher J. et al., 2002). Finally, the immature neurons become newborn neurons, that are characterized by specific expression of neuronal nuclei marker (NeuN) (Donega V. et al., 2013).

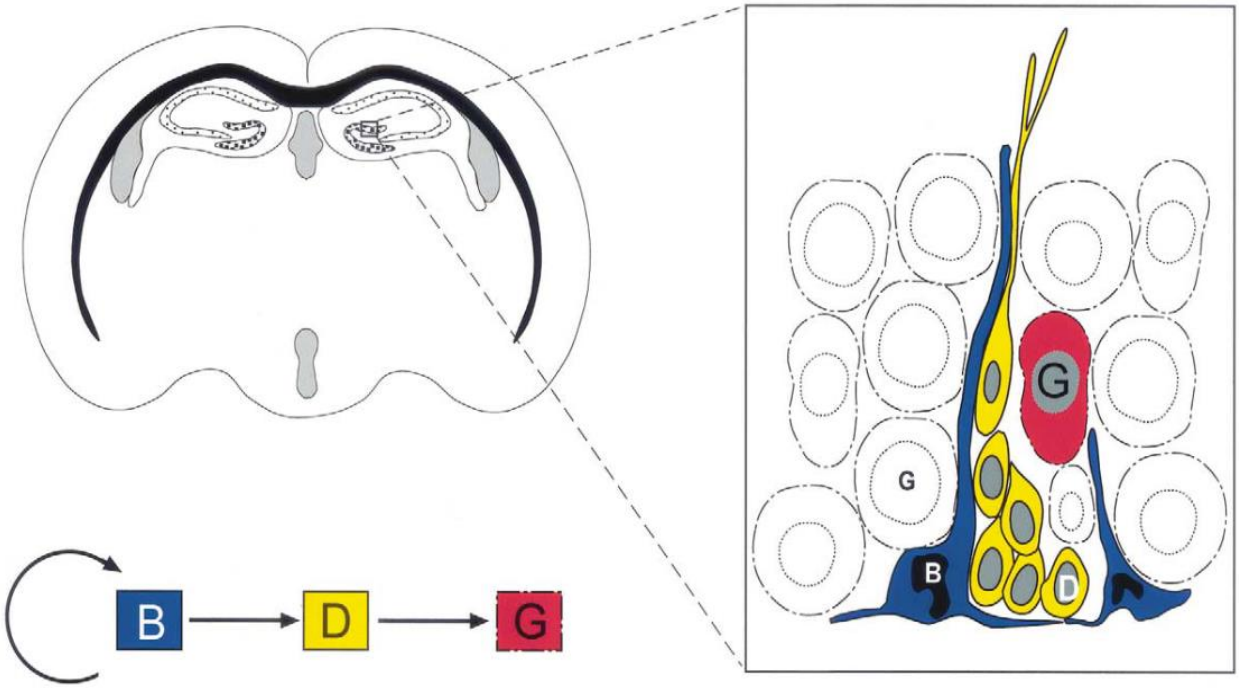

Figure 3. Cell types in the Dentate Gyrus niche (Alvarez-Buyalla A. et al., 2002).
The image shows the cells and architecture of the subgranular layer (SGL). SGL astrocytes (B cells) are characterized by long radial processes that penetrate the granule cell layer and short tangential ones that run parallel to SGL (Alvarez-Buyalla A. et al., 2002). B cells are able to divide themselves and generate immature dark cells (D cells), which mature into new granule neurons (G cells) (Alvarez-Buyalla A. et al., 2002).

1.4.3 NEUROGENESIS ACTIVATION IN HYPOXIC-ISCHEMIC INSULT

There are several studies about the endogenous ability of the neonatal brain to regenerate itself after hypoxic-ischemic insult and the molecular mechanisms involved in this process. In 2004, Plane et al. studied the proliferation of neural stem cells in neonatal SVZ mice, after moderate HI insult. In particular, mice were subjected to ischemia and 45' hypoxia (P10) and sacrificed at different time intervals (P18, P24, P31). The authors observed the increase of neural progenitors in SVZ and in peri-infarct striatum after HI insult (Plane JM. et al., 2004). In 2007, Yang et al. analyzed the differentiation and migration of SVZ progenitors in mice subjected to HI (P6) and monitored from 2 weeks to 5 months of recovery after insult. This study demonstrated that SVZ precursors divided themselves and generated neocortical neurons. In addition, they observed the presence of new cells that colonized cell-sparse columns, composed of reactive astrocytes. This result confirmed a previous Luzzati's study, in which he showed the presence of chains of migrating cells in the SVZ, these cells were connected with the mature brain parenchyma (Luzzati F. et al., 2003). Yang verified that the neurogenesis process was induced by the production of insulin-like growth factor-1 and monocyte chemoattractant factor-1 (Yang Z. et al., 2007). Therefore, the proliferation of neural progenitor cells can be induced by growing factors. Im SH. et al. verified that a continuous intracerebroventricular administration of low dose BDNF and EGF stimulated striatal neurogenesis in hypoxic-ischemic mice. In particular, they showed that the increase of $BrdU^+$/III-$tubulin^+$ (marker of proliferating cells) in SVZ mice after 2 weeks of treatment, and the migration of neuroblasts in the SVZ and the ventricular side of neostriatum mice, 8 weeks after chronic infusion. The new striatal neurons were identified with $BrdU^+$/$NeuN^+$ immunostaining. These effects determined an improvement of motor functions in mice, that were analyzed through Rotarod test (Im SH. et al., 2010). In

2010, Lòpez and his colleagues demonstrated the remyelination of the injured external capsule and the increase of the number of both $NG2^{+}$ types of cells (a marker of early oligodendrocyte progenitors) in HI rats treatment with cannabinoid drug, WIN55212-2, from postnatal days 7 to P14. In particular, in SVZ this drug enhanced cell proliferation and oligodendrogenesis through induction of the transcriptional factor Olig2. This factor activated the differentiation of stem cells characterized by OL phenotype. Moreover, WIN55212-2 induced white matter remyelination and generation of neuroblast cells in the injured striatum 1 week after HI induction (Lòpez DF. et al., 2010). In 2014, Codega et al. identified and isolated quiescent adult NSCs from neurogenic niche. In particular, they performed *in vivo* and *in vitro* analyses to determine the expression profiling of these cells. In ZSV of mice they identified quiescent and activated populations of stem cell astrocytes and their cell cycle properties by using immunohistochemical analyses with specific combination of markers (CD133, GFAP, and EGFR). In addition, they analyzed functional behaviour, *in vivo*, and gene expression profiles of qNSCs and aNSCs *in vitro* (Codega P. et al., 2014). Similarly to SVZ, also in the hippocampus there is evidence of neurogenesis activation after HI injury. Kadam and his colleagues used a mouse model of neonatal stroke to characterize the long-term effects of neurogenesis. They focused their attention on the identification of the newborn cells originated from SGZ and migrated in the ipsilateral and contralateral DG, and newborn cells derived from SVZ and migrated in the ipsilateral and contralateral striatum and neocortex. Then, hypoxic-ischemic mice were subjected to 5 intraperitoneal administration of BrdU, 1 weeks later insult. The analyses of immunohistochemistry were performed on mice (P40) in order to monitor the neuronal and glial cell-lineage markers and BrdU incorporation. These analyses

revealed that the cell-lineage commitment expressed the features similar to controls of 1 week after injury. At P40, the total number of new neurons, derived from SGZ, was reduced in ipsilateral and contralateral hippocampus, while the cells, derived from SVZ, were amplified (Kadam SD. et al., 2008).

Qiu et al. analyzed the effects of HI injury on proliferation and differentiation of neural stem cells in the immature (P9) and juvenile (P21) mouse hippocampus. At first, C57BI/6 mice were subjected to HI insult, after they were subjected to administration of bromodeoxyuridine (50 mg/kg) daily for 7 days after insult. Finally, 5 weeks later, BrdU cells were quantified in DG and CA areas. They observed that the juvenile brain showed a major increase of BrdU labeling than the immature brain after insult. Then, in dentate gyrus the proliferation of NSCs significantly increased in the juvenile brain compared to immature brain, but the total number of $BrdU^+$ cells was less in control juvenile brains than control immature brains. The authors explain this result reporting that during the first phase of physiological development of brain the proliferation and neurogenic abilities are more and decrease with age. Instead, in physiological conditions the juvenile brains had low levels of NSCs proliferation, thus, the damage induced the proliferation in the juvenile brains. In addition, they observed a strong inflammatory response in the juvenile hippocampus, which was correlated with the increase of the proinflammatory cytokine levels (MCP-1, IL-18) and an increase of microglia proliferation 3 days after HI induction.

These divergence results may be correlated with the differences severity of the insult and susceptibility to damage of CD-1 mice compared to C57Bl/6 mice. In addition, in Kadam's study the analysis of BrdU labeling was performed in a period of full development of DG, while in Qiu 's project the cells were detected in the early phase of DG development (Donega V. et al., 2013).

Currently, the long-term survival effects of newborn neuronal cells are not also clear, indeed, it is not known whether these new cells integrate into the local circuitry and evolve into functional cells in the no fully developed brain (Donega V. et al., 2013). Some studies in rodents model of neonatal HI showed that few new neurons survived 6 months after insult (Nakatomi H. et al., 2002; Miles DK. and Kernie SG., 2008; Donega V. et al., 2013), and they had reduced electrophysiological properties (Seri B. et al., 2004; Donega V. et al., 2013).

Nowadays, there are a great number of studies on the fate of newborn cells, originated from SVZ and migrated in cortex, striatum, after HI injury. These studies showed: a significant increase of glial cell-fate commitment in these areas, the differentiation of striatal neuron committed cells in cholinergic neurons or parvalbumin (PV), calbindin (CB), or somatostatin (SOM) interneuron subtypes; and the differentiation of cortical $BrdU^+$ cells into calretinin (CR) interneurons (Yang Z. et al., 2007; Yang Z. et al., 2008; Donega V. et al., 2013). In cortex the major number of interneurons were of the PV subtype. These results suggest that the NSCs originated from the SVZ may differentiate into CR interneurons (Donega V. et al., 2013) .

1.4.4 MOLECULAR AND CELLULAR MECHANISMS INVOLVED IN REGULATION OF NEUROGENESIS IN HYPOXIC-ISCHEMIC BRAIN

Neurogenesis process is not fully understood, in spite of several studies. It is well known that the neurovascular niche has an important role in the regulation, proliferation, differentiation, and survival of newborn cells. Astrocytes are connected to proliferating cells and to blood vessels in DG and produce different factors that are involved in neurogenic processes (Palmer TD. et al., 2000; Song H. et al., 2002; Shen Q. et al., 2004; Donega V. et al., 2013). Growth factors and neurotrophins,

such as Brain-derived neurotrophic factor (BDNF); VEGF; and morphogens as Wnt3; Shh; Noggin regulate cell proliferation and differentiation. The wnt proteins are glycoproteins that act as molecule signals in many cellular processes. They are involved in three different molecular mechanisms: the canonical pathway Wnt/β-catenin; and not canonical pathways of Wnt/JNK and Wnt/Ca^{2+} (Budnik V. et al., 2011). Wnt has an important role in the development of CNS (Ciani L. et al., 2005). Recent evidences suggest that wnt signal may modulate excitatory and inhibitory synaptic transmission (Budnik V. et al., 2011). Wnt-7a increases the synaptic transmission and determines the increase of neurotransmitter release in cerebellar and hippocampal synaptic, through not canonical dependent Camk II pathway. These results suggest that the wnt signal mechanisms may have an essential role in modulation of synaptic plasticity and cellular development. Shh proteins are involved in neuronal cell-fate commitment, while Noggin in neuronal cell differentiation (Zhao C. et al., 2008; Donega V. et al., 2013). Recent data established the crucial regulatory role of membrane receptors Notch1 in distinct phase of neurogenesis after hypoxic-ischemic brain injury (Felling RJ. et al., 2006; Donega V. et al., 2013). Gene expression analyses revealed that overexpression of the intracellular portion of Notch1 enhanced the proliferation of stem cells $GFAP^{+}$ (Schroeter EH. et al., 1998; Donega V. et al., 2013). In contrast, the ablation of Notch1 expression determined a decrease of proliferating cells with astrocytic phenotype and induced the increase of neuron differentiation in ZSG (Breunig JJ. et al., 2007; Donega V. et al., 2013). Then, the expression of Notch1 influenced the regenerative capacity of NSCs, and itself may be regulated by other molecules as gp130, a membrane receptor involved in survival and proliferation of NSCs (Chojnacki A. et al., 2003; Donega V. et al., 2013). As Notch, after hypoxic-ischemic event the ablation of Fibroblast growth factor

receptor 1 (FGFR1) reduced the proliferation of stem cells $GFAP^+$ in ZSV and compromised cortical pyramidal neuron development (Fagel DM. et al., 2009; Donega V. et al., 2013). In 2006, Felling et al. analyzed the mRNA levels of series membranes receptor (Notch1; EGFR; gp130) and observed their increase and a major proliferation of NSCs in the ipsilateral SVZ at 48 hours after insult (Felling RJ. et al., 2006; Donega V. et al., 2013). The interaction of EGFR and its ligands, as EGF1 and TGFa, activated MAPK and Rac pathways, that are implicated in all phase of neurogenesis process (Yarden Y. et al., 2001; Donega V. et al., 2013).

Also, the neurotransmitters seem to be implicated in the regulation of neurogenic process in immature brain after injury. In fact, it has been demonstrated that synapse formation may be regulated by GABAergic innervations, the glutamatergic innervations are involved in neuroblasts survival; dendritic development; and synaptogenesis, while Dopaminergic signaling promoted the proliferation (Pathania M. et al., 2010; Donega V. et al., 2013). Lee S-R et al. studied the role of matrix metalloproteinases (MMPs), a family of zinc endopeptidases, in neuroblast migration through axonal extension (Maddahi A. et al., 2009; Donega V. et al., 2013). Their results established that the migration of neuroblasts into striatum are suppressed with MMPs inhibition (Maddahi A. et al., 2009; Donega V. et al., 2013). In the hippocampus the migration of neuroblasts from SGZ to DG are regulated by radial glial fibers; very -low-density lipoprotein receptor (VLDLR); and apolipoprotein E type 2 receptor (ApoER2) (Tissir F. et al., 2003; Donega V. et al., 2013); and an extracellular matrix serine protease that interrupted migrating neurons signals (D'Arcangelo G. et al., 1995; Donega V. et al., 2013).

1.5 SODIUM/CALCIUM EXCHANGER (NCX)

The Na^+/Ca^{2+} exchanger (NCX) is an important transmembrane protein involved in the regulation of Ca^{2+} and Na^+ homeostasis in neuronal cells and in various cell types. NCX is a member of Ca^{2+} cation antiporter superfamily (CaCA), it catalyzes the exchange of three Na^+ ions and two Ca^{2+} ions across the plasma membrane (Fujioka Y. et al, 2000; Kang TM. and Hilgemann DW., 2004; Reeves JP. and Hale CC., 1984). In mammalian brain there are three dominant genes coding for three isoforms: NCX1 (Nicoll DA. et al, 1990), NCX2 (Li Z. et al, 1994), and NCX3 (Nicoll DA.et al, 1996) proteins. In 1996 Nicoll et al, identified NCX genes localization by mapping mouse chromosomes. NCX1; NCX2; NCX3 are respectively localized on 17, 7, and 12, chromosomes (Nicoll DA. et al, 1996). Currently, more than 15 splice of NCX1 are known (Kofuji P. et al. 1994; Quednau BD. et al. 1997), these variants have a tissue-specific distribution and exchange properties that fulfill the tissue requirements (Quednau BD. et al., 1997). It has been found one NCX2 alternative splicing (Quednau BD. et al., 1997), and three variants for NCX3. The NCX3 gene is composed of 9 exons (Quednau BD. et al., 1997), the exons 2 and 3, also named A and B, respectively, are mutually exclusive (Quednau BD. et al., 1997). Exon 4, also known exon C, is optional. In the rat was observed that the exon A and C are found in skeletal muscle (NCX3-AC), while the exon B is expressed in the brain (NCX3-B and NCX3-BC). The transcription of NCX1 genes is tissue-specific and is independent on intracellular calcium levels (Lee SL et al., 1994; Annunziato L. et al., 2009). Brain NCX1 promoter is characterized by several consensus sequences for specific protein 1 (Sp-1), three binding sites for activator protein 2 (AP-2); putative binding sites for nuclear factor kB (NFkB) and HIF-1 (Annunziato L. et al., 2009). NCX3 genes promoter contains a repressor sequence constituted by a double of

downstream regulatory element (DRE) (Annunziato L. et al., 2009). The specific transcriptional factor called transcription of Ca^{2+}- modulated transcriptional repressor downstream regulatory element antagonist modulator (DREAM), binding to this sequence (Annunziato L. et al., 2009). When the intracellular levels of calcium is high, DREAM is not able to link DRE, then an increase of NCX3 transcription occurs (Gomez-Villafuertes R. et al., 2005; Annunziato L. et al., 2009). Therefore, NCX3 genes transcription depends on calcium intracellular levels (Annunziato L. et al., 2009). Also NCX2 genes transcription depends on calcium intracellular levels (Annunziato L. et al., 2009). All NCX isoforms are constituted of 10 transmembrane (TM) domains (John SA. et al. 2013; Szerencsei RT.et al. 2013), arranged in two clusters separated by a large intracellular loop of 500 residues (Fig. 4). In 2012, Liao et al. analyzed the crystal structure of prokaryotic Methanococcus jannaschii (NCX_Mj), and confirmed the presence of 10 TM segments arranged around a tightly packed core region, in which there are four cation-binding sites. The transmembrane segments can be distinguished into an N-terminal hydrophobic domain and into a C-terminal hydrophobic domain, that are linked through a large hydrophilic intracellular loop of 550 amino acids, named the f-loop (Nicoll DA. et al, 1999). The f-loop is not implicated in Na^{+} and Ca^{2+} translocation, but it regulates NCX activity.

The NCX1 is an ubiquitously protein expressed in brain, heart, skeletal muscle, smooth muscle, kidney, eye, secretory, and blood cells, whereas NCX2 isoform is expressed only in brain and NCX3 protein is expressed only in brain and skeletal muscle (Lee SL. et al., 1994; Annunziato L. et al., 2009). In addition, NCX1 and NCX3 have a different localization in brain and neuronal cells (Quednau BD. et al., 1997; Yu SP. and Colvin DW., 1997; Annunziato L. et al., 2009).

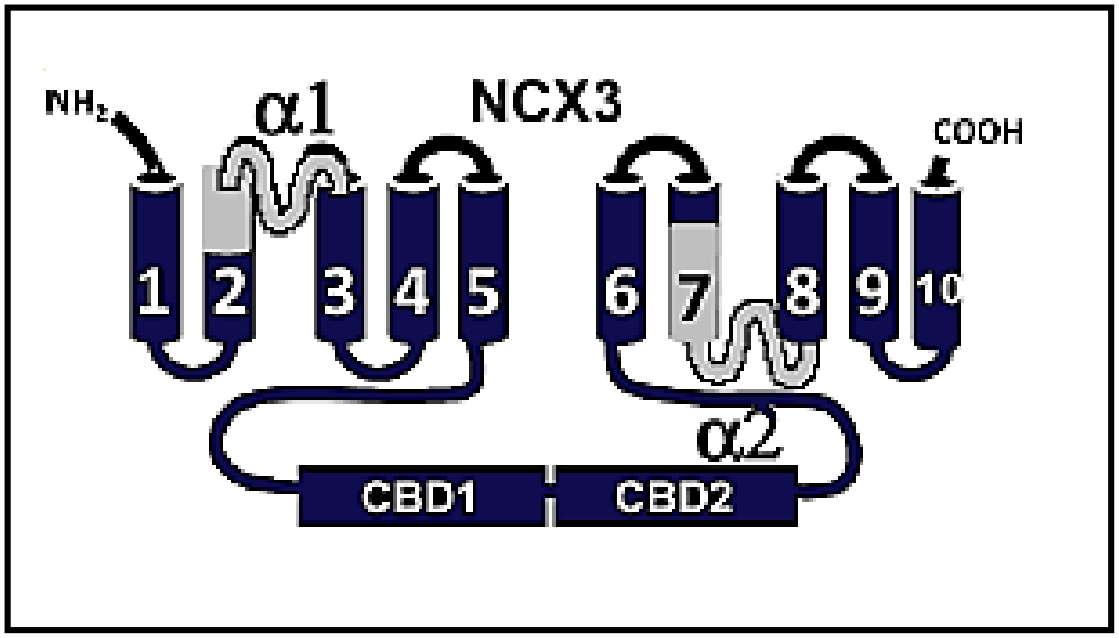

Figure 4. Structure of Na^+/Ca^{2+} exchanger 3 (NCX3). (Secondo A. et al., 2015)
The protein is composed of 10 TM segments arranged around a tightly packed core region, in which there are four cation-binding sites.

1.5.1 REGULATION OF SODIUM/CALCIUM EXCHANGER

The activity of the sodium/calcium exchanger is regulated by different factors: (I) the concentration of the two cations transported, Na^+ and Ca^{2+}; (II) the intracellular pH; (III) cytoplasmic messengers and transductional mechanisms, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC) and protein kinase A (PKA), (IV) reactive oxygen species, reactive nitrogen species (RNS), glutathione reduced (GSH), and glutathione oxidized (GSSG) (Annunziato L. et al., 2004). The concentration of calcium regulates NCX through CBD (Ca^{2+}-binding domain). NCX activity is blocked by the removal of intracellular Ca^{2+} (Nicoll DA. et al., 1999; Hilge M. et al., 2006; Annunziato L. et al., 2009). In the same way, the increase of sodium concentration blocks NCX activity (Annunziato L. et al., 2009). The exchanger activity is also regulated by the intracellular pH. In particular, also the little reductions in pH, as a value of 0.4, can induce an inhibition of NCX of 90% (Annunziato L. et al., 2009). The ATP is able to increase the activity of the exchanger in different ways (Annunziato L. et al., 2009). First, by activating G protein-coupled receptors for endogenous and exogenous ligands second, ATP can stimulate the activity of NCX through the pathway involving PKC or PKA (Annunziato L. et al., 2009). Finally, NCX can be activated by the production of lipid PIP2. This lipid binds the XIP region of the loop "f" which eliminates the inactivation of NCX, thus stimulating the function exchanger (Nicoll DA. et al., 1999; Hilge M. et al., 2006; Annunziato L. et al., 2009). Interestingly, the depletion of ATP determines the inactivation of NCX1 and NCX2 but has no effect on activity of NCX3 (Secondo A. et al, 2007).

1.5.2 PHYSIOLOGICAL ROLE OF SODIUM/CALCIUM EXCHANGER

The $Na^+/Ca2^+$ exchanger protein is involved in several neurophysiological processes. Depending on the intracellular concentrations of Ca^{2+}, $[Ca^{2+}]i$, and Na^+, $[Na^+]i$, NCX can work in two modes: in forward mode four or three Na^+ ions enter cytoplasm and one Ca^{2+} ion exits from the cytoplasm; in reverse mode Na^+ ions exit and Ca^{2+} ion enters. In neurons, NCX is mainly expressed at the level of synapses (Juhaszova M. et al., 1996; Canitano A. et al., 2002; Annunziato L. et al., 2009). During the increase of action potential Ca^{2+} ions enter plasma membrane, this event induces the fusion of synaptic vesicles with the plasma membrane and promotes the neurotransmission. Then, K currents repolarize the plasma membrane and Ca^{2+} ions are distributed in the cytosolic compartment, but the residual of Ca^{2+} ions is extruded by the plasma membrane $Ca^{2+/}$ATPase and by NCX (Annunziato L. et al., 2009).

1.5.3 SODIUM/CALCIUM EXCHANGER ROLE IN ISCHEMIC STROKE

It is well known that NCX isoforms are involved in several pathological conditions, mediating a neuroprotective effect. It has been demonstrated that NCX has an important role in stroke pathogenesis. In 2004, Pignataro et al. analyzed the role of NCX isoforms in brain damaged, after ischemia induction. In particular, they examined the expression of NCX isoforms in different areas and evaluated the measure of brain infarct volume in adult rats in which the focal ischemia was reproduced by using pMCAO technique. The authors performed these analyses also in pMCAO rats in which the NCX gene was silenced by using antisense oligodeoxynucleotides (AS-ODNs) (Pignataro G. et al., 2004). The researchers demonstrated that there was a significant decrease of NCX1, NCX2, and NCX3 from 6 to 72 hours after pMCAO in ischemic core, in contrast in penumbra area there was

only the reduction of NCX3 isoform (Pignataro G. et al., 2004). In addition, they proved that the silencing of NCX1 and NCX3 isoforms determined the increase of damage and worsening of deficits (Pignataro G. et al., 2004). In 2006, Boscia et al. confirmed that NCX expression is regulated in a differential manner after pMCAO, and there was a different expression of NCX isoforms in regions involved in the insult. Also in 2004, Pignataro et al. analyzed the effects of pharmacological inhibition or activation of NCX in brain rats subjected to pMCAO. In particular, they examined the effects of following drugs: the glutamate exchange inhibitory peptide (GLU-XIP), the exchange inhibitory peptide (XIP), diarylaminopropylamine derivative bepridil, the isothiourea derivative (KB-R7943), ferric chloride ($FeCl_3$), and the amiloride derivative 5-(N-4-chlorobenzyl)-20,40-dimethylbenzamil (CB-DMB) (Pignataro G. et al., 2004). They demonstrated that the inhibitors of NCX (GLU-XIP, KB-R7943, and bepridil) determined a significant increase of brain damage in rat subjected to pMCAO compared to control, in contrast, XIP did not cause the rise of infarct volume in rats (Pignataro G. et al., 2004). About KB-R7943, they reported that drug inhibited the NCX in the reverse mode of operation (Pignataro G. et al., 2004). In addition, they confirmed that the administration of $FeCl_3$ functioned as NCX activator and reduced the brain infarct volume in ischemic rats, as Amoroso et al. had already reported in anoxia conditions (Amoroso S. et al., 1997; Pignataro G. et al., 2004). Nevertheless, the authors established that $FeCl_3$ activity had been neutralized by the coadministration of KB-R7943 (Pignataro G. et al., 2004). In 2007, Tortiglione et al. analyzed the different sensitivity to NCX in neuron subtypes of peri-infarct area, after the pharmacological inhibition of protein in rat slices subjected to pMCAO. The researchers demonstrated that the activation of NCX maintained ionic homeostasis in the peri-infarct area after focal ischemia (Tortiglione A. et al., 2007). In particular,

they observed that the administration of bepridil and CB-DMB caused an irreversible electrophysiological alteration of NCX activity in peri-infarct area, related to field potential amplitude (Tortiglione A. et al., 2007). Then, the authors suggested that the inhibition of NCX could trigger a cascade of events leading to neuronal damage (Tortiglione A. et al., 2007). In 2008, Molinaro et al. studied the role of NCX3 protein during severe anoxia condition. In particular, they evaluated the effects of ncx3 ablation on neuronal survival, organotypic hippocampal cultures and primary cortical neurons from ncx3-/- mice were subjected to oxygen glucose deprivation (OGD) plus reoxygenation. In this study, the authors demonstrated that infarct volume increased in the ncx3 knocking-out mice subjected to t-MCAO compared to control and there was a significant increase in cell death of ncx3 knocking-out cortical neurons subjected to OGD. In addition, they showed that hippocampal subregions CA1, CA3, and DG of ncx3 knocking-out had a major vulnerability to anoxia condition (Molinaro P. et al, 2008). Therefore, these results suggested that NCX3 was able to maintain intracellular levels of calcium and protect neurons during hypoxia condition. These data confirmed the results of Secondo's study, in which it has been demonstrated that BHK cells transfected with the brain specific NCX3 gene, unlike NCX1 and NCX2 transfected cells, are able to maintain $[Ca^{2+}]i$ homeostasis during hypoxia plus reoxygenation-induced by Ca^{2+} overload, although ATP concentrations are reduced. NCX worked in the forward mode of operation in the presence of reduced levels of ATP (Secondo A. et al, 2007). Furthermore, the silencing of NCX1 or NCX3 induced a major vulnerability to chemical hypoxia in BHK cells (Secondo A. et al, 2007). In a recent paper published by Pignataro et al, it has been shown that NCX1 and NCX3 represent a new molecular effector involved in a neuroprotective mechanism called "ischemic preconditioning" (Pignataro G. et al, 2012). In particular, they analyzed the

brain infarct volume and the expression of NCX isoforms in rats subjected to preconditioning stimulus (30 min of tMCAO) 72 h before 100 min of tMCAO (Pignataro G. et al, 2012). The results showed that there was a significant reduction of infarct volume in preconditioned rats subjected to harmful ischemia and the ischemic preconditioning induced NCX1 and NCX3 overexpression in ipsilateral temporoparietal cortex of preconditioned rats compared to group subjected alone tMCAO (Pignataro G. et al, 2012). In addition, they demonstrated that the silencing of NCX1 and NCX3 genes prevented the neuroprotective effects mediated by ischemic preconditioning (Pignataro G. et al, 2012). In 2014, Sisalli et al. studied the effects of ischemic preconditioning on primary cortical neurons exposed to 30 minutes of OGD followed 24 hours after stimulus, to 3 hours OGD and reoxygenation for 24 hours. The authors demonstrated that IPC increased NCX1 and NCX3 protein expression 48 hours after OGD plus reoxygenation induction and this effect was mediated by NO production and PI3K/Akt activation (Sisalli MJ. et al., 2014). Therefore, they established that IPC, mediated the upregulation of NCX1 and NCX3 activity, caused the endoplasmatic reticulum refilling and mitochondrial calcium extrusion, thus preventing intracellular calcium dysregulation induced by OGD (Sisalli MJ. et al., 2014). In 2013, it has been developed a potent NCX activator, named neurounina-1. This new compound is 7-nitro-5-phenyl-1- (pyrrolidin-1-ylmethyl)-1H-benzo[e][1,4]diazepin-2(3H)-one, the structure derived from modified structure of SM-15811, a NCX inhibitors (Molinaro P. et al, 2013). In this work the authors analyzed the effects of Neurounina on NCX isoforms activity in the forward and reverse modes of operation by means of Ca^{2+} radiotracer, patch-clamp techniques, Fura-2 microfluorimetry, and thereafter, with the help of chimera strategy, deletion, and site-directed mutagenesis, it has been also identified the molecular determinants of this

compound on NCX structure (Molinaro P. et al, 2013). In addition, they evaluated the protective effects of Neurounina *in vitro* and *in vivo* model of ischemia (Molinaro P. et al, 2013). They demonstrated that Neurounina determined a potent and reversible stimulatory effect on NCX1 and NCX2 in both forward and reverse modes of operation, but it was not able to increase the activity of the NCX3 (Molinaro P. et al, 2013). This compound is characterized by an high lipophilicity, therefore is able to pass blood-brain barrier *in vivo*. In mice subjected to tMCAO, it has been proved that the intraperitoneal administration of neurounina-1 in single doses ranging from 0.003 to 30 mg/kg, 5 hours after ischemia induction, significantly reduced the infarct volume (Molinaro P. et al, 2013).

In 2014, Vinciguerra et al. identified a family of microRNA, miRNA-103/107, is able to modulate NCX1 expression. They found that these microRNAs, by reducing NCX1 expression, worsened ischemic damage induced by tMCAO. The researchers also proved that the administration of anti-miR-103-1 induced a selective upregulation of NCX1 protein expression, which mediated the neurological scores improvement in ischemic rats and a reduction of brain volume infarct (Vinciguerra A. et al., 2014). In 2015, Secondo et al. reported the characterization of the newly synthesized compound 5-amino-N-butyl-2-(4-ethoxyphenoxy)-benzamide hydrochloride, named BED, as a new selective NCX3 inhibitor. (Secondo A. et al., 2015). They suggest that this compound may represent a useful element to distinguish the differential effects of NCX3 isoform in the diseases that require a tight control of Ca^{2+} homeostasis through NCX3 function (Secondo A. et al., 2015).

2 AIM OF THE STUDY

This research project aims to characterize an experimental model of preconditioning in neonatal hypoxia ischemia, in order to identify new molecular targets potentially useful for the development of innovative short and long-term therapies in hypoxic-ischemic encephalopathy. Preconditioning is a phenomenon in which a subliminal injurious stimulus, applied before a longer harmful ischemia, is able to exert a remarkable neuroprotection, thus establishing a state of tolerance to anoxic conditions.

In particular, the purposes of the present study were: (1) to identify preconditioning a reliable and valid stimulus; (2) to evaluate the time window in which the hypoxic preconditioning protected the ipsilateral hippocampus, in mice subjected to hypoxic-ischemic damage. Once characterized a model of preconditioning, the purposes were: (3) to examine the proliferation and differentiation of neural stem cells, in hippocampus dentate gyrus and in subventricular zone of mice subjected to hypoxic preconditioning and/or severe hypoxic-ischemic injury. (4) To verify whether preconditioning stimulus improves the unconditioned reflexes in preconditioned hypoxic-ischemic mice compared with hypoxic-ischemic and finally, (5) to evaluate the relationship between the alteration of ionic homeostasis and the activation of endogenous neurogenesis mediated by plasma membrane NA^{+}/Ca^{2+} exchange (NCX).

3 MATERIALS AND METHODS

3.1 ANIMAL PROCEDURES

Male and female mice C57BL/6 strains, housed under diurnal lighting conditions (12 h darkness/light) were used. All experimental animal protocols were approved by the University of Naples, Federico II. Animal Care and Use Committee and was performed with the highest standards of care under the National Institutes of Health guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2 STUDY DESIGN

In order to accomplish the proposed objectives, the study has been divided into six phases:

Phase 1. Ipsilateral hippocampal damage was evaluated in postnatal day 7 mice subjected to ischemia at different time intervals of hypoxia (10'; 20'; 30'; 60') and sacrificed 24 hours after insult. The temporal course of Ipsilateral hippocampal damage was determined in P7 mice subjected to ischemia plus 60' hypoxia and sacrificed at different time intervals after insult (24 hours; 48 hours; 72 hours; 7 days).

Phase 2. The characterization of preconditioning stimulus was assessed in P7 mice subjected to different stimuli: 10' hypoxia; 20' hypoxia; 30' hypoxia; 60' hypoxia; ischemia; ischemia plus 10' hypoxia; ischemia plus 20' hypoxia. The animals were sacrificed 24 hours after insult. Then, immunohistochemical analyses were performed

on hippocampal mouse sections by using specific antibodies in order to evaluate the Nestin expression changes.

Phase 3. It has been established the time window in which the hypoxic preconditioning protected the ipsilateral hippocampus of mice subjected to hypoxic-ischemic injury (Fig. 5). The experimental groups were organized in three major groups and divided into two subgroups randomized as follows:

Group 1. In the control subgroup, mice were subjected to HI 60' at P9 and sacrificed 24 hours later. In the second subgroup, mice were subjected to 20' hypoxia (PC) at P7, following HI 60' at P9 and sacrificed 24 hours after insult;

***Group 2*.** In the control subgroup, mice were subjected to HI 60' at P10 and sacrificed 24 hours later. In the second subgroup, mice were subjected to 20' hypoxia (PC) at P7, following to HI 60' at P10 and sacrificed 24 hours after insult;

***Group 3*.** In the control subgroup, mice were subjected to HI 60' at P14 and sacrificed 24 hours later. In the second subgroup, mice were subjected to 20' hypoxia (PC) at P7, following to HI 60' at P14 and sacrificed 24 hours after insult.

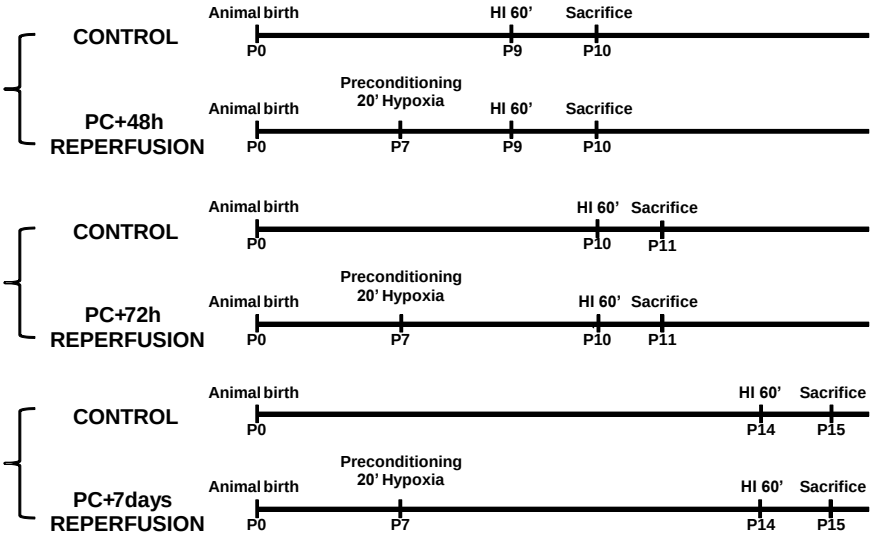

Figure 5. Phase 3 experimental program.

Phase 4. The effects of hypoxic preconditioning on endogenous neurogenesis were examined in hippocampus dentate gyrus and in subventricular zone of mice subjected to hypoxic-ischemic injury. In particular, the differentiation of NSCs was followed through the analysis of proliferating $BrdU^+$ cells. Then, immunohistochemical analyses were performed on hippocampal and striatum mouse sections by using specific antibodies. P7 mice were subjected to two intraperitoneal administrations of BrdU, with time frame of 90' minutes. Subsequently, the experimental groups were randomized as following (Fig. 6):

Group 1. P7 naïve mice were subjected to two intraperitoneal administrations of BrdU and sacrificed at P11.

Group 2**.** P7 mice were subjected to two intraperitoneal administrations of BrdU and 20' hypoxia in the same day. At P10 they were subjected to HI 60' and sacrificed at P11.

Group 3**.** P7 mice were subjected to two intraperitoneal administrations of BrdU. At P10 they were subjected to HI 60' and sacrificed at P11.

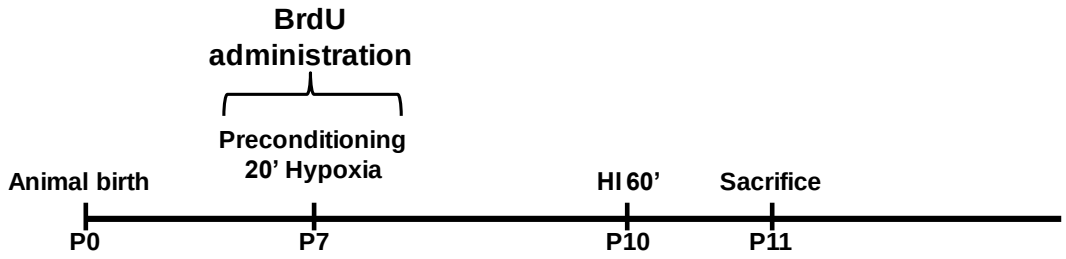

Figure 6. Phase 4 experimental program.

Phase 5. It has been verified whether preconditioning stimulus could improve the unconditioned reflexes in preconditioned hypoxic-ischemic pups compared to hypoxic-ischemic pup mice. Then, mice were monitored with several behavioral tests

at different time intervals (Fig. 7). The experimental groups were randomized as follows:

Group 1. Naïve pup group from postnatal day 7 to until the day of sacrifice (P11) for behavioral testing.

Group 2. PC+HI pup group from postnatal day 7 to until the day of sacrifice (P11) for behavioral testing.

***Group 3*.** HI pup mice group from postnatal day 7 to until the day of sacrifice (P11) for behavioral testing**.**

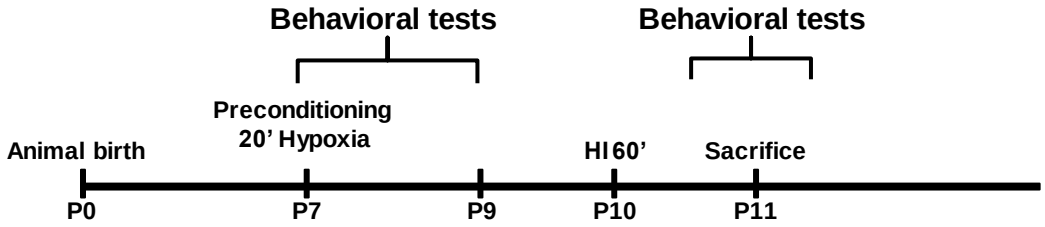

Figure 7. Phase 5 experimental program.

Phase 6. It was evaluated the relationship between the alteration of ionic homeostasis and the activation of endogenous neurogenesis mediated by NCX. Protein expression analyses of NCX1 and NCX3 were performed in hippocampus mice. In addition, the cell count of $NCX1^+$ and $NCX3^+$ cells were performed in dentate gyrus mice. The experimental groups were randomized as following:

Group 1. P11 naïve mice were sacrificed and tissues analyzed as previously described.

Group 2. P11 preconditioned hypoxic-ischemic mice were sacrificed and tissues analyzed as previously described.

Group 3. P11 hypoxic-ischemic mice were sacrificed and tissues analyzed as previously described.

3.3 HYPOXIC-ISCHEMIC SURGICAL PROCEDURE

The ipsilateral ischemic injury has been induced in according to Rice-Vannucci model (Rice JE. et al., 1981; Vannucci SJ. et al., 2004). Postnatal day seven C57BL/6 mice were anesthetized with 1.5% sevoflurane, and 98.5% O_2 (Oxygen concentrator, Mod. LFY-I-5). The body temperature of the animal was maintained at 37±0.5°C during the whole procedure with a heating pad. Under a surgical stereo microscope, a midline skin incision (0.5 cm) was made on the neck, and the right common carotid artery (CCA) was exposed, and double ligated with a suture thread (6-0), in order to isolate the region respectively upstream and downstream of the area to be cut. CCA was cut between the two knots by using specific micro-clipper. The incision was rinsed with 1% lidocaine and sutured with a 6.0 polypropylene (Prolene) suture. The surgery involves a maximum duration of 10 minutes anesthesia. Animals were returned to their dams and monitored continuously during a recovery period of 1 hour. After that, the pups were subjected to different time intervals of hypoxia (10; 20; 30; 60 minutes). The hypoxic insult was reproduced by placing animals into a hypoxic chamber, perfused with an equilibrated gaseous mixture (8% O_2 and 92% N_2) which composition was monitored by using an Oxygen monitor. The hypoxic chamber was placed in a water bath heated to 37°C. At the end of the procedure, the pups were returned to their dams. The animals were monitored continuously for 30 minutes and then checked every 30 minutes for 2 hours and then daily until they were sacrificed, 24 hours; 48 hours; 72 hours; 7 days after insult.

3.4 HYPOXIC PRECONDITIONING

Postnatal day seven C57BL/6 mice were subjected to 20 minutes of hypoxia, into a hypoxic chamber, perfused with an equilibrated gaseous mixture (8% O_2 and 92% N_2) monitored by using an Oxygen monitor. The hypoxic chamber was placed in a water bath heated to 37°C. Control pups not subjected to hypoxic preconditioning were exposed to room air in a chamber, which was placed in a water bath heated to 37°C, for the same time intervals. At the end of the procedure, all pups were returned to their dams. The animals were monitored continuously for 30 minutes and then checked every 30 minutes for 2 hours and then daily until the induction of hypoxic-ischemic injury. Following, the pups were subjected to HI, as previously described, at different time intervals (P9; P10; P14) and sacrificed 24 hours after insult.

3.5 PROPIDIUM IODIDE LABELING, TISSUE PREPARATION AND BRAIN DAMAGE ANALYSIS

Pup mice were anesthetized with 1.5% sevoflurane and 98.5% O_2 gaseous mixture (Oxygen concentrator, Mod. LFY-I-5). The body temperature of the animal was maintained at 37°C during the whole procedure with a heating pad. Under a surgical stereo microscope, a midline sagittal skin incision (0.5 cm) was made on the skull. After identification of Bregma and Lambda coordinates, two microliters of Propidium Iodide (PI, 1 mg/mL in distilled water, Sigma) were injected into the right lateral brain ventricle of newborn mice. Twenty minutes after injection, pups were deeply anesthetized and transcardially perfused with saline solution containing 0.01 ml heparin, followed by 10 ml of 4% paraformaldehyde in 0.1 mol/L PBS in saline solution. Brains were rapidly removed on ice and postfixed overnight at +4°C and

cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB) with 0.02% sodium azide for 24 hours at 4°C. The brains were cut on a cryostat into coronal sections (thickness 100 μm) in rostrum-caudal direction. Brain damage was assessed with Propidium Iodide technique, a method that detects degenerative cells (Carloni S. et al, 2007; Unal Cevik I. and Dalkara T., 2003). Propidium Iodide solution penetrates in the cells whose cellular membrane is damaged and allows to distinguish dark vital tissue from white necrotic and apoptotic tissue. Consecutive sections were analyzed with fluorescence microscope (Nikon E400) and acquired with software Image Pro-Plus. The PI-stained infarct area was calculated with image analysis software (Image-J) (Bederson et al.,1986). Ischemic volume was obtained by the ratio between the sum of the infarction hippocampal areas of brain section and the sum of the total areas of the respective ipsilateral hippocampus, multiplied for thickness cerebral slides. The total infarct volume, corrected for edema, was expressed as percentage of the volume of the hippocampus ipsilateral to the lesion.

3.6 TISSUE PROCESSING, IMMUNOSTAINING, AND CONFOCAL IMMUNOFLUORESCENCE

Immunostaining and confocal immunofluorescence procedures were performed as previously described (Anzilotti S. et al., 2015). Pups were anesthetized and transcardially perfused with saline solution containing 0.01 ml heparin, followed by 10 ml of 4% paraformaldehyde in 0.1 mol/L PBS in saline solution. Brains were rapidly removed on ice and postfixed overnight at +4°C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB) with sodium azide 0.02% for 24h at 4°C. Next, they were sectioned frozen on a sliding cryostat at 40 μm thickness, in rostrum-

caudal direction. Afterwards, free floating serial sections were incubated with PB Triton× 0.3% and blocking solution (0.5% milk, 10% FBS, 1% BSA) for 1 hours and 30 minutes. So sections were incubated overnight at +4°C with following the primary antibodies: rabbit polyclonal anti-NeuN (1:1000, Millipore); mouse monoclonal anti-Nestin (1:200, Millipore); rabbit polyclonal antibody glial anti-GFAP (1:500, Novus Biologicals). The next day, the primary antibodies were removed, sections were washed with 0.1 M phosphate buffer (PB) and incubated with the corresponding florescent-labeled secondary antibodies (Alexa 488/Alexa 594 conjugated antimouse/antirabbit IgGs). Nuclei were counterstained with Hoechst. Images were observed using a Zeiss LSM510 META/laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Single images were taken with an optical thickness of 0.7 m and a resolution of 1024 × 1024. In double-labeled sections, the pattern of immune reactivity for both antigens was identical to that seen in single-stained material. Control double-immunofluorescence staining entailed the replacement of the primary antisera with normal serum (data not shown). To minimize a possible cross-reactivity between IgGs in double immunolabeling experiments, the full complement of secondary antibodies was maintained but the primary antisera were replaced with normal serum or only one primary antibody was applied (data not shown). In addition, the secondary antibodies were highly preadsorbed to the IgGs of numerous species. Tissue labeling without primary antibodies was also tested to exclude autofluorescence.

3.7 BrdU ADMINISTRATION AND IMMUNOHISTOCHEMISTRY

For the acute BrdU labeling experiment, BrdU (Sigma, 100 mg/kg body weight, 10 mg/ml in 0.007 N NaOH in 0.9% NaCl) was administered by intraperitoneal injection twice at postnatal day 7, with a time frame of 90 minutes. The dose (50µl) was administered in two times to facilitate absorption. Pups were subjected to hypoxic preconditioning and/or hypoxic-ischemic surgery, as previously described. At postnatal day 11, the mice were anesthetized and transcardially perfused, as earlier reported. The tissues were processed in according to protocol. Afterwards, free floating serial sections were incubated with 2M HCl for 30 minutes, following the sections were washed with 0.1 M PB, three times for 10 minutes. Then, the immunohistochemistry and confocal immunofluorescence was performed in according to procedure. It has been used the following primary antibodies: rat polyclonal anti-BrdU (1:300, Abcam); mouse monoclonal anti-PSANCAM (1:300, Millipore).

3.8 DIAMINOBENZIDINE (DAB) IMMUNOSTAINING, AND CONFOCAL IMMUNOFLUORESCENCE

Tissue processing was performed as previously described. Diaminobenzidine (DAB) immunostaining was performed with the Ultra Tek HRP kit (anti-polyvalent), in according to the manufacturer's protocol (Scy Tek Laboratories). Immunohistochemical staining protocol: (1) free floating serial slides were incubated in hydrogen peroxide for 15 minutes at temperature room; (2) they were washed twice for 10 minutes with 0.1 M phosphate buffer (PB); (3) sections were incubated in super block for 10 minutes at temperature room; (4) they were washed once for 10 minutes with 0.1 M PB buffer; (5) tissues were incubated overnight at +4°C with

following primary antibodies: mouse monoclonal anti-NCX1 (1:500; Swant, Bellinzona, Switzerland), rabbit polyclonal anti-NCX3 (1:3000; Swant, Bellinzona, Switzerland). The next day, sections were washed four times for 5 minutes with 0.1 M PB buffer; (7) they were incubated with Ultra Tek Anti-Polyvalent for 10 minutes at room temperature; (8) sections were washed four times for 5 minutes with 0.1 M PB buffer; (9) sections were incubated with Ultra Tek HRP for 10 minutes at room temperature and rinsed four times in 0.1 M PB buffer; (10) under chemical hood, tissues were incubated with DAB Chromogen to DAB Substrate [120 µl] for 3 minute at room temperature; (11) DAB incubation was interrupted with pure water and sections were placed on polysine slides and stored in dark room overnight at room temperature; (12) slides were dehydrated with four passages in ethanol (50%; 70%; 95%; 100%) for 5 minutes each one and by a single step in xylene for 10 minutes. Finally, slides were closed with Eukitt glue and cover glass. Images were observed using a Zeiss LSM510 META/laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Single images were taken with an optical thickness of 0.7 mm and a resolution of 1024 × 1024.

3.9 INTENSITY OF NESTIN IMMUNOSTAINING

The double-labeled tissue (Fig. 10, 12) was used to evaluate the fluorescence intensity of Nestin in hippocampus dentate gyrus. The consecutive and identical sections were selected starting from bregma −1.46 mm, interaural +2.34 in order to analyze the dentate gyrus. During the phase of image acquisition by confocal microscope, standard conditions were regulated (Pinhole 35 µm; Laser intensity 6.6 Units; Dimensions (x512, y512); Image size 1277.25 µm; Digital offest 0; Digital gain

1; Master gain 678). One image for each section was acquired. Three-four sections were analyzed for each mouse. Image J software was used to measure the fluorescence intensity of Nestin (Anzilotti S. et al., 2012).

3.10 CELL-COUNTING ANALYSIS

The number of BrdU and PSANCAM positive cells was determined in the dentate gyrus of the hippocampus and subventricular zone of C57BL/6 mice at postnatal day 11, by manual counting at × 10 magnification. The consecutive and identical sections were selected starting from bregma −1.46 mm, interaural +2.34 in order to analyze the dentate gyrus. While the consecutive and identical sections were selected starting from bregma 0.14 mm, interaural +3.94 for the analysis of subventricular zone. Only BrdU and PSANCAM positive cells with clearly visible cell bodies and profiles were counted. Six mice per group were included in the study of dentate gyrus, and four slices for each mouse were analyzed. Five mice per group were included in the study of subventricular zone, and three-four slices for each mouse were analyzed.

The number of NCX1 and NCX3 positive cells was determined in the dentate gyrus of the hippocampus of C57BL/6 mice at postnatal day 11, by manual counting at × 10 magnification. The consecutive and identical sections were selected starting from bregma −1.46 mm, interaural +2.34. Only NCX1 and NCX3 positive cells with clearly visible cell bodies and profiles were counted. Three mice per group were included in the study, and four slices for each mouse were analyzed (Anzilotti S. et al., 2015).

3.11 WESTERN-BLOT ANALYSIS

Western-blot analyses were performed in according to protocol of Pignataro' s study (Pignataro G. et al., 2012). Hippocampal samples were harvested from brains of mice subjected to (a) hypoxic preconditioning plus HI, (b) HI, and (c) control. Under all the experimental conditions, ten groups of ipsilateral and contralateral hippocampal slices were obtained after 24 hours of hypoxic-ischemic injury for analysis of NCX1 protein, while for NCX3 analyses 6 groups of ipsilateral and contralateral hippocampal slices were obtained. Mouse brain samples were homogenized in a lysis buffer (0.5 M Hepes ph 7.4, 1 M NaF, 100 Mm Na_3O_4V; 1 M Naazide, 25% Triton X-100) containing protease and the phosphatase inhibitor. After centrifugation at 11,000 g at 4 °C for 30 minutes, the supernatants were collected. Protein concentration was estimated using the Bradford reagent. Then, 50 µg of protein was mixed with a Laemmli 4x sample buffer. The samples were resolved by 8% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were probed with antibodies anti-NCX1 (mouse monoclonal; Swant, Bellinzona, Switzerland; 1:1000 dilution), anti-NCX3 (rabbit polyclonal; Swant, Bellinzona, Switzerland; 1:2000), and anti-β-Tubulin (Sigma-Aldrich mouse monoclonal 1:10000 dilution). After, nitrocellulose membranes were first washed with 0.1% Tween 20 and then incubated with the corresponding secondary antibodies for 1 hour (GE Healthcare, Little Chalfont, UK). Immunoreactive bands were detected with the ECL (GE Healthcare). The optical density of the bands (normalized with β-Tubulin) was determined by Chemi-Doc Imaging System (Bio-Rad, Segrate, Italy)

3.12 BEHAVIORAL TESTS

Experiments were performed under diurnal lighting conditions between 10:00 A.M. and 5:00 P.M. Pups mice were analyzed in behavioral reflex tests in according to Ten protocol (Ten VS. et al., 2003). Reflex tests were performed at different time intervals: (1) twice at postnatal day 7; (2) at postnatal day 9; (3) at postnatal day 11, before sacrifice.

3.12.1 RIGHTING REFLEX TEST

In according to Ten's protocol mice were placed in a supine position, and the time (in seconds) required to flip to the prone position was measured (Ten VS. et al., 2003). It has been given to each pup three attempts with a maximum time of 30 seconds for each trial, the mean time to perform the reflex was written down. The analyses were performed in control group (n=12), hypoxic-ischemic group (n=11) and preconditioned hypoxic-ischemic group (n=11).

3.12.2 CLIFF AVOIDANCE REFLEX TEST

In according to Ten's protocol pups were placed with their forepaws and chest extending over the board of table tend to move away by backing up with forepaws or their body and head turning sideways (Ten VS. et al., 2003; Fan LW. et al., 2010). In according to Fan, it has been considered the time required (in seconds) to come back from the edge was recorded, but it has been chosen up to a maximum observation time of 30 seconds; if the mouse was unable to perform the reflex within the allotted time or fell off from the table, the maximal time was assigned (Ten VS. et

al., 2003; Fan LW. et al., 2010). Each pup was given three attempts with a maximum time of 30 seconds for each trial, the mean time to perform the reflex was written down. The analyses were performed in control group (n=12), hypoxic-ischemic group (n=13) and preconditioned hypoxic-ischemic group (n=13).

3.12.3 GEOTAXIS REFLEX TEST

In according to Ten's protocol, pups were placed head downward on an inclined board (40°) (Ten VS. et al., 2003). In according to Ten, it has been considered the time (in seconds) required for the pups to rotate their bodies head up (>90°rotation) was recorded, but it has been chosen up to a maximum observation time of 30 seconds; if the mouse was unable to perform the reflex within the allotted time or fell off from the table, the maximal time was assigned. Each pup was given three attempts with a maximum time of 30 seconds for each trial, the mean time to perform the reflex was written down. The analyses were performed in control group (n=12), hypoxic-ischemic group (n=14) and preconditioned hypoxic-ischemic group (n=14).

3.13 STATISTICAL ANALYSIS

The data were evaluated as mean ± S.E.M. Statistically significant differences among means were determined by ANOVA followed by Newman-Keuls post-hoc test. For the comparison between the percentage of hippocampal damage in preconditioned hypoxic-ischemic and hypoxic-ischemic mice statistically significant differences between means were determined by t-Test. Statistical significance was accepted at

the 95% confidence level ($p<0.05$). Statistical analyses were performed by using GraphPad Prism 5 software and graphs with Microsoft Excel.

4 RESULTS

4.1 EFFECT OF ISCHEMIA AND DIFFERENT DURATION OF HYPOXIA (HI) ON HIPPOCAMPAL DAMAGE EVAULATED 24 HOURS AFTER INJURY

The damage was evaluated through Propidium Iodide administration *in vivo*, in four groups of animals (P7), subjected to several time intervals of hypoxia (10'; 20'; 30'; 60') following ischemia induction. Hippocampal damage significantly increased according to the duration of hypoxia (Fig. 8). The damage was localized in the ipsilateral hippocampus to the CCA, mainly in CA1 and CA3 areas.

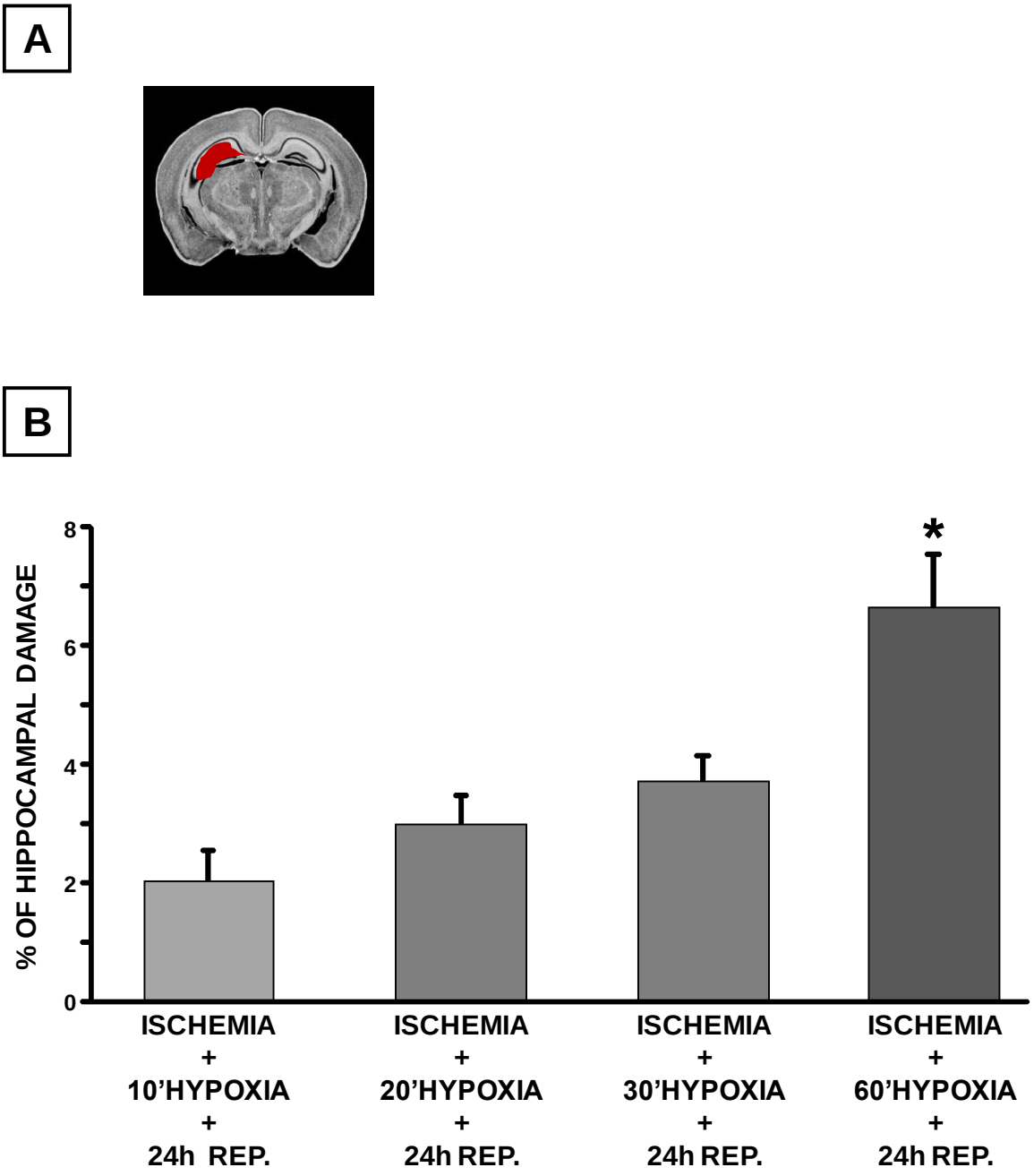

Figure 8. (A) The image shows the area analyzed for each brain slice in hypoxic-ischemic mice. (B) The hippocampal damage significantly increases in HI 60' mice compared to HI 10' group, 24 hours after induction of insult. Each column represents the mean ± S.E.M of data obtained from ischemic brain volume analysis of seven-day-old mice subjected to ischemia and to several time intervals of hypoxia (10, 20, 30, 60 minutes). The total infarct volume, corrected for edema, was expressed as percentage of the volume of the hippocampus ipsilateral to the lesion. **P*<0.05 versus HI 10' group. The analyses were performed in seven mice for each group.

4.2 TIME COURSE OF HIPPOCAMPAL DAMAGE INDUCED BY ISCHEMIA AND HYPOXIA 60’ (HI)

The evolution of ipsilateral hippocampal damage was evaluated through Propidium Iodide administration *in vivo*, in four groups of animals (P7) subjected to ischemia plus 60’ hypoxia and sacrificed at different time intervals after insult (24h; 48h; 72h; 7 days). Hippocampal damage significantly increased 7 days after induction of insult, with a mean value of 42.5%. The damage was localized in CA1 and CA3 hippocampal areas as confirmed by immunofluorescence analyses (Fig. 9).

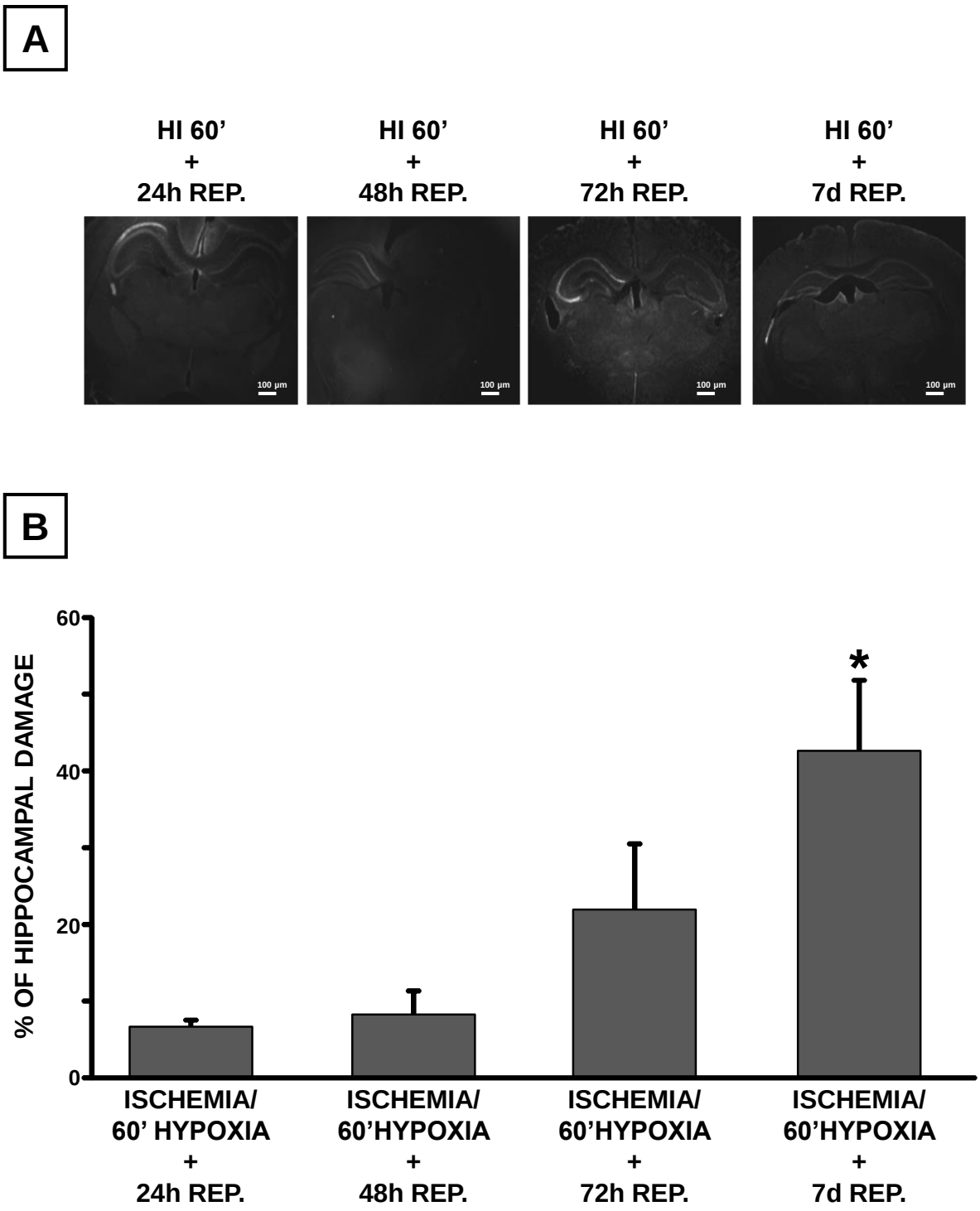

Figure 9. (A) Representative images of Propidium Iodide staining in the hypoxic-ischemic groups have been shown. Scale bar 100 µm. (B) The hippocampal damage significantly increases in HI 60' mice 7 days after induction. Each column represents the mean ± S.E.M of data obtained from ischemic brain volume analysis of seven-day-old mice subjected to ischemia plus 60' hypoxia and sacrificed at different time intervals (24 hours, 48 hours, 72 hours, 7 days). The total brain infarct volume, corrected for edema, was expressed as percentage of the volume of the hippocampus ipsilateral to the lesion. **P*<0.05 versus HI 60' plus 24 hours of reperfusion. The analyses were performed in the following groups: HI 60' and 24h reperfusion (n=7), HI 60' and 48h reperfusion (n=5), HI 60' and 72h reperfusion (n=5), HI 60' and 7 days reperfusion (n=5).

4.3 FLUORESCENCE INTENSITY OF NEURAL STEM CELL (NSC) MARKER, NESTIN, INCREASES AFTER 20’ OF HYPOXIA FOLLOWED BY 24 HOURS OF RECOVERY

In order to analyze the activation of NSCs in dentate gyrus niche, after a specific stimulus, fluorescence analysis was performed in different mice groups. Pup mice (P7) were divided into the following eight groups: control, ischemia, 10’ hypoxia, 20’ hypoxia, 30’ hypoxia, 60’ hypoxia, ischemia plus 10’ hypoxia, ischemia plus 20’ hypoxia. All mice were sacrificed 24 hours after stimulus induction. The fluorescence analysis was performed through immunohistochemistry by using specific antibodies: anti-NeuN, anti-Nestin, and marker of nuclei Hoechst. NeuN antibody identifies the mature neurons, while Nestin antibody allows to identify an intermediate filament protein type VI (IFN), that is a component of neural progenitor cytoskeleton. Then, anti-Nestin marks the NSCs localized in neurogenic niches. The analysis of Nestin fluorescence intensity showed that there was a significant increase of Nestin in mice group subjected to 20’ hypoxia compared to control group. In addition, there was a significant reduction of Nestin in mice groups subjected to 30’ and 60’ hypoxia compared to control and 20’ hypoxic groups (Fig. 10, 11). Furthermore, the analysis of Nestin showed that there was not a significant change in mice subjected to a stronger insult (Fig. 12, 13).

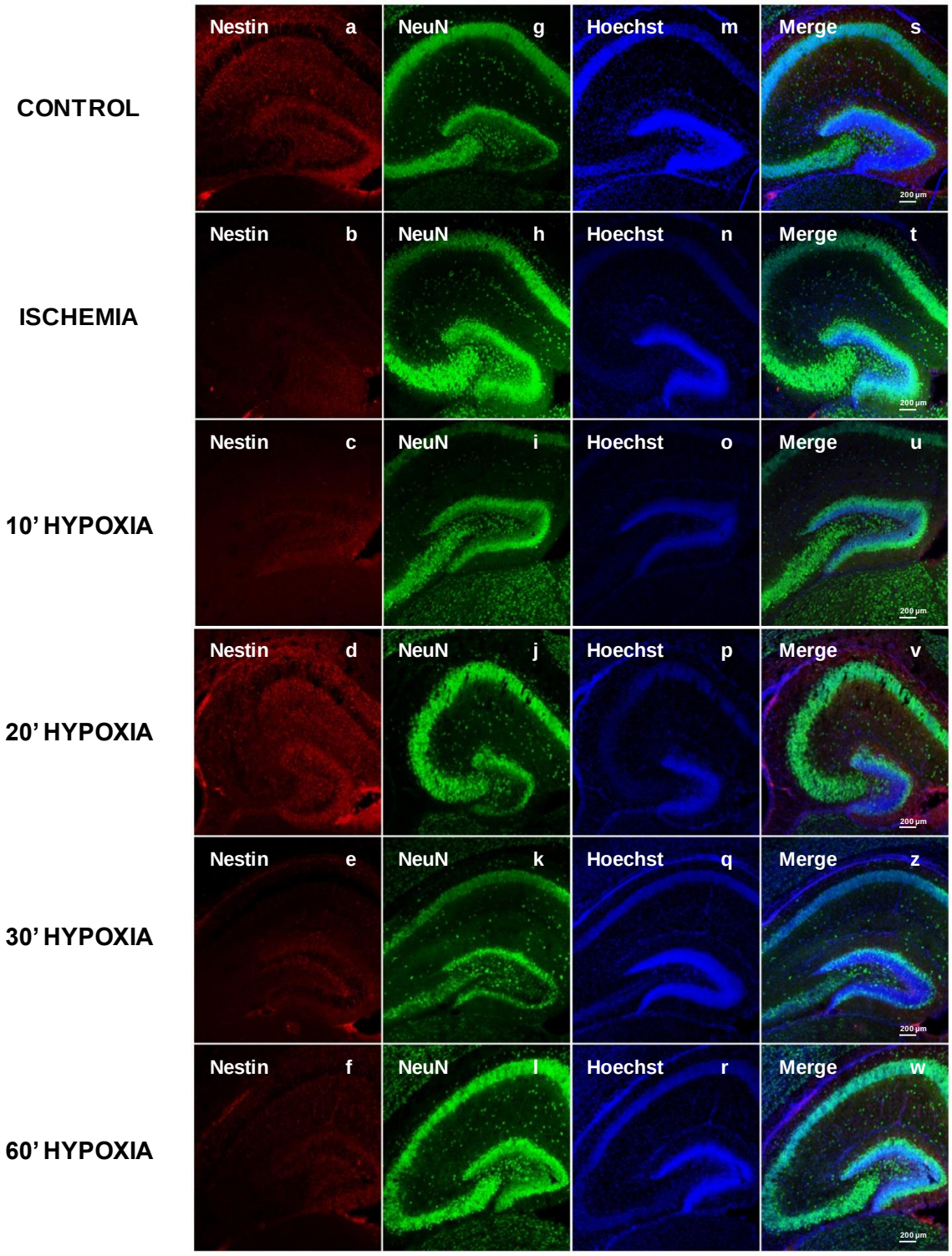

Figure 10. Distribution of Nestin and NeuN positive cells in mouse dentate gyrus. Single staining of Nestin (a-f); NeuN (g–l), Hoechst (m-r) in brain sections from control (a, g, m, respectively), ischemic (b, h, n, respectively), 10' hypoxic (c, i, o, respectively); 20' hypoxic (d, j, p, respectively); 30' hypoxic (e, k, q, respectively); 60' hypoxic (f, l, r, respectively) groups. Double-staining of Nestin and NeuN (s-t-u-v-z-w) in the dentate gyrus of six groups. Scale bar 200 µm. Standard conditions: Pinhole 35 µm; Laser intensity 6.6 Units; Dimensions (x512, y512); Image size 1277.25 µm; Digital offest 0; Digital gain 1; Master gain 678.

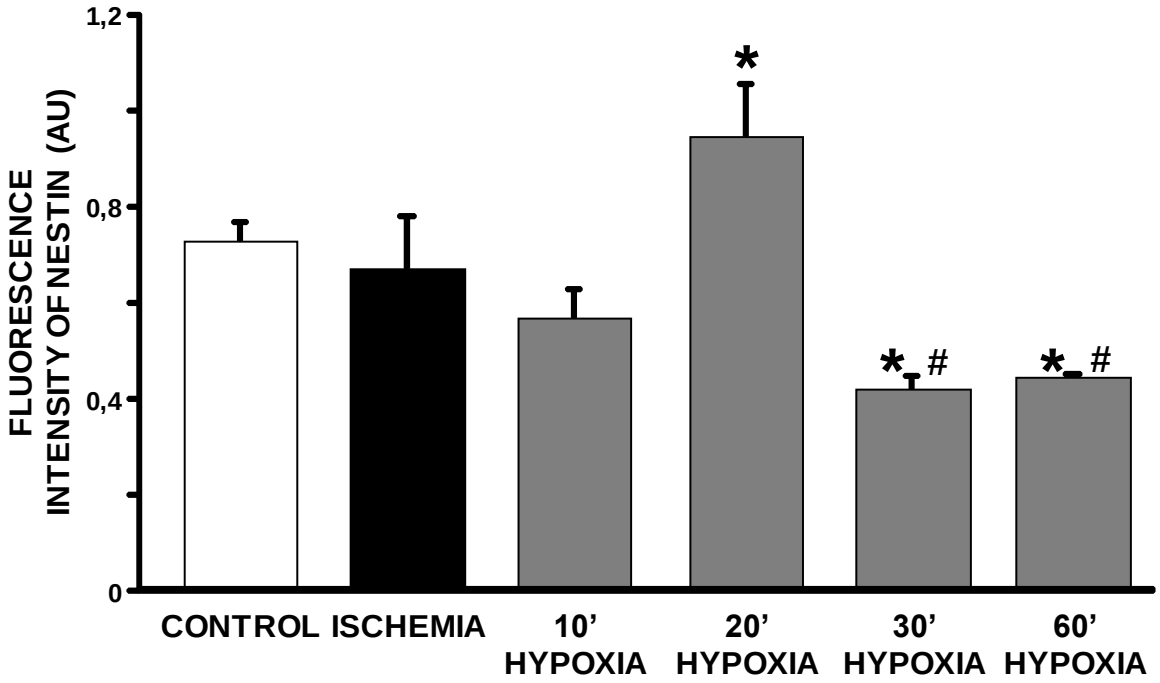

Figure 11. Fluorescence intensity of Nestin increases in mice subjected to 20' hypoxia compared to control mice. In addition, the fluorescence intensity of Nestin decreases in mice subjected to 30' and 60' hypoxia compared to control and 20' hypoxic groups. Each column represents the mean ± S.E.M of data obtained from measurements of fluorescent levels of Nestin in the dentate gyrus, for each mouse group (control, ischemia, 10' hypoxia, 20' hypoxia, 30' hypoxia, 60' hypoxia). Quantification of fluorescence intensity (expressed in arbitrary units, AU). **P*<0.05 versus control group; $^{\#}$*P*<0.05 versus 20' hypoxic group. The analyses were performed in five mice for each treatment group, except 60' hypoxic mice group (n=4).

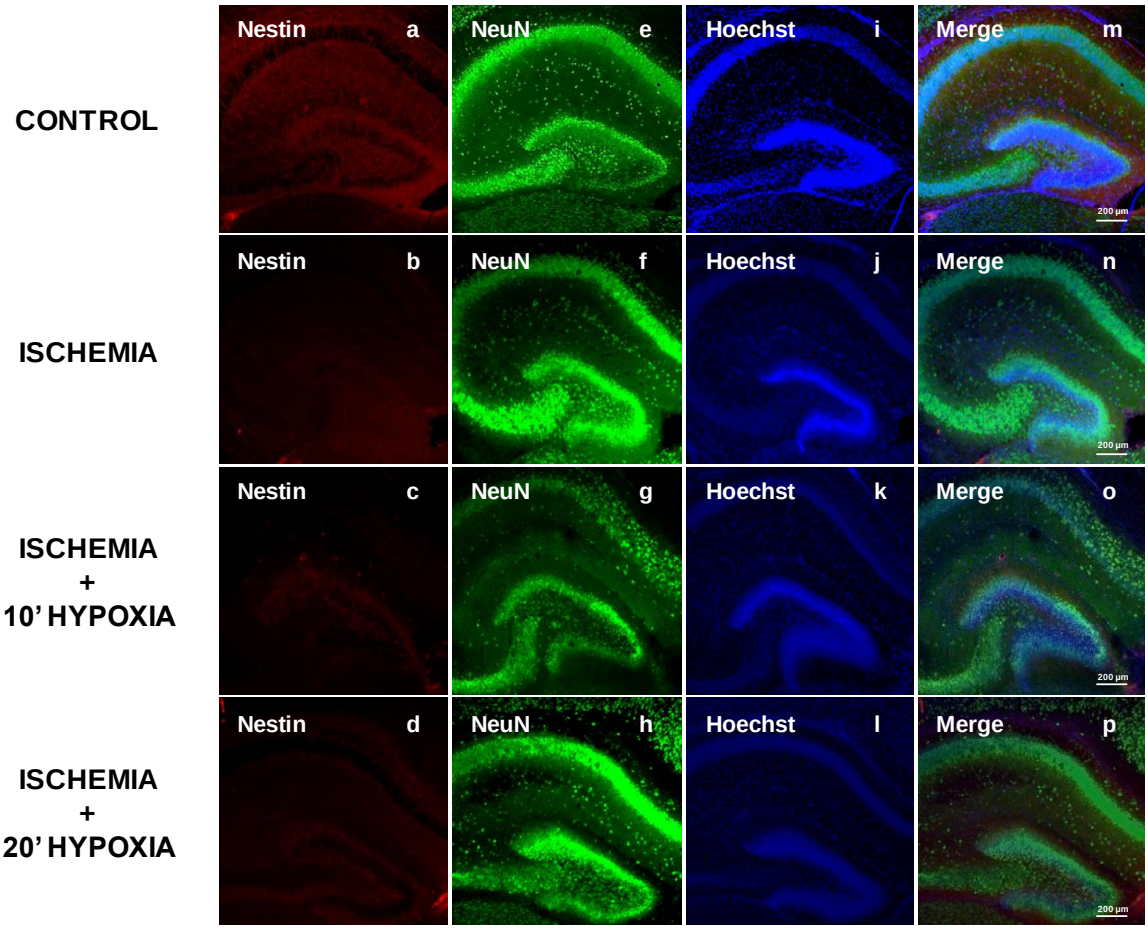

Figure 12. Distribution of Nestin and NeuN positive cells in the mouse dentate gyrus. Single staining of Nestin (a-d); NeuN (e-h), Hoechst (i-l) in brain sections from control (a, e, i, respectively), ischemic (b, f, j, respectively), HI 10' (c, g, k, respectively); HI 20' (d, h, l, respectively) groups. Double-staining of Nestin and NeuN (m-n-o-p) in the dentate gyrus of four groups. Scale bar 200 µm. Standard conditions: Pinhole 35 µm; Laser intensity 6.6 Units; Dimensions (x512, y512); Image size 1277.25 µm; Digital offest 0; Digital gain 1; Master gain 678 .

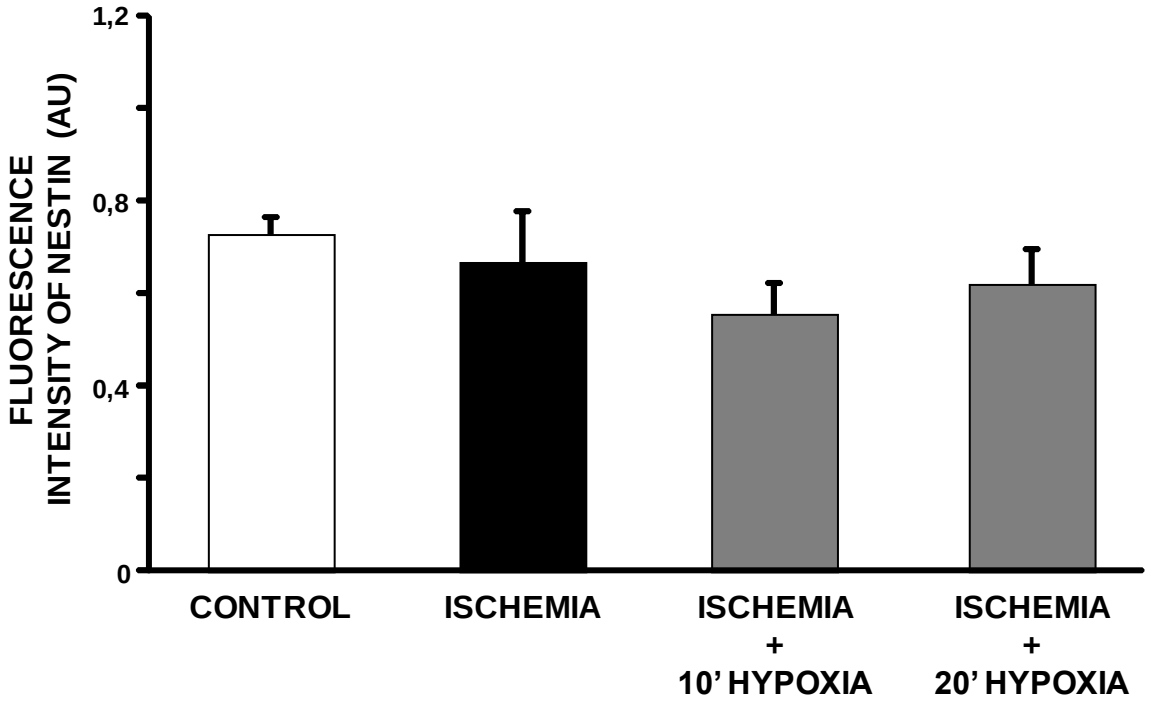

Figure 13. Fluorescence intensity of Nestin does not increase in mice subjected to ischemia and ischemia plus hypoxia compared to control mice. Each column represents the mean ± S.E.M of data obtained from measurements of fluorescent levels of Nestin in the dentate gyrus for each mouse group (control, ischemia, ischemia plus 10' hypoxia, ischemia plus 20' hypoxia). Quantification of fluorescence intensity (expressed in arbitrary units, AU).The analyses were performed in control group (n=5), ischemic group (n=5), HI 10' group (n=3), HI 20' group (n=4).

4.4 EVALUATION OF THE TIME WINDOW FOR HYPOXIC PRECONDITIONING NEUROPROTECTION

The damage was evaluated through Propidium Iodide administration *in vivo*, in three major groups of animals (P7) in turn divided into two subgroups. The first group was subjected to hypoxic-ischemic insult at postnatal day nine (P9); the second group was subjected to hypoxic-ischemic insult at postnatal day ten (P10); the third group was subjected to hypoxic-ischemic insult at postnatal day fourteen (P14). In each group seven-day-old mice were divided into the first subgroup where the animals were untreated and in the second one the mice were subjected to hypoxic preconditioning, subsequently, both subgroups were subjected to HI insult at different time intervals (P9, P10, P14). All animals were sacrificed 24 hours after injury. The percentage of brain infarct volume has been valued for all subgroups. Then, the mean value of the first and second subgroup of each experimental condition was compared by using t-Test. Paired analysis demonstrated that there was a significant decrease of hippocampal damage in preconditioned mice, subjected to HI 60' three days after 20' hypoxia stimulus and sacrificed 24 hours later compared to mice subjected to only HI insult at P10 (Fig. 14).

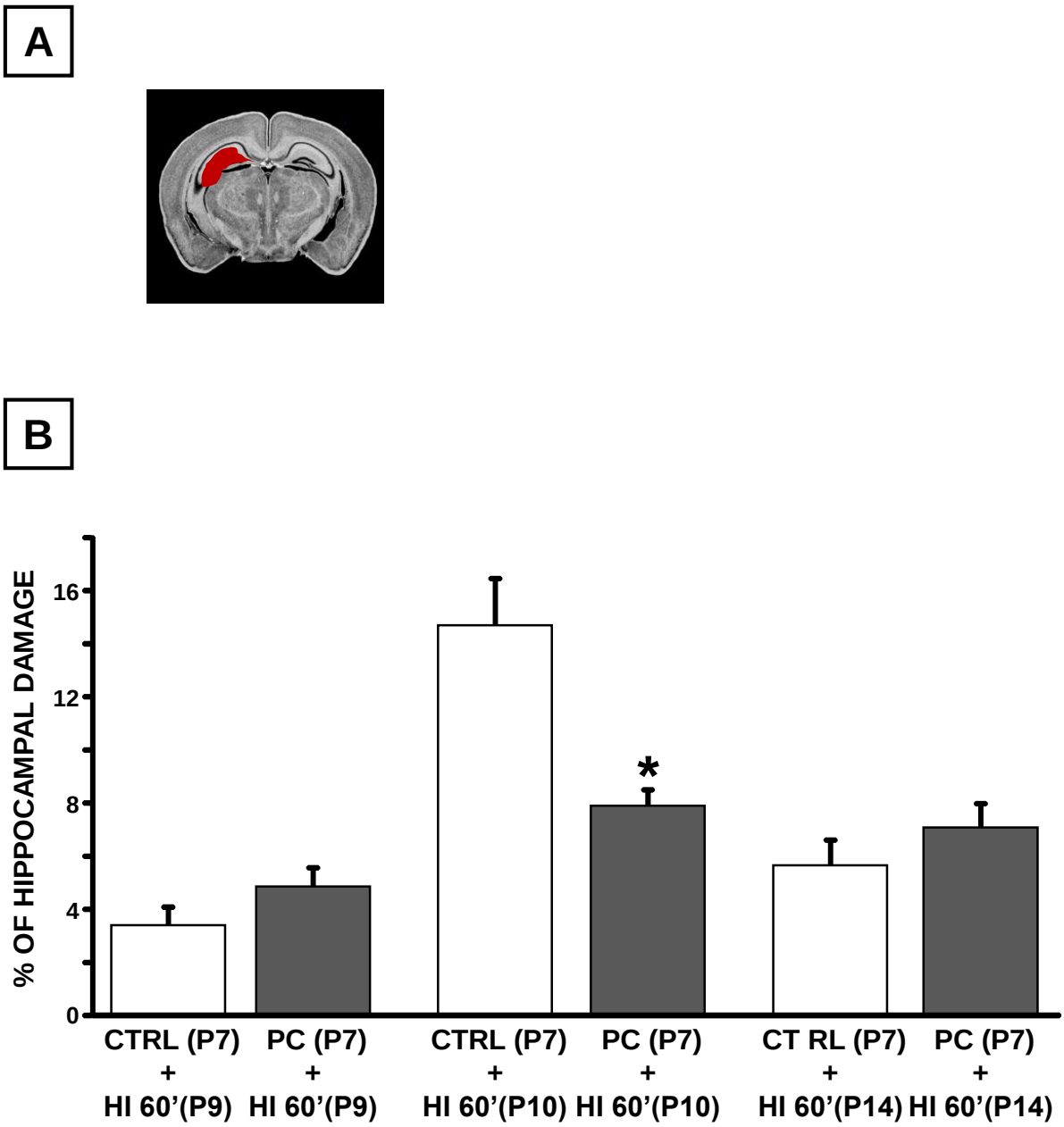

Figure 14. (A) The image shows the area analyzed for each brain slice in hypoxic-ischemic and preconditioned hypoxic-ischemic mice (subjected to preconditioning at P7 plus HI 60' at P10). (B) The hippocampal damage significantly decreases in preconditioned hypoxic-ischemic mice (P10) compared to hypoxic-ischemic mice (P10), sacrificed 24 hours after insult induction. Data represent the mean ± S.E.M. **P*<0.05 versus CTRL (P7)+ HI 60' (P10). The analyses were performed in: CTRL (P7)+ HI 60' (P9) group (n=4), PC (P7)+ HI 60' (P9) group (n=7), CTRL (P7)+ HI 60' (P10) group (n=6), PC (P7)+ HI 60' (P10) group (n=7), CTRL (P7)+ HI 60' (P14) group (n=7), PC (P7)+ HI 60' (P14) group (n=11).

4.5 HYPOXIC PRECONDITIONING INCREASES THE TOTAL NUMBER OF PSANCAM POSITIVE CELLS IN SUBVENTRICULAR ZONE OF MICE SUBJECTED TO HYPOXIC-ISCHEMIC INJURY

The effect of preconditioning stimulus (20' hypoxia) on endogenous neurogenesis was examined in subventricular zone of mice subjected to hypoxic-ischemic injury. In particular, the differentiation of NSCs was monitored through the analysis of proliferating $BrdU^+$ cells. As previously described, all P7 mice were subjected to two intraperitoneal administrations of BrdU, and eventually, they were divided into three experimental groups: control, hypoxic-ischemic; and preconditioned hypoxic-ischemic mice. Then, the analysis of proliferating NSCs was performed by using immunohistochemistry with specific antibodies: anti-BrdU, anti-PSANCAM, and marker of nuclei Hoechst. BrdU antibody identifies the proliferating cells, which have incorporated the BrdU dose, while PSANCAM antibody allows to identify a membrane-bound glycoprotein expressed by neuroblast cells. Then, double-staining of BrdU and PSANCAM marks the proliferating neural progenitors localized in neurogenic niches. The analysis of PSANCAM staining in subventricular zone showed that there was a significant increase of PSANCAM positive cells in preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic group. In addition, the analysis of BrdU/PSANCAM double staining demonstrated that there was a significant decrease of BrdU/PSANCAM positive cells in hypoxic-ischemic and preconditioned hypoxic-ischemic mice compared to control group (Fig. 15, 16).

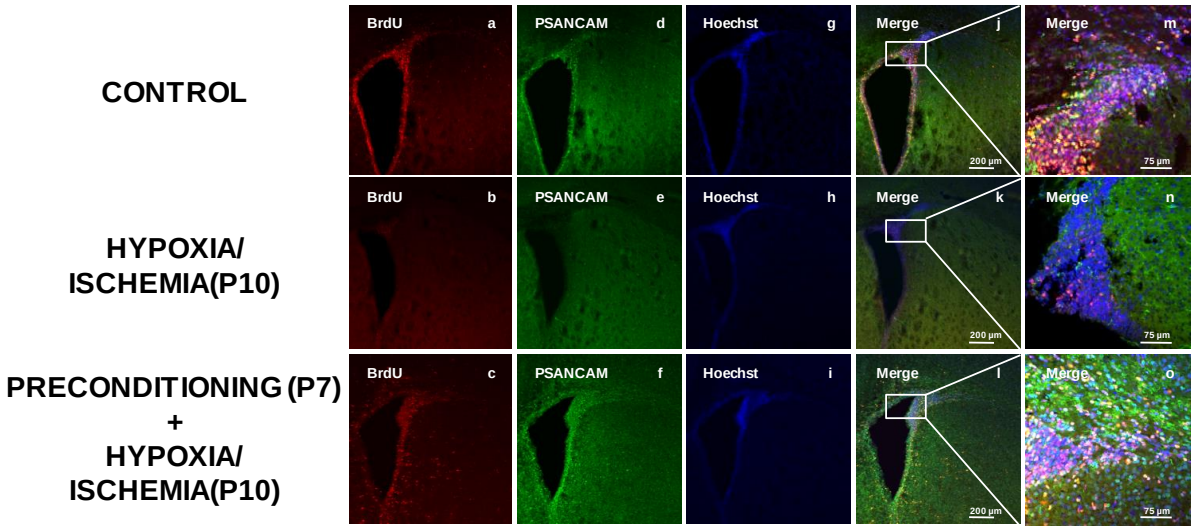

Figure 15. Distribution of PSANCAM and BrdU/PSANCAM positive cells in the mouse subventricular zone. Single staining of BrdU (a-c); PSANCAM (d-f), Hoechst (g-i) in brain sections from control (a, d, g, respectively), hypoxic-ischemic (b, e, h, respectively), preconditioned hypoxic-ischemic (c, f, i, respectively) mice. Double-staining of BrdU and PSANCAM (j-k-l) in the subventricular zone of three groups. Scale bar 200 µm. High magnification of portions of dentate gyrus (m-n-o) Scale bar, 75 µm.

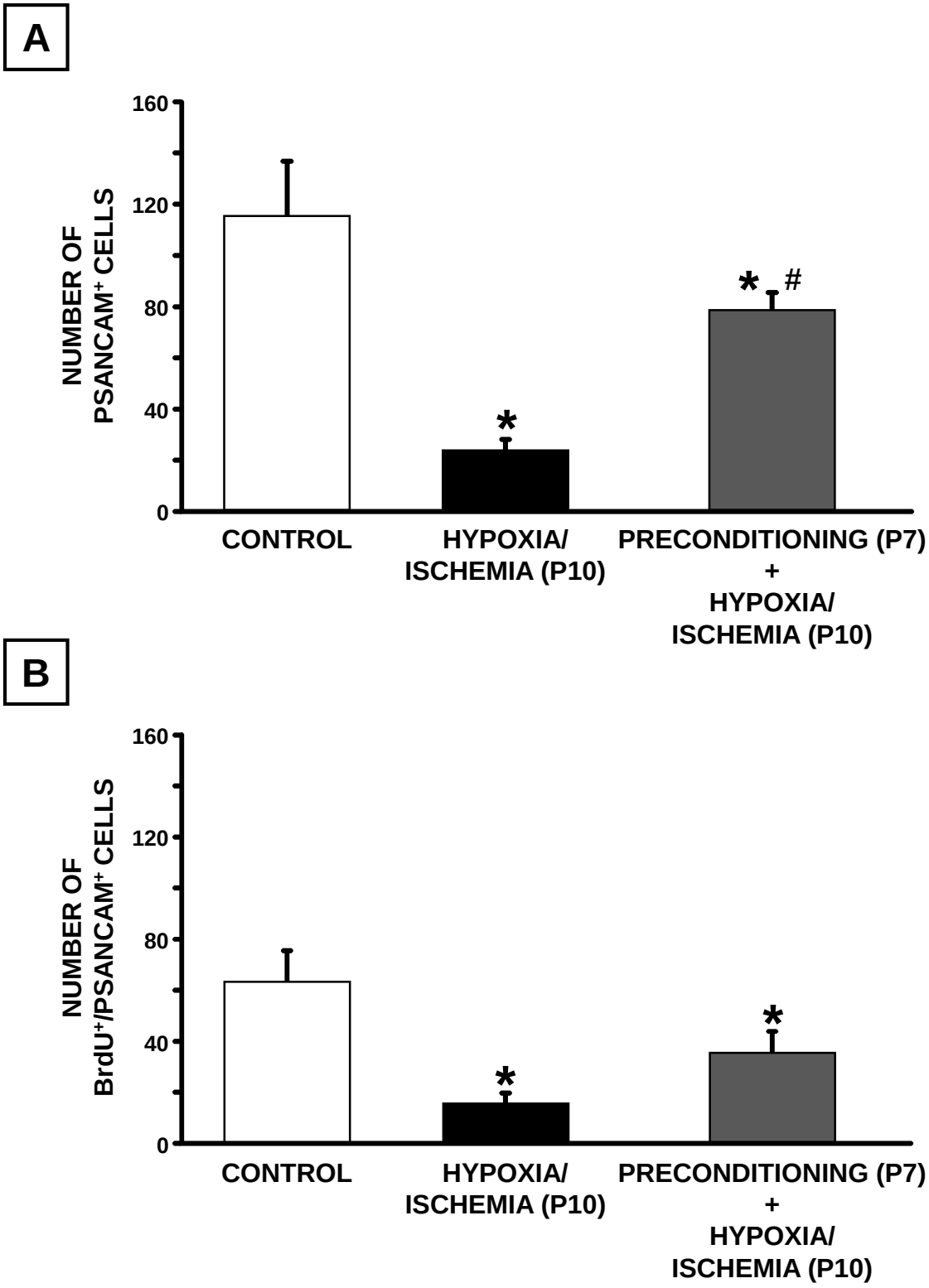

Figure 16. (A) Preconditioning induces the increase of total number of PSANCAM-positive cells in subventricular zone of hypoxic-ischemic mice. (B) The number of BrdU/PSANCAM-positive cells decreases in the subventricular zone of hypoxic-ischemic and preconditioned hypoxic-ischemic mice compared to control group. Data represent the mean ± S.E.M. **P*<0.05 versus control group; $^{\#}P$<0.05 versus hypoxic-ischemic group. The analyses were performed in five animals for each group.

4.6 HYPOXIC PRECONDITIONING INCREASES THE TOTAL NUMBER OF PSANCAM POSITIVE AND BrdU/PSANCAM POSITIVE CELLS IN DENTATE GYRUS OF MICE SUBJECTED TO HYPOXIC-ISCHEMIC INJURY

The effect of preconditioning stimulus (20' hypoxia) on endogenous neurogenesis was examined in dentate gyrus of mice subjected to hypoxic-ischemic injury. In particular, the differentiation of NSCs was detected through the analysis of proliferating $BrdU^+$ cells. As previously described, all P7 mice were subjected to two intraperitoneal administrations of BrdU, and eventually, they were divided into three experimental groups: control, hypoxic-ischemic; and preconditioned hypoxic-ischemic mice. Then, the investigation of proliferating NSCs was performed by using immunohistochemical analyses with specific antibodies: anti-BrdU, anti-PSANCAM, and marker of nuclei Hoechst. BrdU antibody identifies the proliferating cells, which have incorporated the BrdU dose, while PSANCAM antibody allows to identify a membrane-bound glycoprotein expressed by neuroblast cells, double-staining of BrdU and PSANCAM marks the proliferating neural progenitors localized in neurogenic niches. The analysis of PSANCAM staining in dentate gyrus showed that there was a significant increase of PSANCAM positive cells in preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic group. In addition, the analysis of BrdU/PSANCAM double staining demonstrated that there was a significant increase of BrdU/PSANCAM positive cells in preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic group (Fig. 17, 18)

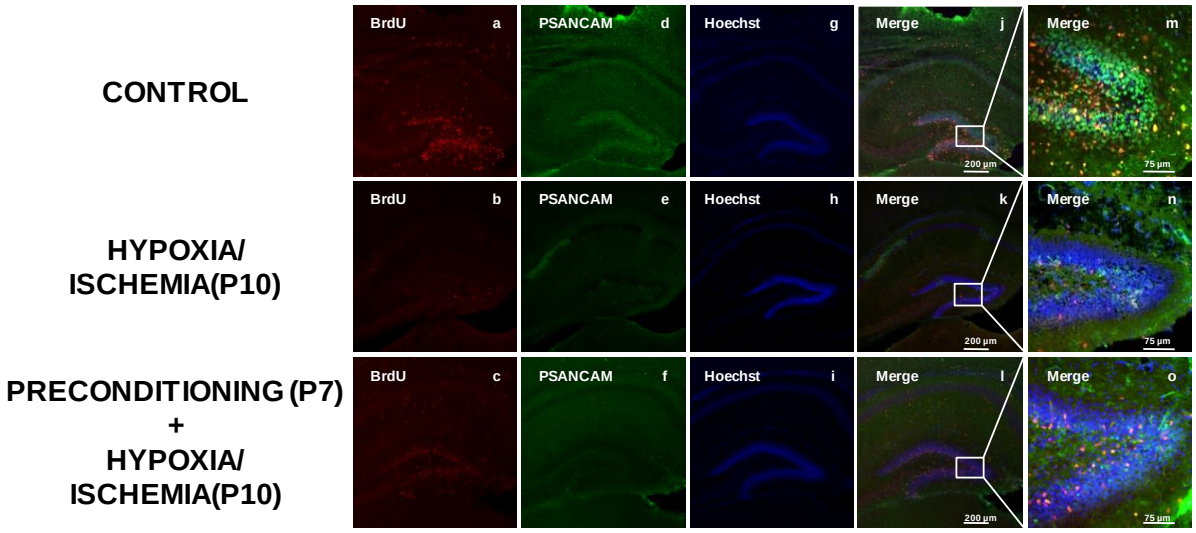

Figure 17. Distribution of PSANCAM and BrdU/PSANCAM positive cells in the mouse dentate gyrus. Single staining of BrdU (a-c); PSANCAM (d-f), Hoechst (g-i) in brain sections from control (a, d, g, respectively), hypoxic-ischemic (b, e, h, respectively), preconditioned hypoxic-ischemic (c, f, i, respectively) mice. Double-staining of BrdU and PSANCAM (j-k-l) in the dentate gyrus of three groups. Scale bar 200 µm. High magnification of portions of dentate gyrus (m-n-o) Scale bar, 75 µm.

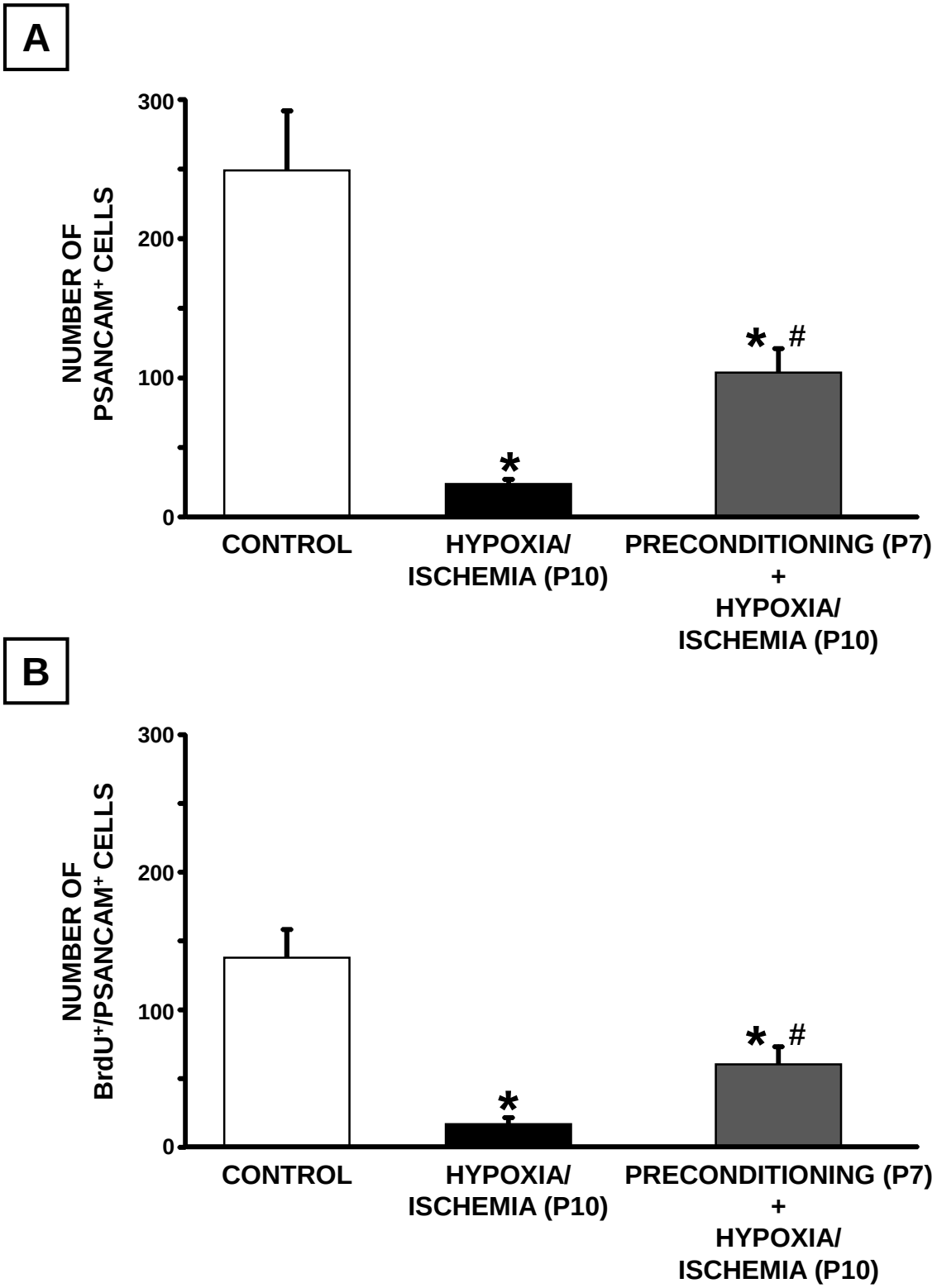

Figure 18. (A) Preconditioning induces the increase of total number of PSANCAM-positive cells in dentate gyrus of hypoxic-ischemic mice. (B) Preconditioning induces the increase of total number of BrdU/PSANCAM positive cells in dentate gyrus of hypoxic-ischemic mice. Data represent the mean ± S.E.M. **P*<0.05 versus control group; $^{\#}P$<0.05 versus hypoxic-ischemic group. The analyses were performed in six animals for each group.

4.7.1 HYPOXIC PRECONDITIONING DOES NOT IMPROVE PERFORMANCE IN RIGHTING REFLEX TEST OF MICE SUBJECTED TO HYPOXIA ISCHEMIA

Short-term neurobehavioral functions were evaluated in pup mice by using specific neurobehavioral tests. Righting reflex test was performed in three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic animals. Mice performance was monitored at different time intervals. In particular, mice were analyzed twice at postnatal day seven, with a time frame of 60 minutes. Afterwards, the test was replicated at postnatal day nine and before the sacrifice (P11). In the early phase of hypoxic preconditioning induction no difference was observed among three groups. At postnatal days eleven again no difference among three groups occurred. The animals always spent a similar time (sec) to perform trial (Fig. 19).

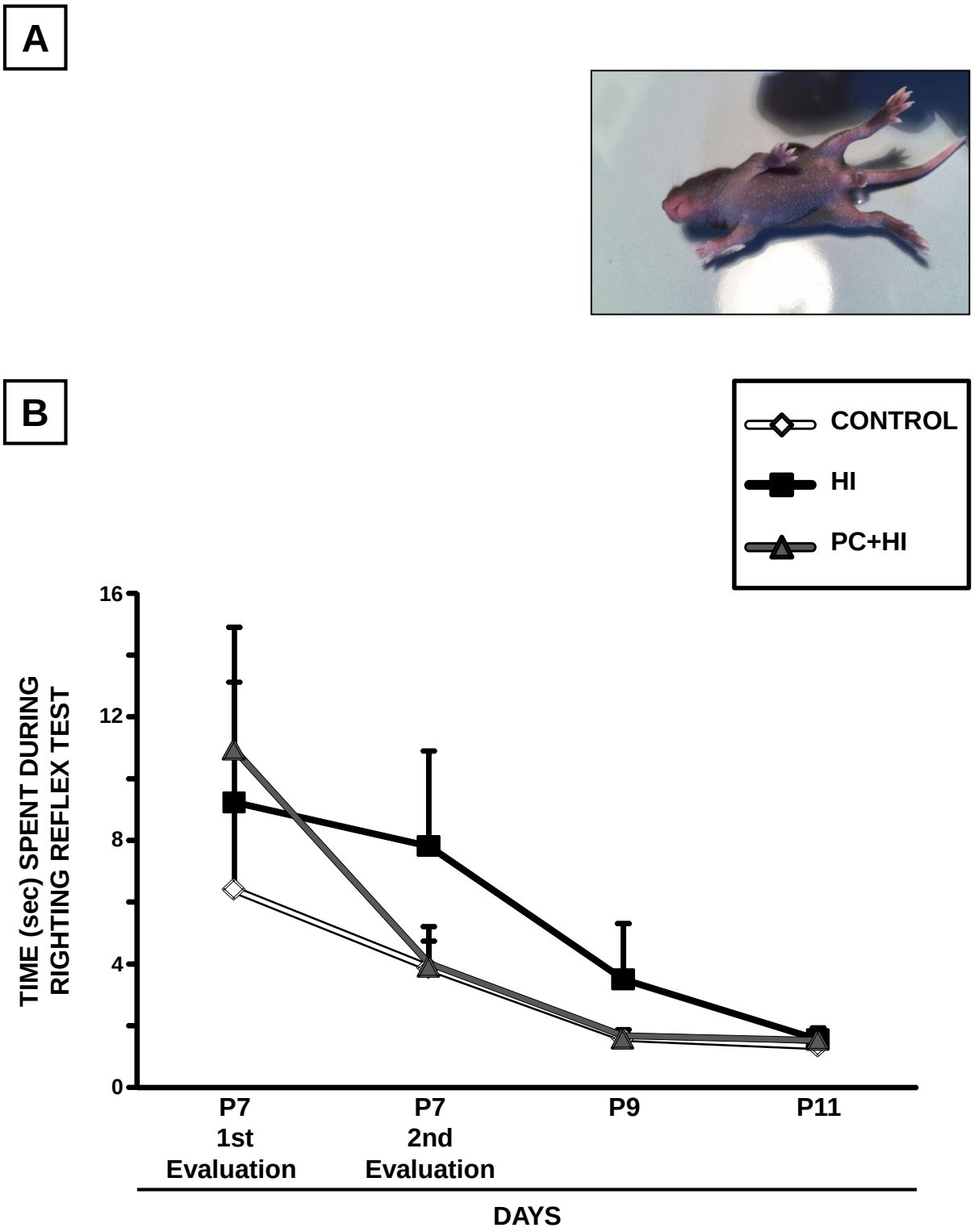

Figure 19. (A) The picture shows the pup placed in supine position, the time (in seconds) required to flip to the prone position was measured (B) Time course of righting performance in three experimental groups: control, hypoxic-ischemic, preconditioned hypoxic-ischemic mice. Data represent the mean ± S.E.M. The analyses were performed in control mice (n=12); hypoxic-ischemic mice (n=11); preconditioned hypoxic-ischemic mice (n=11).

4.7.2 HYPOXIC PRECONDITIONING IMPROVES PERFORMANCE IN CLIFF AVOIDANCE REFLEX TEST OF MICE SUBJECTED TO HYPOXIA ISCHEMIA

The development of sensorimotor reflexes was evaluated in pup mice by using specific neurobehavioral tests. Cliff avoidance test was performed in three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic hypoxic-ischemic animals. Mice performance was monitored at different time intervals. In particular, mice were analyzed twice at postnatal day seven, with a time frame of 60 minutes. Afterwards, the test was replicated at postnatal day nine and before the sacrifice (P11). In the early phase of hypoxic preconditioning induction no difference was observed among three groups. At postnatal day eleven preconditioned hypoxic-ischemic mice spent less time (sec) to perform trial than hypoxic-ischemic mice (Fig. 20).

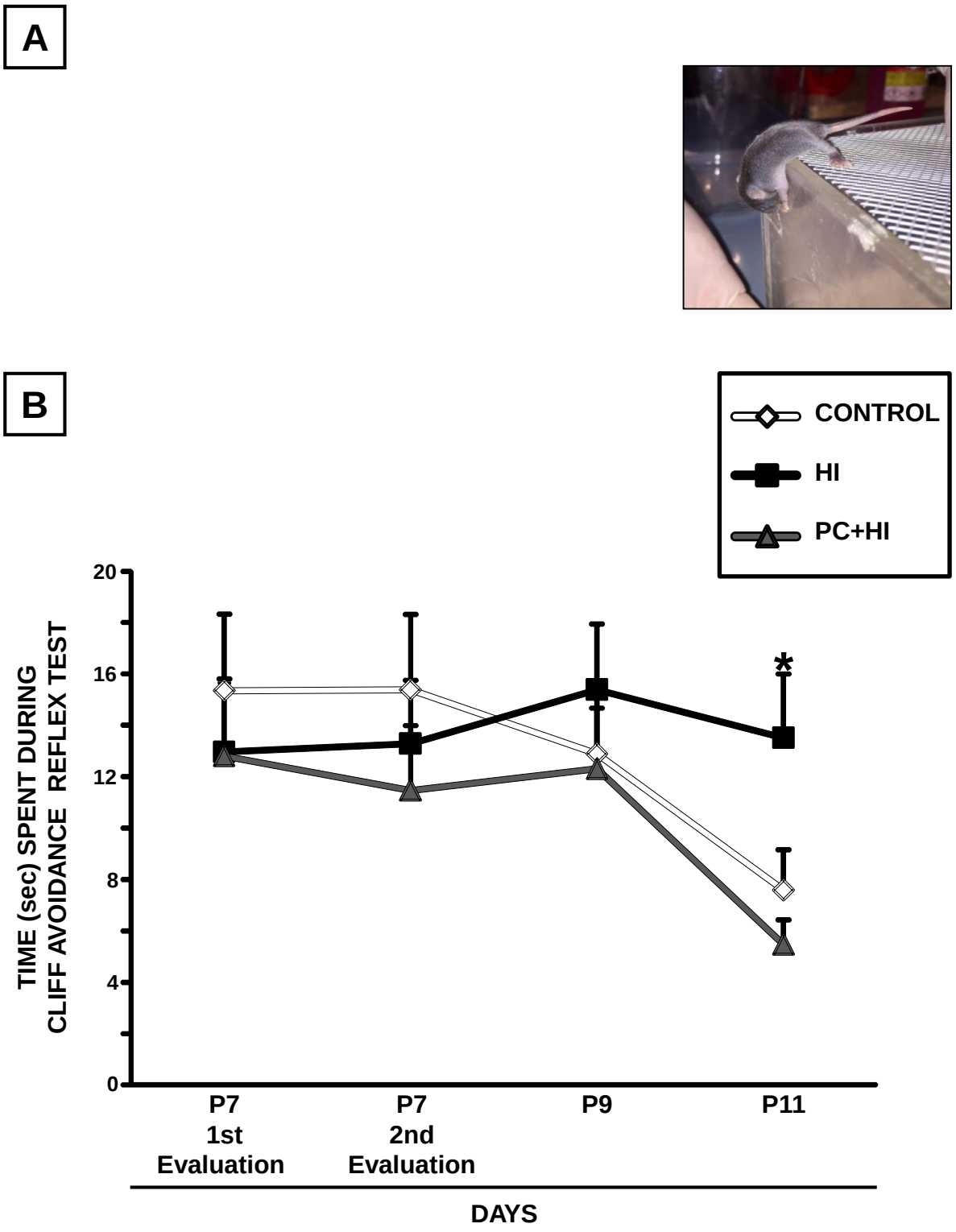

Figure 20. (A) The picture shows the pup placed with its forepaws and chest extending over the board of table. The time (in seconds) required for pup to came back from the edge was measured (B) Time course of cliff avoidance performance in three experimental groups: control, hypoxic-ischemic, preconditioned hypoxic-ischemic mice. Data represent the mean ± S.E.M. *P<0.05 versus control and preconditioned hypoxic-ischemic groups. The analyses were performed in control mice (n=12); hypoxic-ischemic mice (n=13); preconditioned hypoxic-ischemic mice (n=13).

4.7.3 HYPOXIC PRECONDITIONING IMPROVES PERFORMANCE IN GEOTAXIS REFLEX TEST OF MICE SUBJECTED TO HYPOXIA ISCHEMIA

The development of sensorimotor reflexes was evaluated in pup mice by using specific neurobehavioral tests. Geotaxis reflex test was performed in three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic animals. Mice performance was monitored at different time intervals. In particular, mice were analyzed twice at postnatal day seven, with a time frame of 60 minutes. Afterwards, the test was replicated at postnatal day nine and before the sacrifice (P11). In the early phase of hypoxic preconditioning induction no difference was observed among three groups. At postnatal day eleven preconditioned hypoxic-ischemic mice spent less time (sec) to perform trial than hypoxic-ischemic mice (Fig. 21).

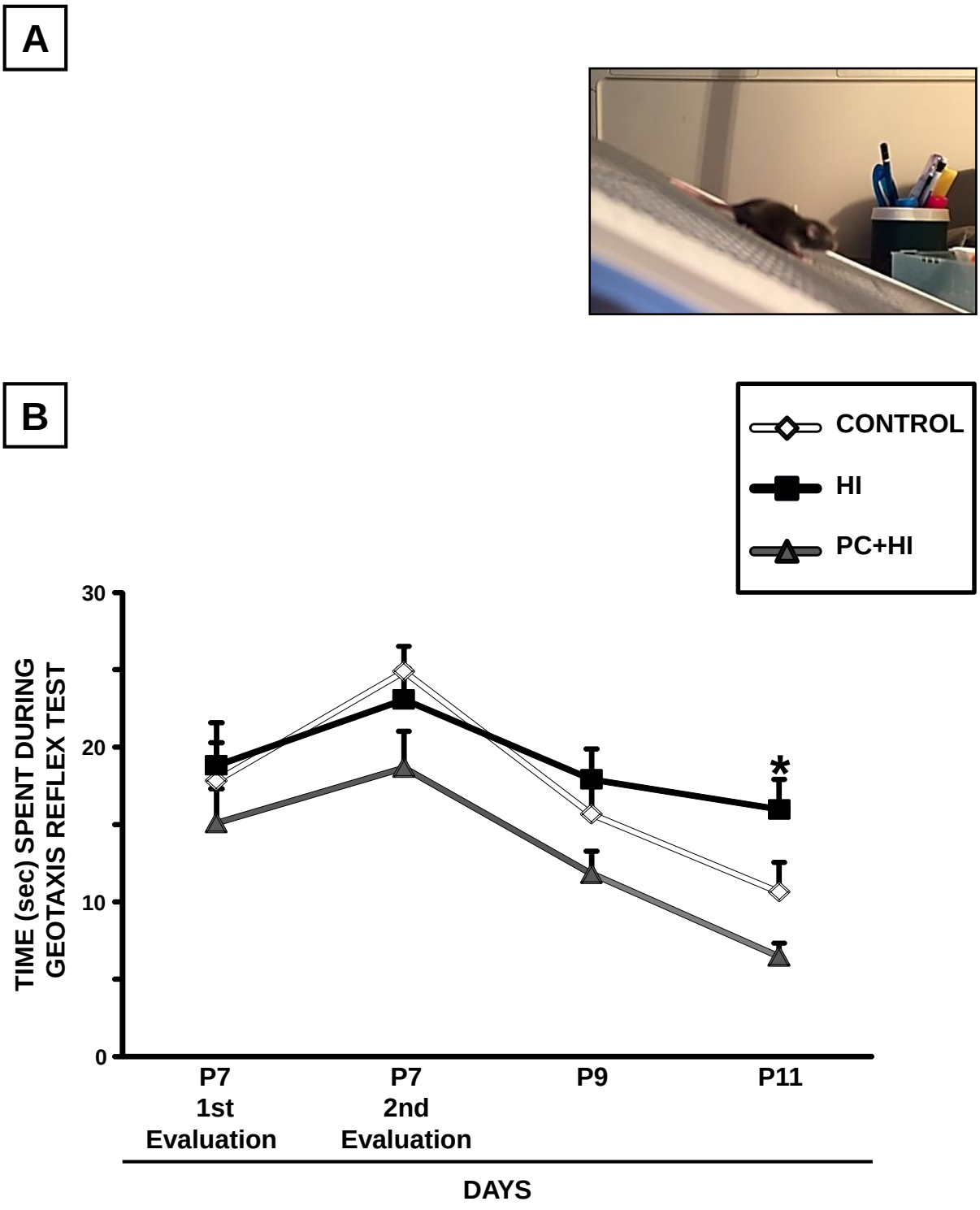

Figure 21. (A) The picture shows pup placed head downward on an inclined board (40˚). The time (in seconds) required for the pup to rotate their bodies head up (>90˚rotation) was measured (B) Time course of geotaxis performance in three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic animals. Data represent the mean ± S.E.M. **P*<0.05 versus control and preconditioned hypoxic-ischemic groups. The analyses were performed in control mice (n=12); hypoxic-ischemic mice (n=14); preconditioned hypoxic-ischemic mice (n=14).

4.8 EXPRESSION LEVELS OF NCX1 AND NCX3 PROTEINS DO NOT CHANGE IN THE IPSILATERAL HIPPOCAMPUS OF PRECONDITIONED HYPOXIC-ISCHEMIC AND HYPOXIC-ISCHEMIC MICE

NCX1 and NCX3 expression was evaluated in the ipsilateral hippocampus of three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic mice, sacrificed at postnatal day eleven. Analyses were performed by using a western blotting technique.

Data about protein expression of two NCX isoforms did not show any significant difference among three groups (Fig. 22).

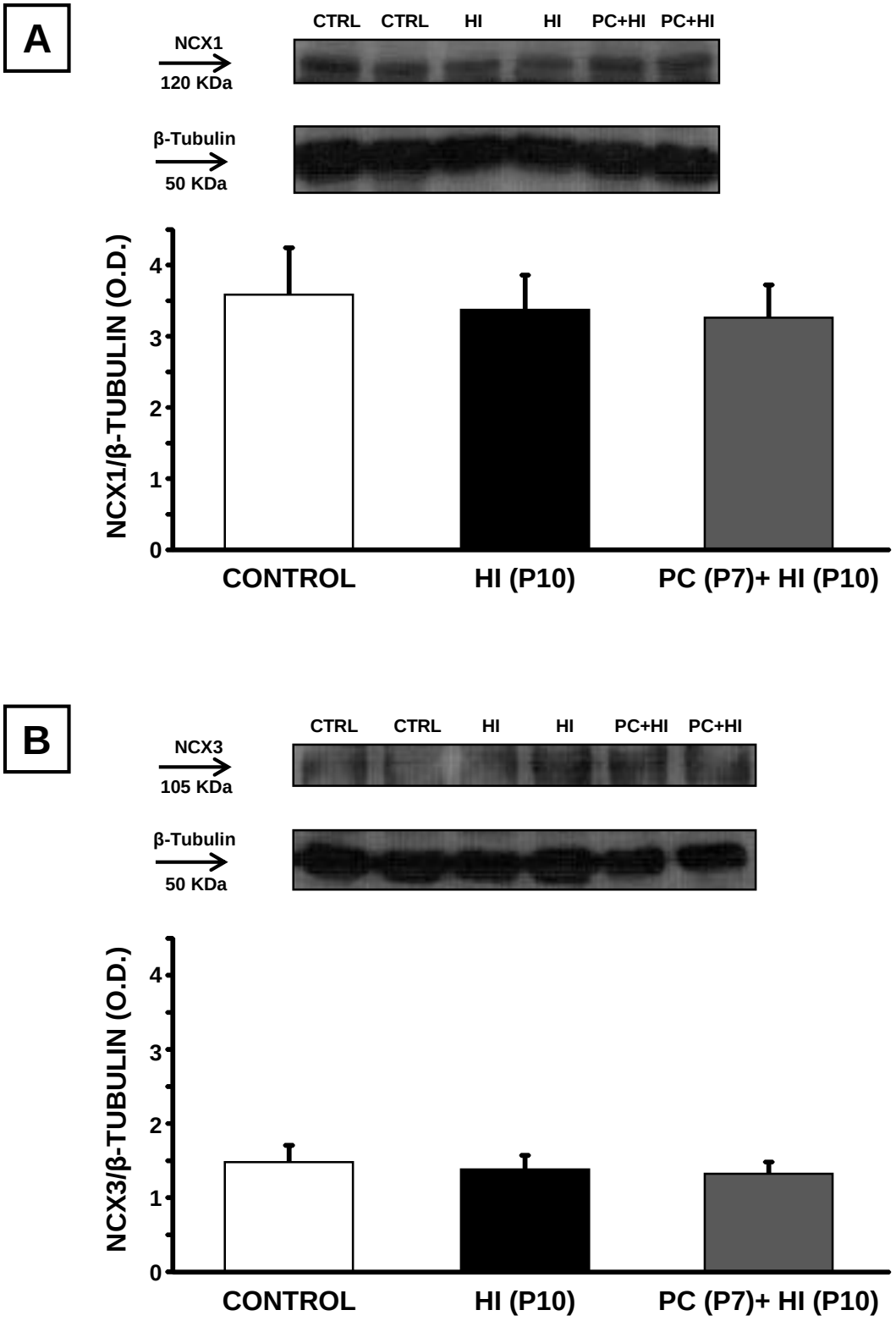

Figure 22. (A) No difference of NCX1 expression levels exists between control and treated mice. (B). No difference of NCX3 expression levels exists between control and treated mice. Data represent the mean ± S.E.M. Analyses of NCX1 expression were performed in ten animals for each group. Analyses of NCX3 expression were performed in six animals for each group.

4.9 HYPOXIC PRECONDITIONING INDUCES NCX1 AND NCX3 OVEREXPRESSION IN DENTATE GYRUS OF MICE SUBJECTED TO HYPOXIC-ISCHEMIC INJURY

NCX1 and NCX3 expression was evaluated in ipsilateral dentate gyrus of three experimental groups: control, hypoxic-ischemic, and preconditioned hypoxic-ischemic mice. Analyses were performed by using immunohistochemical technique. 3,3′-diaminobenzidine (DAB) staining, performed by using specific antibodies direct against NCX1 and NCX3 proteins, indicated a different condition in a specific area of ipsilateral hippocampus. Interestingly, counting analysis revealed that there was a pronounced increase of NCX1 and NCX3 positive cells in preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic group. Cells are mainly localized in layer under granular cells, subgranular zone (Fig. 23, 24).

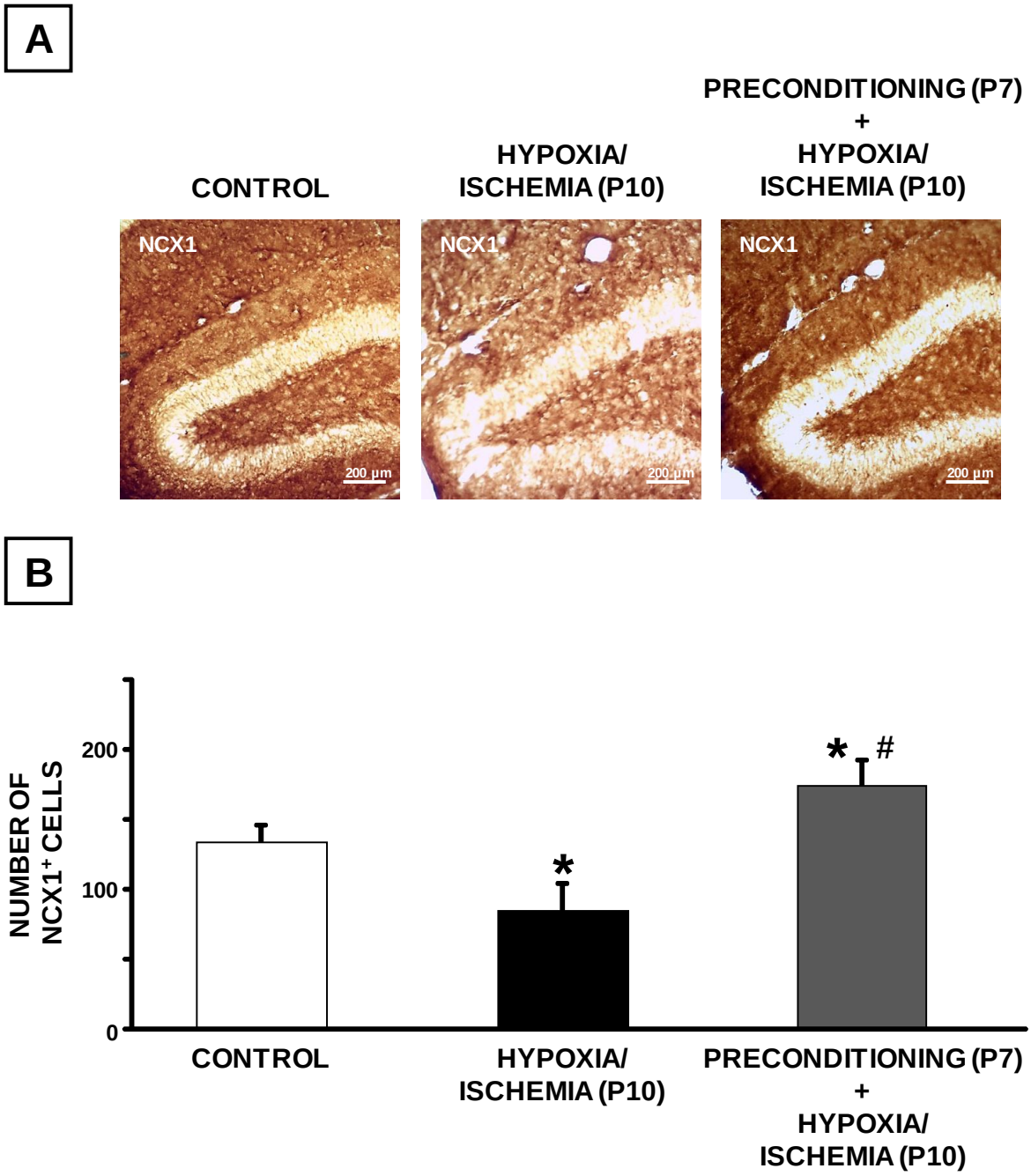

Figure 23. (A) Representative images of NCX1 positive staining of three mice groups have been shown. Scale bar 200 µm. (B) The number of NCX1 positive cells increases in the dentate gyrus of preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic animals, sacrificed after 24 hours. Data represent the mean ± S.E.M. **P*<0.05 versus control group; $^{\#}$*P*<0.05 versus hypoxic-ischemic group. The analyses were performed in three animals for each group.

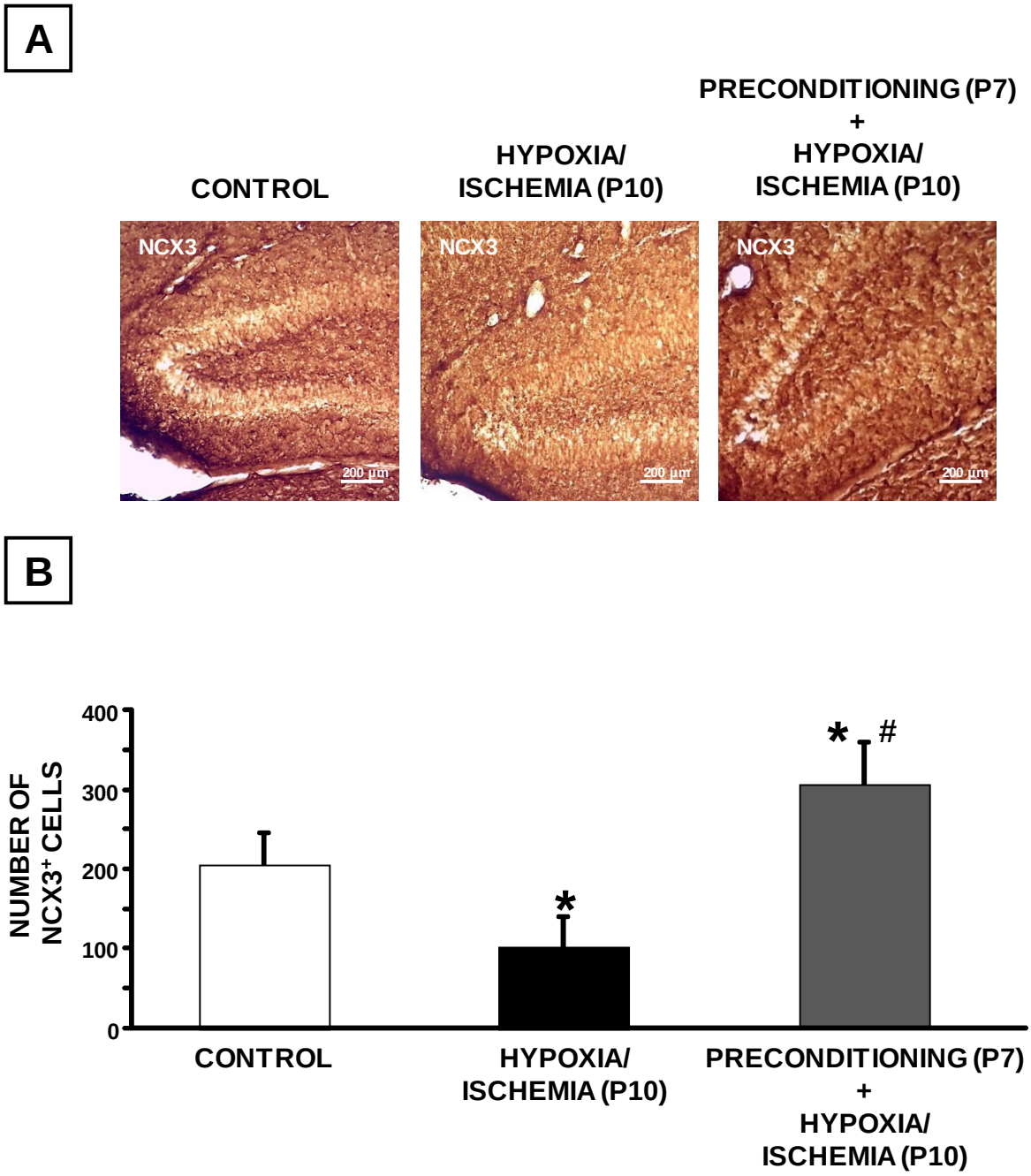

Figure 24. (A) Representative images of NCX3 positive staining of three mice groups have been shown. Scale bar 200 µm. (B) The number of NCX3 positive cells increases in the dentate gyrus of preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic animals, sacrificed after 24 hours. Data represent the mean ± S.E.M. **P*<0.05 versus control group; $^{\#}P$<0.05 versus hypoxic-ischemic group. The analyses were performed in three animals for each group.

5 DISCUSSION

In this project, we demonstrated that 20' hypoxia may represent a reliable and valid preconditioning stimulus in a mouse model of neonatal hypoxia ischemia. In fact, our data showed that this hypoxic preconditioning (HPC) delivered before a harmful insult is associated to endogenous neurogenesis in immature brain. Furthermore, since HPC induced NCX upregulation, the increase of neurogenic process may be linked to an ionic homeostasis maintenance, regulated by NCX proteins.

In previous studies different times of hypoxia had been applied. Thus, in 2006, Pourié et al. demonstrated that a brief 5 minutes' hypoxic exposition, applied between 10 and 24 hours after birth, was able to work as a preconditioning stimulus. In their study they showed that the hypoxic preconditioning prevented apoptosis induction in CA1 and DG hippocampal regions at different time intervals after hypoxia induction and induced an increase of cell density in these hippocampal regions 3 weeks after hypoxia induction (Pourié G. et al., 2006). The researchers also observed a transient increase of caspase-3 at 3 days after hypoxia, and according to Garnier et al. (Garnier P. et al., 2003), they reported that the activation of caspase-3 is crucial for triggering neuroprotection in ischemic preconditioning models. Finally, they observed the increase of neurogenesis in subventricular zone and in dentate gyrus of rats at P20 and P27 and suggested that these events were linked to an early activation of the ERK1/2 pathway (Pourié G. et al., 2006).

In 2007, Yin et al. demonstrated, in a mouse model of neonatal hypoxia ischemia, that HPC induced the activation of the PI3-K/Akt signaling pathway and suppression of inflammation and these events protected cortex, hippocampus, and striatum against white matter injury, determining the significant reduction of tissue loss 24

hours and 7 days after HI induction (Yin W. et al., 2007). Our results obtained with 20' hypoxia agree with Yin and Pourié's findings, since we observed a reduction of hippocampal damage in preconditioned hypoxic-ischemic mice 24 hours after a severe insult. Moreover, 3 days after hypoxia induction a severe hypoxic-ischemic insult induced a less brain infarct in preconditioned hypoxic-ischemic mice 24 hours after injury.

Another interesting finding of our study that deserves to be underlined is that in DG there was an increase of neural stem cells marker, Nestin, already 24 hours after the induction of the stimulus.

On this regard, it should be mentioned that in 2013 Ara and his colleagues demonstrated, in a piglet model of neonatal hypoxia ischemia, that hypoxic preconditioning increases neurogenesis in subventricular zone and new neuroblast cells migrate in striatum and cortex, 3 weeks after insult (Ara J. et al.,2013). In accordance to Ara et al. (2013), we observed a significant increase of neuroblast cells in the subventricular zone and a great number of proliferating neuroblast cells in dentate gyrus of preconditioned hypoxic-ischemic mice. Interestingly, in preconditioned hypoxic-ischemic animals the enhancement of neural progenitors was also associated with the improvement of the reflex performance. In particular, 24 hours after severe insult the animals spent less seconds to perform geotaxis and cliff avoidance trials compared to hypoxic-ischemic mice. The improvement of neurobehavioral functions in preconditioned hypoxic-ischemic mice had been also observed in Sun's study (Sun HS. et al., 2015). In 2003, Ten and his colleagues analyzed the neonatal sensorimotor reflexes in a mouse model of hypoxia ischemia and demonstrated that the worsening in the performances could be associated with infarct volume enlargement (Ten VS. et al., 2003). In the present work we confirm

this result. In summary, in the first part of our study has been shown that 20' hypoxia functions as a preconditioning stimulus in neonatal hypoxic-ischemic mice, determining the reduction of hippocampal damage, the increase of neuroblast cells in neurogenic niches, and the improvement of sensorimotor reflexes.

In the second part of the project, we investigated the relationship between the alteration of ionic homeostasis and the activation of endogenous neurogenesis mediated by NCX. Particularly, we focused our attention on hippocampal dentate gyrus because we observed the greatest increase of proliferating premature neurons ($BrdU^+/PSANCAM^+$) in this brain region.

The choice of NCX lays on our previous works, showing that NCX1 and NCX3 represent two new molecular effectors involved in adult brain preconditioning (Pignataro G. et al, 2012). In particular, we demonstrated that there was a significant reduction of infarct volume in preconditioned rats subjected to harmful ischemia and that the ischemic preconditioning induced NCX1 and NCX3 overexpression in the ipsilateral temporoparietal cortex (Pignataro G. et al, 2012). In addition, it has been demonstrated that the silencing of NCX1 and NCX3 genes prevented the neuroprotective effects mediated by ischemic preconditioning (Pignataro G. et al, 2012). According to this previous study, we performed the counting of NCX1 and NCX3 positive cells in hippocampus and we demonstrated that a pronounced increase within dentate gyrus of preconditioned hypoxic-ischemic mice compared to hypoxic-ischemic group occurred. In particular, we observed that NCX1 and NCX3 positive cells were mainly localized in the layer under granular cells, the so-called subgranular zone. The overexpression of NCX1 and NCX3 positive cells, induced by hypoxic preconditioning, probably represents a mechanism to reduce the intracellular alteration of ionic homeostasis. These findings are in accordance with previous

studies, in which it has been demonstrated that in neurons and glial cells there was an upregulation of NCX protein 72 hours after preconditioning induction (Pignataro G. et al., 2012). We speculate that this event occurs in order to counteract the dysregulation of intracellular Na^+ and Ca^{2+} homeostasis triggered by harmful ischemia.

The pivotal role played by NCX during preconditioning has been further highlighted in other recent papers. In particular, Sisalli et al. (2014) demonstrated, in primary cortical neurons, that IPC increased NCX1 and NCX3 protein expression 48 hours after OGD plus reoxygenation induction and this effect was mediated by NO production and PI3K/Akt activation. Therefore, the upregulation of NCX1 and NCX3 activity mediated the endoplasmatic reticulum refilling and mitochondrial calcium extrusion, thus preventing intracellular calcium dysregulation induced by OGD (Sisalli MJ. et al., 2014). Furthermore, in 2015, Boscia et al. analyzed, by using both *in vitro* and *in vivo* models of adult ischemia, whether the sodium/calcium exchanger NCX1 and calretinin, a Ca^{2+}-binding protein, cooperate to confer neurons greater resistance to degeneration. They demonstrated that the neuroprotective effect of calretinin was correlated to NCX1 expression in striatal interneurons. Indeed, the silencing of calretinin in brain cells prevented the neuroprotection mediated by ischemic preconditioning and reduced the expression of NCX1 protein. In preconditioned brain was also observed an increase of AKT phosphorylation (pAKT) protein levels (Boscia F. et al., 2015). Moreover, in our laboratory, it has been demonstrated that treatment of PC12 cells and cortical neurons with neurotrophin NGF induced the overexpression of neuronal splicing from NCX1, NCX1.4. The expression and activity of this isoform reached the peak after 7 days of treatment, and this evolution was correlated with neuronal differentiation and neurite outgrowth (Secondo A. et al.,

2015). In addition, in this paper it has been analyzed the correlation between the NCX1.4 overexpression and the activation of Akt-pathway. During neuronal differentiation induced by using of NGF treatment on PC12 cells, the authors proved that the great activation of sodium voltage-gated channels occurred. Then, the increase of intracellular levels of Na^+ determined the activation of NCX1 reverse mode operation. Consequently, the increase of intracellular levels of Ca^{2+} and ER Ca^{2+} refilling occurred. These events determined the phosphorylation of Akt, that translocated into the nucleus and induced the transcription of molecules involved in the neuronal differentiation (Secondo A. et al., 2015). Therefore, it is possible to speculate that overexpression of NCX proteins might be involved in the increase of neuronal differentiation in this *vivo* model of hypoxic preconditioning.

In conclusion, our results indicate that the hypoxic preconditioning is associated to endogenous neurogenesis in immature brain after insult. Indeed, this stimulus is able to trigger the proliferation of neural progenitor cells in the neurogenic niches. The increase of neurogenic process might be related to ionic homeostasis maintenance, regulated by NCX proteins. Therefore, NCX proteins can represent potential pharmacological targets for the treatment of brain damage associated to neonatal hypoxic-ischemic encephalopathy.

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