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PERINATAL BRAIN INJURY: MECHANISMS AND POTENTIAL PHARMACOLOGICAL THERAPIES

The role of SIRT1 in the neuroprotective effect of Melatonin and of Endothelin-1 in Oligodendrocyte Progenitor Cell Development

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1 *Perinatal Brain Injury*

According to the World Health Organization (WHO), the perinatal period commences at 22 completed weeks (154 days) of gestation and ends seven completed days after birth. During this stage, the brain is highly vulnerable and may be subjected to several conditions that can affect normal neurological development, such as neonatal hypoxia-ischemia, neonatal asphyxia, placenta inflammation, brain trauma, metabolic diseases and genetic malformations. With an estimated incidence of 1.5 per 1000 live term newborns, hypoxic-ischemic encephalopathy (HIE) is the most frequent type of acquired neonatal brain injury (Kurinczuk et al., 2010). The manifestation of brain injury differs depending on the developmental status of the brain at the time of the insult (Rocha-Ferreira and Hristova, 2016). In pre-term infants (<37 completed weeks of gestation, according to WHO), white matter injury is predominant due to the maturation-dependent vulnerability of the oligodendrocyte (OL) lineage and to the presence of a relatively abundant developing oligodendrocyte progenitor cell (OPC) population, which is very vulnerable to excitotoxicity and neuroinflammation induced by hypoxia-ischemia (Back, 2017). In contrast, in term babies, in which the maturation of the white matter is more advanced and the presence of oligodendrocyte progenitors is reduced, neuronal degeneration is the most commonly observed manifestation of injury (McQuillen and Ferriero, 2004). These conditions lead to long lasting sequelae, including seizures, cognitive and motor skills impairment, learning deficits and cerebral palsy (Pierrat et al., 2005). Despite the high level of infants affected every year by perinatal brain injuries and the severe long-term outcomes associated with these pathologies, therapies available for these patients are very limited. Therefore, there is a need to better understand the mechanisms underlying neurological disorders in infants to guide strategies that will prevent the injury and/or lead to functional recovery of the brain.

This thesis aims to gain more insight on the mechanisms underlying neurodevelopment and neuroprotection after injury in the early postnatal brain.

The dissertation is divided into two parts. The first part, entitled “The role of SIRT1 on the Beneficial Effects of Melatonin Administration Following Hypoxia-Ischemia Brain

Injury" was carried out at the Department of Biomolecular Sciences, University of Urbino Carlo Bo. In this study, we used a model of hypoxia–ischemia brain injury in neonatal rats to address the mechanisms underlying the neuroprotective effects of Melatonin, a naturally occurring neurohormone secreted by the pineal gland with antioxidant, anti-apoptotic and anti-inflammatory effects. In particular, we investigated the role of the Silent Information Regulator 1 (SIRT1) in the early phase of the injury and the consequences of its modulation on autophagy. SIRT1 is a NAD-dependent deacetylase protein belonging to the class III of histone deacetylase protein family that play an important role in the signaling cascades activated during HIE, including cell death, inflammation, and oxidative stress. We found that Melatonin strongly and rapidly modulates SIRT1 activity, indicating that SIRT1 modulation is an important player for its neuroprotective effect.

The second part of this dissertation entitled "Elucidating the Functional Role of Endothelin-1 in the Developing Subventricular Zone" was carried out at the Center for Neuroscience Research, at Children's National Medical Center, in Washington, DC. In this study, we analyzed the role of Endothelin-1 (ET-1) in OPC development in the developing postnatal subventricular zone (SVZ). In premature infants, hypoxic-ischemic damage is often associated with white matter brain injury (WMBI), characterized by loss of OLs and OPC differentiation failure (Back, 2017). The mechanisms regulating OPC proliferation and differentiation are still poorly understood but recent studies have identified ET-1 as an endogenous peptide that may regulate their development. To study the role of ET-1 in this context, we used both organotypic brain slices and an *in vivo* mouse model of ET-1 overexpression. We found that ET-1 regulates OPC proliferation in the developing SVZ by enhancing the overall proliferative ratio. In addition, we also characterized the expression pattern of ET-1 and its receptors in the piglet SVZ.

2 The Role of SIRT1 in the Beneficial Effects of Melatonin Administration Following Hypoxia-Ischemia Brain Injury

Introduction

2.1.1 Hypoxic Ischemic Encephalopathy

Hypoxic-ischaemic encephalopathy (HIE) is a leading cause of perinatal brain injury in neonates and is the result of impaired cerebral blood flow and oxygen delivery to the brain during the prenatal, intrapartum or postnatal period. HIE affects 1.5 per 1000 live births in developed countries with incidence even higher in underdeveloped countries (Kurinczuk et al., 2010). By the age of 2 years, more than 40% of neonates with HIE will have severe long-term neurodisabilities including seizures, cognitive and motor skills impairment, learning deficits and cerebral palsy (Pierrat et al., 2005).

Pathophysiology of Hypoxic Ischemic Encephalopathy

During a hypoxic-ischemic (HI) episode, the lack of oxygen and glucose leads to significantly less oxidative phosphorylation and thus to decrease in adenosine triphosphate (ATP) production. This low level of ATP causes failure of many ion channels and enzymes that maintain cell integrity, particularly the sodium/potassium (Na^+/K^+) pump. When the Na^+/K^+ pumps fail, an excessive influx of positively charged Na^+ ions drastically precipitate depolarization of neurons, leading to release of glutamate, a key excitatory neurotransmitter. Excessive activation of glutamate-gated ion channel receptors and voltage-dependent ion channels results in neuronal dysfunction and death, a process called excitotoxic neurodegeneration. The prolonged activation of glutamate receptors induces an influx of intracellular calcium (Ca^{2+}) into the cells, activating Ca^{2+}-sensitive proteases, phosphatases, phospholipases and nitric oxide synthase (NOS), eventually leading to intracellular acidification, mitochondrial and endoplasmic reticulum damage and disruption of the cytoskeleton and cell membrane. Furthermore, toxic reactive oxygen species (ROS), generated through activation of NOS, can further damage proteins, nucleic acids, and lipids and potentially open the mitochondrial permeability transition pore, which in turn promotes even more ROS production and induces the release of pro-apoptotic factors such as cytochrome c into the cytoplasm. The developing brain is especially vulnerable to oxidative imbalance, as the antioxidant capacity of immature neurons is easily overwhelmed by hypoxia-induced ROS. The overproduction of free radicals and the downregulation of

anti-oxidant mechanisms result in neuronal cell death (Allen and Brandon, 2011; McLean and Ferriero, 2004).

The pattern of injury depends on the developmental stage of the brain and on the severity of the insult, which both affect the selective regional vulnerability, as well as the subsequent clinical manifestations. In preterm infants, periventricular white matter is particularly vulnerable to HI, and results in a selective pattern of injury characterized by motor, cognitive, and sensory deficits. In term infants, HI causes selective damage to sensorimotor cortex, basal ganglia and thalamus, resulting in severe motor disability and speech difficulties (Rocha-Ferreira and Hristova, 2016). Both necrosis and apoptosis have been reported as types of cell death occurring after HI. Cellular necrosis occurs in severe cases of HI whereas apoptosis occurs in milder injury. Necrosis is characterized morphologically by cytoplasmic swelling, breakdown of the plasma membrane, nuclear dissolution (karyolysis) and lysis. Upon rupture, cellular content is released causing inflammation of the surrounding tissue and the migration of mediators, like neutrophils, microglia/macrophages and reactive astrocytes, resulting in glial scar formation (Allen and Brandon, 2011). Apoptosis is a programmed cell death that causes cytoplasmic condensation, cell shrinkage, DNA fragmentation, and general preservation of the cell membranes with no associated inflammation. Apoptosis requires ATP and is therefore often observed in the penumbra of an ischemic area, which is a ring of mild to moderately ischemic tissue, supplied with blood by collateral arteries (Shih et al., 2009). However, even cells in this region will die if reperfusion is not established during the early hours after HI since collateral circulation is inadequate to maintain the neuronal demand for oxygen and glucose indefinitely (Castillo et al., 2016). In addition to apoptosis and necrosis, brain cells may undergo autophagy following HI (Carloni et al., 2008). Autophagy is an adaptive process that allows for the recycling and degradation of proteins and organelles through lysosome machinery (He and Klionsky, 2009). Autophagy is considered to be a homeostatic, nonlethal stress response protecting the cell from low nutrient supplies (Northington et al., 2011). If the cell injury is repairable, the cell might undergo autophagy to sequester the damaged organelles. However if the damage cannot be repaired or removed, autophagic cell death might occur or the intrinsic apoptosis pathway might be initiated.

The different types of cell death discussed above demonstrate the complexity of neonatal HI injury. These relevant mechanisms should be taken into account to design pharmacological treatments against HIE.

Neuroprotective Treatments to Manage Hypoxic Ischemic Encephalopathy

Despite the high number of infants affected every year by HIE and the severe long-term outcomes associated with this pathology, there are very few preventative or protective treatments available for patients. The majority of treatments target one or more of the following mechanisms: inhibition of glutamate release, blocking glutamate receptors, inhibition of inflammation, and/or reducing ROS production.

Hypothermia is one of the most prominent treatments against HIE and acts by decreasing cerebral metabolism and oxygen demand, reducing glutamate excitotoxicity, free radical production and thus cell death. Current protocols suggest either selective head or whole body cooling, with a temperature ranging around 33.5 ± 0.5°C and 34.5 ± 0.5°C (Davidson et al., 2015; Jacobs et al., 2013). Timing and duration of the treatment are critically important for brain recovery: a preclinical study in 7-day-old rats showed that mild hypothermia (decreasing temperature by 2 to 4°C), started 2 hours after the end of HI surgery for 48 hours, was associated with significant improvements in oligodendrocyte precursor cell survival, oligodendrocyte maturation, microglia activation and cell death (Xiong et al., 2013). Potential use of stem or progenitor cells to reduce brain damage or promote regeneration in combination with mild hypothermia is another promising strategy against HIE (Wang et al., 2014). Neural stem cells (NSCs) have the potential to generate both glial and neuronal cell lineages following loss of endogenous cells due to cerebral HI. In a preclinical study carried out by Wang et al., mice received combined therapy of severe hypothermia (27–28°C for 24 hours) and NSC transplantation after HI intervention. The results displayed a significant reduction in infarct volumes and cell death in the hypothermia + NSCs group compared to the hypothermia, HI, and NSC groups at 1, 2 and 4 weeks after cell transplantation, and significant improvements in the overall functional neuronal activity (Wang et al., 2014).

Erythropoietin (EPO) is a glycoprotein hormone and a well-known regulator of erythropoiesis, the process of generating new red blood cells. During fetal development, EPO is produced mainly in the liver; however, following birth, the kidney accounts for ~80% of its production. After HI brain injury, EPO and its receptors (EpoR) are widely

upregulated throughout the central nervous system (CNS) in several cell types, including progenitor cells, astrocytes, oligodendrocytes, and microglia (Sugawa et al., 2002). Many animal models have shown that EPO treatment is associated with decreased apoptosis, excitotoxicity and inflammation, improving neurogenesis, axon regeneration and cognitive outcomes, although the exact mechanism is still unknown (Wang et al., 2004). A clinical trial in term infants with moderate/severe HIE found that EPO administration (300 U/kg or 500 U/kg) every other day for 2 weeks reduced the risk of disability at 18 months and improved long-term outcomes in infants with moderate HIE but not with severe HIE (Zhu et al., 2009).

The treatments discussed above appear to be promising agents to ameliorate the effects of HIE. However, they have limited applicability in neonates and potential side effects when translated from preclinical animal models to clinical use. For example, hypothermia has negative effects on hemostasis and leads to increased risk of bradycardia and hypotension (Diederen et al., 2018). Furthermore, the partial neurological protection obtained with current hypothermia protocols is highly dependent on starting hypothermia within the optimal window of opportunity (i.e. 90-120 minutes from insult) (Davidson et al., 2015). The significance and severity of adverse effects related to EPO administration also remain controversial. As EPO is used primarily as an erythropoietic agent, it affects red blood cell formation and can induce hypertension and thrombotic events (Santhanam et al., 2010).

Recently, Melatonin - an indoleamine constitutively secreted by the pineal gland - was found to be neuroprotective in several models of perinatal brain damage, including cerebral HI (Carloni et al., 2008; Lin et al., 2018; Zhao et al., 2018).

2.1.2 Melatonin

Melatonin (N-acetyl-5-methoxytriptamine) is a natural neurohormone primarily secreted by the pineal gland during the nighttime in a circadian and circannual manner, and its concentration is highest during childhood, significantly decreasing throughout life (Reiter, 1995). Melatonin regulates circadian rhythm via activation of two membrane-bound receptors belonging to the G protein-coupled family, named MT1 and MT2 (Dubocovich and Markowska, 2005; Jilg et al., 2005). In the CNS, MT1 and MT2 are localized in the suprachiasmatic nucleus, thalamus, hypothalamus and hippocampus,

and are particularly expressed by cholinergic, dopaminergic and glutamatergic neurons (Evely et al., 2016; Weaver et al., 1989).

Once synthesized, Melatonin, due to its lipophilic profile, can easily diffuse into the cerebrospinal fluid towards the pineal recess, a third ventricle evagination penetrating into the pineal gland, or it can be released from cerebrospinal fluid-contacting pinealocytes (Tricoire et al., 2002). Melatonin is also released into the blood circulation where it is distributed to all tissues. Circulating Melatonin is mainly metabolized by hepatic degradation through P450 enzymes to generate 6-hydroxy Melatonin, which is then conjugated with sulfuric or glucuronic acid to produce the principal metabolite, 6-sulfatoxy-Melatonin, eliminated by the urine. Alternatively, Melatonin can be metabolized through the oxidative pyrrole-ring cleavage, which appears to be the major metabolic pathway in the CNS (Slominski A. et al., 2012).

Beneficial effects of Melatonin

Currently, Melatonin is regarded as an important multitasking molecule involved in immunomodulation, inflammation, oxidative stress and aging (Reiter et al., 2010).

Melatonin is a potent free radical (FR) scavenger, as well as an indirect antioxidant. As a scavenger, Melatonin removes singlet oxygen (O2), superoxide anion radical (O^{-}_{2}), hydroperoxide (H_2O_2), hydroxyl radical (·OH) and the lipid peroxide radical (LOO·) (Rodriguez et al., 2004). Melatonin also acts as an indirect antioxidant by upregulating antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), and by increasing the efficiency of mitochondrial electron transport (Tomas-Zapico and Coto-Montes, 2005). These protective effects of Melatonin result in the significant inhibition of mitochondrial apoptosis and mitophagy.

Melatonin is also highly effective in protecting cells from inflammation and acts on the immune system by inhibiting cytokine production, neutrophil infiltration and NOS activation (Rancan et al., 2018; Tomas-Zapico and Coto-Montes, 2005).

ROS production and inflammation are common features in the pathogenesis and development of a great number of neurodegenerative diseases and injuries, including Alzheimer's (Simoncini et al., 2015), Parkinson's disease (Golpich et al., 2017), traumatic brain injury (Ansari et al., 2008) and HIE (Zhao et al., 2016). Based on its ability to function as a potent antioxidant agent and regulator of the immune system, it has been suggested that Melatonin may act as a neuroprotective agent against HI brain

injury. Furthermore, Melatonin is safe for administration during pregnancy or to newborn infants (Gitto et al., 2013; Miller et al., 2014) and is well tolerated even at high doses (Seabra et al., 2000). Thus, Melatonin is a molecule of high clinical interest.

Previous studies performed in our laboratory found that Melatonin provides significant protection against brain damage in a neonatal rodent model of HI. The beneficial effect was observed with: 1) a single dose of 15 mg/kg Melatonin administered 5 minutes before HI or 2) when the same dose was given after HI and repeated 24 and 48 hours later. This latter schedule of administration also significantly improved the long-lasting behavioral outcomes, reducing learning deficits induced by HI brain injury (Carloni et al., 2008).

Recently, an important role in mediating the effects of Melatonin was ascribed to the Silent Information Regulator 1 (SIRT1). SIRT1 is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase which is implicated in several biological functions, such as cell survival, DNA repair, aging, stress response, and the regulation of the circadian rhythm machinery (Jung-Hynes and Ahmad, 2009; Longo and Kennedy, 2006; Nakahata et al., 2008). Recent experiments performed in our laboratory reported that Melatonin prevents SIRT1 depletion induced by HI, indicating a functional interaction between the indoleamine and SIRT1 in ischemic conditions (Carloni et al., 2014).

2.1.3 The Silent Information Regulator 1

Sirtuins are a family of NAD^+-dependent protein deacetylases and/or ADP-ribosyltransferases involved as crucial regulators in a variety of cellular processes (Blander and Guarente, 2004). Within this family, SIRT1 is a nuclear metabolic sensor that directly couples the cellular metabolic status of the cell (via NAD^+) to the regulation of gene expression through deacetylation of histones and transcription factors. In mammals, SIRT1 orchestrates diverse biological processes including cell differentiation, apoptosis and cellular senescence (Longo and Kennedy, 2006; Luo et al., 2001), autophagy (Lee et al., 2008), and circadian rhythms (Nakahata et al., 2008). Accumulating evidence also suggests that SIRT1 plays critical roles in several brain functions by regulating learning and memory (Koronowski and Perez-Pinzon, 2015), neuronal and glial differentiation (Jablonska et al., 2016; Libert et al., 2008),

participating in neuronal protection (Raval et al., 2008) and preventing neurodegeneration in mouse models of neuronal diseases (Kim et al., 2007; Qin et al., 2006).

SIRT1 can modulate apoptotic cell death by acting on the tumor suppressor protein p53, which is a well know regulator of apoptosis. p53 is activated in response to stress-induced DNA damage, which eventually leads to activation of cell cycle arrest, apoptosis and autophagy to facilitate DNA repair mechanisms (Zilfou and Lowe, 2009). p53 transcriptionally regulates pro-apoptotic molecules, such as Bak (Graupner et al., 2011) and Bax (Miyashita and Reed, 1995), which are important players in inducing mitochondrial outer membrane permeabilization in response to death stimuli. SIRT1 physically interacts with p53 and deacetylates Lys382 residue of p53 in a NAD^+-dependent manner (Vaziri et al., 2001). SIRT1-mediated deacetylation of Lys382 decreases p53-mediated transcriptional activation of pro-apoptotic molecules, leading to apoptosis inhibition (Reed and Quelle, 2014).

In addition, SIRT1 plays a role in regulation of autophagy, a key catabolic process that allows for the recycling and degradation of proteins and organelles through lysosome machinery. SIRT1 regulates the autophagy-lysosome pathway by deacetylation of autophagy-related genes (Atg) which are critically important for autophagosome formation (Atg5, Atg7, Atg8) (Ng and Tang, 2013) or by negatively regulating mTOR, an autophagy inhibitor complex (Jeong et al., 2018).

Recent experiments performed in our laboratory, reported that Melatonin prevents HI-induced SIRT-1 depletion, and reduces endoplasmic reticulum stress 24 hours after the insult (Carloni et al., 2014). However, additional studies are required to address the mechanistic link between SIRT1 and Melatonin. Therefore, in this study, we sought to further characterize the effects of HI and Melatonin on SIRT1 regulation and its downstream targets in the early phase of the injury (1 hour after HI), along with Melatonin's impact on cell death and autophagy.

Material and Methods

2.2.1 Cerebral hypoxia-ischemia (HI)

All surgical and experimental procedures were carried out in accordance with the Italian regulation for the care and use of laboratory animals (according to Directive 86/609/EEC), and were approved by the Animal Care Committee of the University of Urbino "Carlo Bo". Pregnant Sprague-Dawley rats were housed in individual cages and the day of delivery was considered day 0. Neonate rats from different litters were randomized, normalized to ten pups per litter and kept in regular light/dark cycles (lights on 8 am–8 pm). On postnatal day 7, after anesthesia with 5% isoflurane in N2O/O2 (70/30%) mixture, pup rats underwent unilateral ligation of the right common carotid artery via a midline neck incision. After artery ligation, the wound was sutured and the animals were allowed to recover for 3h under a heating lamp. Pups were then placed in an airtight jar and exposed for 2.5 h to a humidified nitrogen–oxygen mixture (92% and 8%, respectively) delivered at 5–6 L/min (HI). The jar was partially submerged in a 37°C water bath to maintain a constant thermal environment.

2.2.2 Drug administration

Melatonin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted in normal saline solution to a final concentration of 5% DMSO (vehicle). The Melatonin solution was injected intraperitoneally to pup rats 5 min after HI at the dose of 15 mg/kg. Control animals received the same volume of vehicle. Propidium iodide (PI; 0.5 μL of a 1 mg/mL solution in distilled water; Sigma-Aldrich) was injected 40 minutes after HI into the right lateral ventricle. Twenty minutes after PI injection, animals were sacrificed and perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS.

2.2.3 Western blot analysis

Pups were anesthetized and euthanized by decapitation. Brains were rapidly removed and the cytosolic, mitochondrial and nuclear fractions prepared from cerebral cortices according to Nijboer et al (2007). Samples were stored at -80°C until use. After mixing with sodium dodecyl sulfate gel-loading buffer and heating 4 min at 95°C, samples (20-50 μg protein) were electrophoresed onto sodium dodecyl sulfate–polyacrylamide gel and proteins transferred to a PVDF membrane. ColorBurstTM electrophoresis marker (3

μL/gel, Sigma-Aldrich) was used for qualitative molecular mass determinations and for visual confirmation of blot transfer efficiency. Blots were then blocked with non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.6, plus 0.1% Tween-20) and probed with the following primary antibodies: anti-SIRT1 (Santa Cruz Biotechnology, 1:1000), anti-cytochrome c (Santa Cruz Biotechnology, 1:1000), anti-BAX (Santa Cruz Biotechnology, 1:500), anti-p53 (Santa Cruz Biotechnology, 1:2000), anti-acetyl-p53 (Cell Signaling Technology, 1:500), anti-LC3 (Cell Signaling Technology, 1:1000) and anti-p62 (Sigma-Aldrich, 1μg/mL). A monoclonal antibody against β-actin (Santa Cruz Biotechnology, 1:4000) or against Hsp60 (Santa Cruz Biotechnology, 1:1000) and a polyclonal antibody against Lamin B (Santa Cruz Biotechnology, 1:1000) were used as controls for protein gel loading. Data were normalized to β-actin or Hsp60 or lamin B, and expressed as % of control.

2.2.4 Immunohistochemistry

One hour after HI, pups were deeply anesthetized with 5% isoflurane in N2O/O2 (70/30%) mixture and perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS. Brains were rapidly removed on ice and processed for antigen retrieval by immersing overnight in 10 mmol/L sodium citrate buffer (pH 6.0, 4°C) and boiling in the same buffer for 3 min. After boiling, brains were cryoprotected with 30% sucrose/PBS (72 h, 4°C). Brain sections (thickness 12 μm) were incubated with 1.5% normal blocking serum for 1 h at room temperature, and then overnight at 4°C with anti-SIRT1 (Santa Cruz Biotechnology, 1:50). Fluorescein IsoThiocyanate-conjugated mouse anti-rabbit IgG (Santa Cruz Biotechnology, 1:200) and Texas Red anti-mouse IgG (Santa Cruz Biotechnology, 1:200) were used to demonstrate immunoreactivity of SIRT-1 as green fluorescence. The specificity of the reactions was evaluated in some slices by omitting the primary antibody from the incubation medium. Peroxidase activity was amplified by 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H2O2 at the appropriate stage. Images were acquired on a Leica TCS SP5 II confocal microscope (Leica Microsystem).

2.2.5 Quantitative real time PCR

Total RNA was extracted from 100 μl of rat homogenates using an RNA purification kit (Norgen Biotek Corporation). Samples were evaluated for nucleic acid quality and quantity using the Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies).

RNA was stored at − 80 °C until use. The levels of mRNA were determined from cDNA by using the following primers:

SIRT1-forward (f), 5'- TTTATGCTCGCCTTGCTGTG -3', SIRT1-reverse (r), 5'-TGTCCGGGATATATTTCCTTTGC-3'; β-actin-forward (f), 5'-CCCGCGAGTACAACCTTCTTG-3', β-actin-reverse (r), 5'-GTCATCCATGGCGAACTGGTG-3'. The pairs of forward and reverse primers were purchased from Sigma-Aldrich. Synthesis of cDNA was carried out using a reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative polymerase chain reaction real time (RT-qPCR) was performed with the SYBR Green PCR master mix (Applied Biosystems) on an ABI Prism 7500 Real Time PCR System (Applied Biosystems). Beta-actin has been used as an endogenous control to determine relative mRNA expression. Product specificity was examined by dissociation curve analysis. Data were analyzed by a 7500 system software (1 1.4.0) with the automatic comparative threshold (Ct) setting for adapting baseline. Detection thresholds were set at 35 Ct. Results are expressed in the figures as fold change related to control (CTRL) and the relative amount of SIRT1 was calculated using the Ct method:

$$\Delta E = Ct\ SIRT1\ (treated\ samples) - Ct\ beta\text{-}actin\ (treated\ samples);$$

$$\Delta Ct = Ct\ SIRT1\ (CTRL) - Ct\ beta\text{-}actin\ (CTRL);$$

$$\Delta\Delta Ct = \Delta E - \Delta Ct$$

$$Fold\ change = 2^{-\Delta\Delta Ct} \quad (2)$$

2.2.6 Cell counting

Cell counting after PI injection was conducted in the cerebral cortex and in the CA1 and CA2/CA3 areas of the hippocampus on 20X microscopic images using a BX-51 Olympus microscope. Positive cells were counted in three separate fields of each area in slices cut at the level A 3750 of the Konig and Klippel stereotaxic atlas. Five animals per group were analyzed.

2.2.7 Data analysis

The relative intensity of the bands detected by Western blot was analyzed using the Image J 1.45 software (https://imagej.nih.gov/ij/). Statistical analyses were performed

using Prism Computer program (GraphPad Software Inc.). The Bartlett's test was used to determine data homogeneity. One-way ANOVA followed by the Newman-Keuls multiple comparison test was used to determine differences between single treatment groups. Results were considered to be significant when $P \leq 0.05$.

Results

2.3.1 Melatonin reduces necrosis and the early stages of apoptotic cell death

To address the early effects of Melatonin administered 5 min after a neonatal HI insult, we assessed cellular necrosis and apoptosis 1 hour after HI. Necrotic cell death was assessed using propidium iodide (PI). Many PI-positive cells were observed in the CA1 and CA2/CA3 areas of the ischemic hippocampus (CA1 area: 123.20 ± 2.46; CA2/CA3 area: 114.60 ± 2.34, Fig. 1A, B). Less PI-positive cells were detected in the ipsilateral cerebral cortex (62.00 ± 9.69), particularly in the deep layers. In Melatonin-treated animals the number of PI-positive cells was significantly lower both in the hippocampus and in the cortex (CA1 area: 73.20 ± 4.39; CA2/CA3 area: 70.40 ± 4.15; cerebral cortex: 24.00 ± 1.81; Fig. 1A, B), indicating a beneficial effect of Melatonin in reducing necrotic cell death after the insult. We then performed experiments to study the effect of Melatonin administration on apoptotic cell death occurring after a HI insult. HIE results in mitochondrial impairment related to glutamate excitotoxicity and excessive intracellular accumulation of Ca^{2+}, NO and ROS. These mitochondrial alterations trigger a shift in localization of pro-apoptotic proteins BCL2 associated X (Bax) and cytochrome C (CytC) within the cell. Specifically, upon initiation of apoptotic signaling, Bax translocates to mitochondria, leading to a loss in membrane potential and the release of the CytC into the cytosol. Therefore, we evaluated the mitochondrial release of CytC and the translocation of Bax from the cytosol to mitochondria. The ischemic cortex showed a significant lower expression of CytC in the mitochondrial fraction (65.7 ± 16.6; Fig. 2A) and a higher expression in the cytosolic fraction (181.9 ± 13; Fig. 2B), compared to both the control and the contralateral cortex of the brain. Melatonin injection significantly preserved the mitochondrial expression of the protein (81.3 ± 12.1; Fig. 2A) and consequently reduced it in the cytosol (94.8 ± 14.1; Fig. 2B), indicating a reduced outer membrane permeabilization in Melatonin-treated animals. This effect was concomitant with a reduction in Bax translocation to the mitochondria (Fig. 2C, D). Taken together, these results show that immediate Melatonin administration reduces necrotic and apoptotic cell death occurring acutely after HI brain injury.

2.3.2 Melatonin preserves SIRT1 expression

To examine the mechanism underlying the early therapeutic effect of Melatonin on HI brain injury, we performed SIRT1 immunostaining in the cortex region of the brain. We previously found that Melatonin treatment increased SIRT1 expression at 24 hours post injury (Carloni et al., 2014). Therefore, we next asked whether changes in SIRT1 expression could be observed as early as 1 hour post injury. Few SIRT1+ cells were detectable in the injured cortex of the ischemic animals (Fig. 3A, panel a). Interestingly, more SIRT1+ cells were observed in the cortex of Melatonin-treated ischemic animals, compared to HI animals (Fig. 3A, panel d). We confirmed these immunostaining results with western blots for SIRT1 protein in control, HI, and Melatonin-treated HI cortical tissue. No changes in SIRT1 expression were found in vehicle-treated and Melatonin-treated control animals nor in the contralateral cortex of vehicle-treated and Melatonin-treated ischemic animals (Fig. 3B). Conversely, SIRT1 expression was significantly reduced in the injured cortex of ischemic animals (46.8 ± 11.4; Fig. 3B) compared to vehicle-treated animals (104.7 ± 5.4; Fig. 3B). SIRT1 expression was preserved in Melatonin-treated ischemic animals (91.1 ± 14.4 compared to vehicle-treated animals). SIRT1 is mainly expressed in the nucleus where it acts as transcription factor but it can also shuttle from the nucleus to the cytoplasm (Yanagisawa et al., 2018). Base on this evidence, we next assessed SIRT1 expression level in both the nuclear and cytosolic subcellular fractions to address any possible differences in the subcellular localization after HI. In vehicle treated animals, SIRT1 was expressed both in the cytosolic and nuclear fractions (Fig. 3C, D), although integrated optical density (OD) measurements showed that the highest amount was observed in the nucleus (Fig. 3C, D). In control conditions, Melatonin did not affect SIRT1 expression in either subcellular fractions (Fig. 3C, D); however, after HI, SIRT1 expression was significantly decreased in both subcellular fractions. Melatonin treatment following HI restored SIRT1 protein levels in both fractions (Fig. 3C, D). Lastly, these findings were confirmed by real time PCR for *Sirt1* mRNA levels in rat whole brain samples. *Sirt1* mRNA levels were decreased 0.46 fold following HI, compared to the vehicle-treated animals; Melatonin treatment completely rescued *Sirt1* mRNA levels.

Together, these results indicate that Melatonin treatment is able to preserve SIRT1 expression after HI brain injury.

2.3.3 Melatonin modulates p53 by acting on SIRT1

To determine whether Melatonin treatment following HI affects SIRT1's downstream pathway, we addressed the expression of the tumor suppressor protein p53. As shown in figure 4, the total expression of p53 was increased following HI in both cytosol and nucleus (cytosolic fraction: 165.2 ± 17.6; nuclear fraction: 175.3 ± 8.47; Fig. 4A, B) compared to vehicle-treated animals (cytosolic fraction: 93.2 ± 9.6; nuclear fraction: 99.7 ± 0.45; Fig. 4A, B). Conversely, the level of total p53 were significantly reduced in Melatonin-treated ischemic animals, in both fraction (cytosolic fraction: 123.2 ± 19.5; nuclear fraction: 104.8 ± 17.8; Fig. 4A, B) compared to vehicle-treated animals.

To determine whether Melatonin treatment following HI affects SIRT1's deacetylase activity, we then assessed the acetylation status of p53. We found that acetylated p53 was increased in the cytosol and nucleus fractions of whole brain tissue following HI (cytosolic fraction: 167.5 ± 31.3; nuclear fraction: 182.3 ± 12.1; Fig. 4C, D). However, Melatonin injection after HI, restored acetylated p53 expression in both fractions (cytosolic fraction: 88.2 ± 9.88; nuclear fraction: 102.61 ± 17.5; Fig. 4C, D).

These results suggest that Melatonin treatment is effective in decreasing p53 expression following HI.

2.3.4 Melatonin affects autophagy activation

SIRT1 also plays a role in autophagy activation. Therefore, we next investigated whether increased SIRT1 expression following Melatonin treatment modulates autophagy in the HI-injured brain. To evaluate autophagy, we analyzed the expression level of two proteins: the microtubule-associated protein 1A/1B-light chain 3 (LC3)-II and p62. LC3-II is a lipidated protein recruited into the autophagosomal membranes after autophagy activation and is often use to track the fusion between lysosomes and autophagosomes (Yoshii and Mizushima, 2017). Degradation of p62 is another widely used marker to monitor autophagic activity because p62 directly binds to LC3-II and is selectively degraded by autophagy before the fusion between lysosomes and autophagosomes (Yoshii and Mizushima, 2017). We found that HI significantly increased LC3 II expression (133.9 ± 16.9, compared to vehicle-treated animals: 97.1 ± 6.2; Fig. 5A) and decreased p62 expression (56.71 ± 11.03, compared to vehicle-treated animals: 101.04 ± 1.7; Fig. 5B), indicating autophagy activation. Interestingly, Melatonin significantly increased the HI-induced LC3 II expression and further reduced

expression of p62 (Fig. 5 A, B, respectively). These effects indicate autophagy over-activation after Melatonin treatment in ischemic conditions.

Discussion

This study examined the neuroprotective benefits of Melatonin administration in response to HI brain injury in p7-day-old rats. Here, we report that SIRT1 expression rapidly decreased in the brain 1 hour after neonatal HI, resulting in p53 acetylation and induction of apoptosis. Furthermore, these effects were concomitant with autophagy activation, demonstrated by changes in LC3 II and p62 expression. Pharmacological doses of Melatonin, administered five minutes after the end of the HI insult, reversed the effects of HI on SIRT1 expression, apoptosis, and necrosis. Interestingly, Melatonin treatment also potentiated autophagy. Overall, these results demonstrate that Melatonin treatment immediately following HI is able to prevent the initiation of multiple signaling cascades that lead to severe brain damage.

Protein acetylation is emerging as an evolutionarily conserved regulatory mechanism involved in coordinating a variety of metabolic pathways in response to different conditions, including glycolysis, fatty acid synthesis, gluconeogenesis, cell cycle, DNA repair, cell survival and differentiation, mitochondrial biogenesis, and autophagy (Rodriguez et al., 2013; Zhao et al., 2010). This is achieved by epigenetic modulation of histone and non-histone proteins in the nucleus, to regulate gene expression, and by modulation of specific protein substrates within the mitochondria and the cytoplasm (Martinez-Redondo and Vaquero, 2013). SIRT1 is a member of the class III group of histone deacetylases and its deacetylase activity is dependent on the ratio of NAD^+/NADH in the cell. During HI brain injury, the lack of oxygen and glucose leads to mitochondrial respiratory chain impairments and a decrease in the NAD^+/NADH ratio, thereby affecting SIRT1 activity (Koronowski and Perez-Pinzon, 2015). We found that SIRT1 expression rapidly decreased following HI and this effect was seen both in the nucleus, where it is predominantly localized, but also in the cytosol, where it is also present in significant amounts. Concomitantly, we observed increased expression of acetylated p53, which is indicative of a reduction in SIRT1 deacetylase activity following HI. Melatonin treatment was able to rescue SIRT1 protein levels 1 hour after HI in both cell fractions thus affecting p53 acetylation. The distribution and modulation of SIRT1 following HI and Melatonin treatment supports a role of the sirtuin in controlling cell activity and neurodegeneration in both cellular compartments. Recent studies have shown that cytoplasm-localized SIRT1 was

associated with increased sensitivity to apoptosis and this effect was independent of its deacetylase activity, but dependent on Caspases (Jin et al., 2007). These findings support a role of SIRT1 in regulating cell processes and in protecting the brain in both the nucleus and the cytosol.

SIRT1 plays a key role in cerebral protection by regulating the acetylation level of p53 to modulate apoptotic cell death. Activated p53 triggers cell cycle arrest and apoptosis by inducing transcription of cell cycle regulators and proapoptotic genes (ex: p21, BAX and PUMA) and repressing transcription of the antiapoptotic BCL-2 family of proteins (Fridman and Lowe, 2003). In addition to its functions as a transcription factor, p53 also promotes cell death by directly targeting the apoptotic pathway. Specifically, p53 can translocate into mitochondria where it induces mitochondrial outer membrane permeabilization through direct interactions with BCL-2 family members, promoting the activation of Bax (Reed and Quelle, 2014). Post-translational modifications of p53 are critical in modulating its tumor suppressive functions. Acetylation increases p53 protein stability and activity, leading to activation of apoptosis and senescence (Reed and Quelle, 2014). Conversely, p53 deacetylation, which is carried out by histone deacetylase (HDACs) such as SIRT1, can also play an important role in p53 regulation by inhibiting p53 activity and preventing its induction of cell cycle arrest and/or apoptosis. In agreement with these findings, we show here that SIRT1 downregulation following HI brain injury was concomitant with increased expression of acetyl-p53 followed by activation of the apoptosis cascade. Melatonin treatment was able to restore the level of SIRT1 protein and thus its deacetylase activity on p53, leading to an overall reduction of apoptotic cell death.

Sirtuins can directly influence autophagy via deacetylation of Atg proteins, or through the activation of FoxO transcription factor family members, the AMP-dependent kinase, or the mammalian target of rapamycin (Hariharan et al., 2010; Ng and Tang, 2013). Recent evidence suggests that activation of autophagy following cerebral ischemia protects neurons from death. Wang et al. (2012) reported that neuronal survival was promoted during cerebral ischemia when autophagy was induced by nicotinamide phosphoribosyltransferase (Nampt), which is the rate-limiting enzyme in mammalian NAD^+ biosynthesis and regulates the TSC2-mTOR-S6K1 signaling pathway (Wang et al., 2012). Furthermore, autophagy activation during focal cerebral

ischemic preconditioning contributed to tolerance to subsequent lethal ischemic injury, reducing infarct volume, brain edema and motor deficits induced by permanent focal ischemia (Sheng et al., 2010). In agreement with these findings, we showed that the protein levels of LC3 II were increased after HI brain injury and this HI induced-autophagy activation was even more pronounced after Melatonin injection. Importantly, this increase in autophagy activation correlated with an overall reduction in necrotic and apoptotic cell death. The relationship between autophagy and cerebral ischemia is still unclear, yet some laboratories have suggested that autophagy may function as a primary energy source in the early phase of ischemia to prevent or delay cell death. Like apoptosis, autophagy mainly occurs in the penumbra after focal ischemia. Here, it may provide a source of ATP by recycling misfolded/unfolded proteins and damaged organelles and delay apoptotic cell death through the inhibition of caspase-8 activation (Song et al., 2017). For example, Sarkar et al. have shown that trehalose, an mTOR-independent autophagy activator, led to enhanced clearance of autophagy substrates, such as mutant huntingtin and the A30P and A53T α-synuclein mutants, associated with Huntington's disease and Parkinson's disease, respectively (Sarkar et al., 2007). Moreover, other studies found that rapamycin (an mTOR inhibitor and autophagy activator) protected cells against pro-apoptotic insults by inducing autophagy activation, resulting in mitochondria clearance and reducing cytosolic cytochrome c release and downstream caspase activation (Ravikumar et al., 2006). Taken together, these results indicate that Melatonin treatment promotes autophagy following HI injury, possibly via maintenance of SIRT1 expression, and this increase in autophagy likely plays an important role in protecting the brain.

Neuroplasticity is the brain's ability to reorganize itself by forming new neural connections throughout life and is essential to make adaptive changes on both morphological and functional levels during normal development and following brain injuries. Cell regeneration after CNS injuries, such as traumatic brain injury, ischemic stroke and hypoxia, largely depends on the generation of new neurons and/or glia from proliferative progenitor cells that respond to various insults by expansion of their pool. Emerging evidence suggests that SIRT1 may also regulate cellular proliferation and differentiation in response to brain injury. SIRT1 overexpression, achieved by transfected Sirt1 or pharmacological agonist resveratrol, was able to alleviate

astrogliosis both in primary cortical astrocyte cultures and in an *in vivo* model of traumatic brain injury, thereby improving neurobehavioral function (Li et al., 2017). Moreover, in a mouse model of neonatal hypoxia that reproduces the diffuse white matter injury seen with premature infants, SIRT1 was found to regulate the proliferation and differentiation of oligodendrocyte progenitor cells (OPCs) by modulating the activity of cell cycle regulatory proteins, in particular the Cyclin-dependent kinases 2 (Cdk2) (Jablonska et al., 2016). Furthermore, it has been reported that SIRT1 inactivation ameliorated remyelination and delayed paralysis in a mouse model of demyelinating injuries by increasing *Pdgfrα* expression, a known regulator of OPC proliferation and function, both in neural stem cells and neural progenitors (Rafalski et al., 2013). Further studies are required to better define the role of SIRT1 in the regulation of neuronal and glial progenitor cells after brain injury, especially HI.

A strong body of evidence now supports that Melatonin is neuroprotective for acute HI perinatal brain injury, mediated via its anti-oxidant, anti-apoptotic, and anti-inflammatory properties (Hassell et al., 2015). In our model of HI in 7 days-old-rats, administration of pharmacological doses of Melatonin immediately after the ischemic insult rescued SIRT1 expression and activity, increased autophagy and reduced necrotic and apoptotic cell death, providing further evidence about its neuroprotective effect. Several other studies have documented the neuroprotective effect of Melatonin against brain injuries. Melatonin has been proven to be effective in attenuating endoplasmic reticulum stress occurring after ischemia/reperfusion in cultured neurons exposed to oxygen glucose deprivation. In addition, animals treated with melatonin after transient focal cerebral artery occlusion had significantly reduced infarction volumes and individual cortical lesion sizes, as well as increased numbers of surviving neurons (Lin et al., 2018). Melatonin treatment also effectively decreased neuronal apoptosis resulting from oxygen glucose deprivation (Lin et al., 2018). Furthermore, in a mouse model of transient middle cerebral ischemic/reperfusion injury, Melatonin treatment significantly improved the survival rates and neural outcomes of the injured mice by preserving blood–brain barrier (BBB) integrity via a reduction in stroke-induced free radical production (Chern et al., 2012). Moreover, Melatonin treatment after stroke dramatically enhanced endogenous neurogenesis and cell proliferation in the peri-infarct

region (Chern et al., 2012). Overall, these results confirm the role of Melatonin as neuroprotective agent against several types of brain injury.

Melatonin is a well-tolerated molecule, even at high doses. Melatonin is safe for administration during pregnancy or to the newborn after birth, with no known side effects (Aversa et al., 2012; Gitto et al., 2004). Furthermore, because of its lipophilic properties, Melatonin easily crosses most biological barriers, including the placenta and the blood-brain barrier. Therefore, because of its safety profile and multiple pre-clinical studies showing neuroprotective efficacy, clinical trials should be performed to test Melatonin as a potential therapeutic treatment for traumatic CNS injuries.

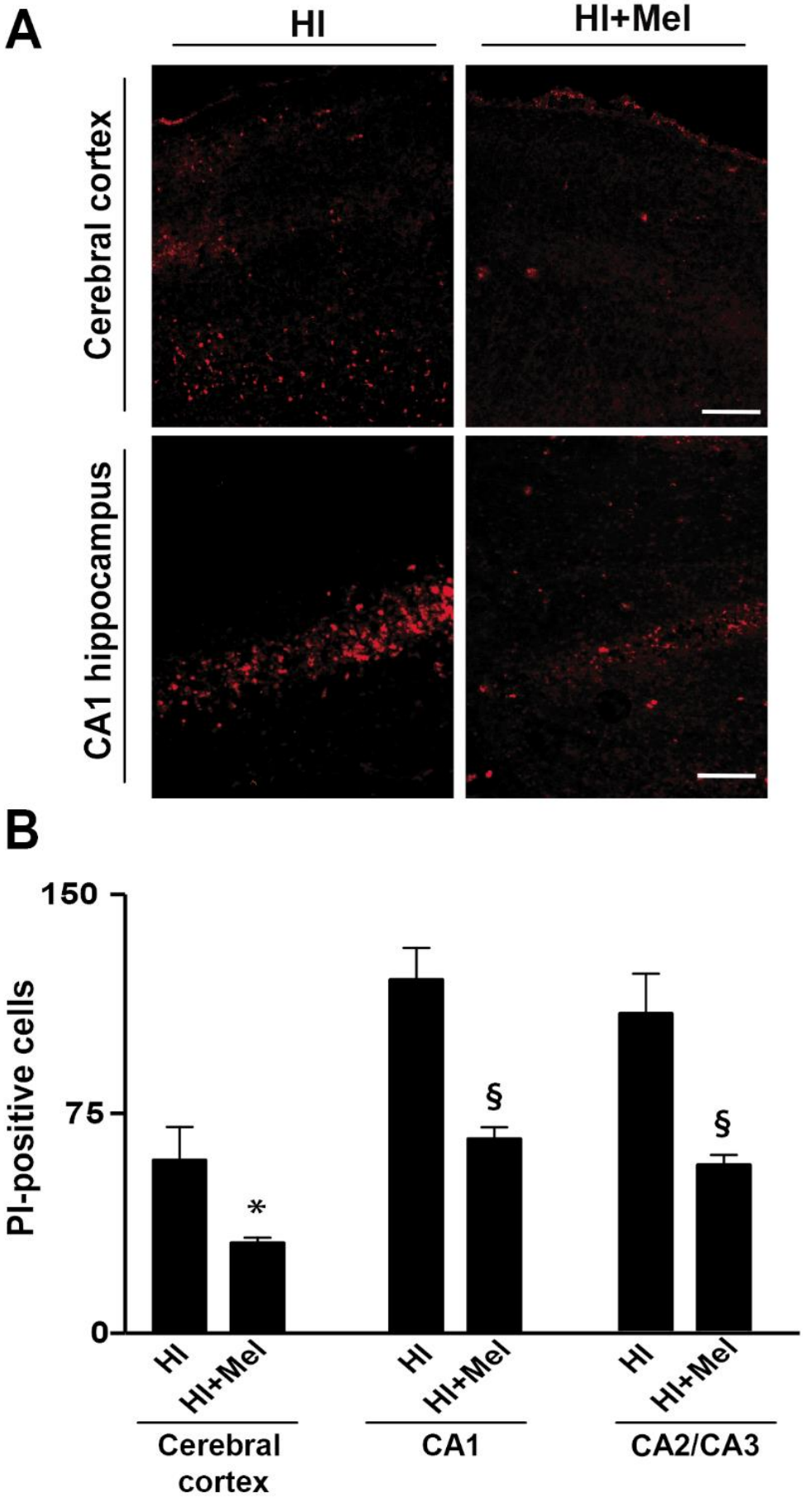

A
HI
HI+Mel
Cerebral cortex
CA1 hippocampus
B
PI-positive cells
150
75
0
*
§
§
HI
HI+Mel
HI
HI+Mel
HI
HI+Mel
Cerebral cortex
CA1
CA2/CA3

FIGURE 1. Effect of neonatal hypoxia-ischemia and melatonin on propidium iodide (PI) uptake. (A) Representative images showing cells labeled with PI (red) in the lesioned cerebral cortex and lesioned CA1 region of the hippocampus of vehicle-treated (HI) and melatonin-treated (HI+Mel) ischemic animals. PI was injected icv 20 min before the sacrifice of the animals. Scale bar, 50 μm. (B) PI-positive cells counting in the cerebral cortex, CA1 and CA2/CA3 regions of the hippocampus. Counting was performed as described in Methods and data reported as the mean ± s.e.m. (N=5/group). Significantly different from vehicle-treated ischemic animals (one-way ANOVA followed by Newman-Keuls multiple comparison test, **P*<0.05, § *P*<0.001).

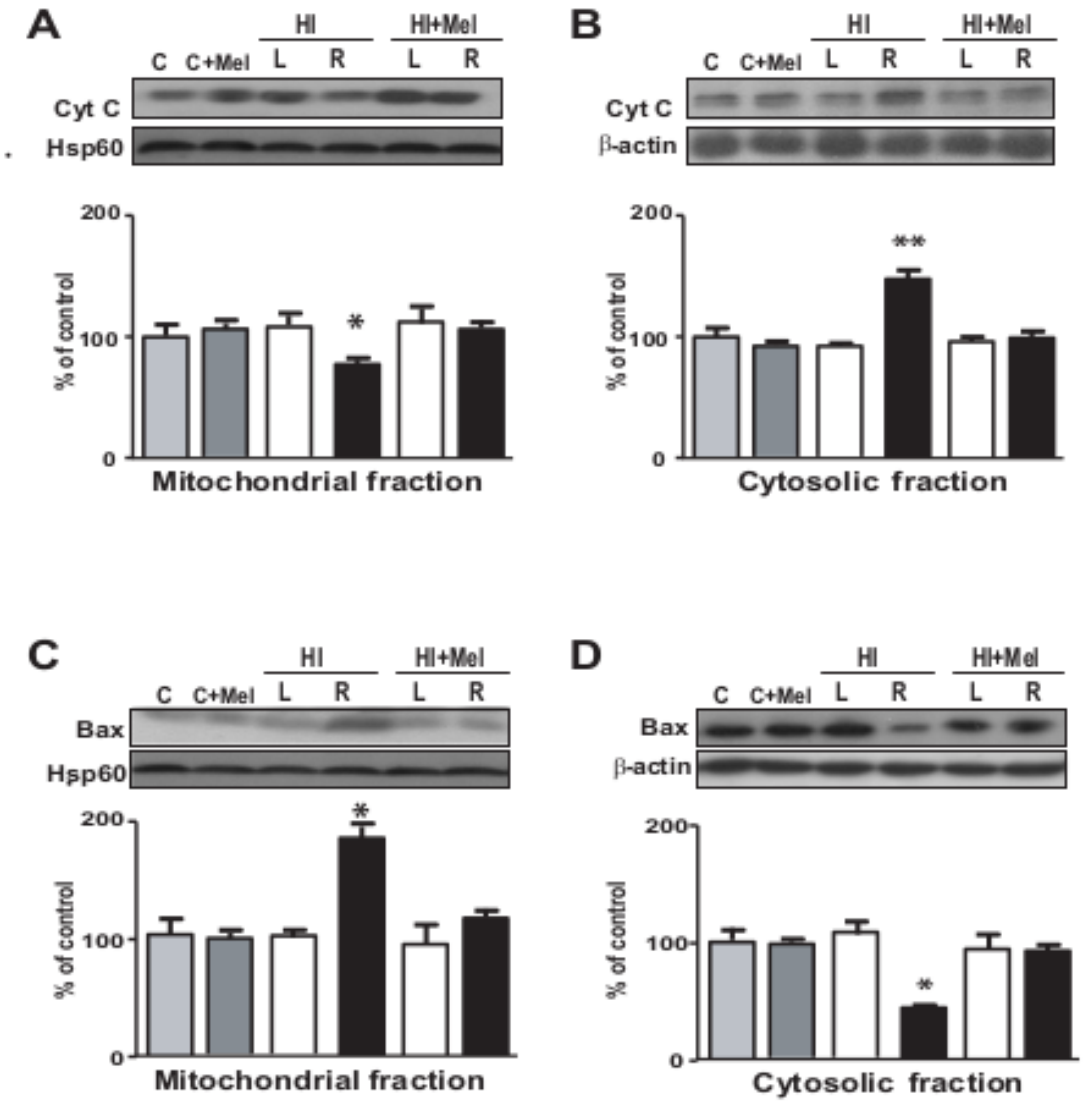


FIGURE 2. Effect of neonatal hypoxia-ischemia and melatonin on mitochondrial apoptosis. Representative Western blots and quantitative evaluation of Cytochrome C (A, B) and Bax (C, D) expression in the mitochondrial and the cytosolic fraction of the cerebral cortex of vehicle-treated (C) or melatonin-treated (C+Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals sacrificed 1 h after HI. β-actin or Hsp60 was run as loading controls for the cytosolic and the mitochondrial fraction, respectively. Data are expressed as % of control and are the mean ± s.e.m. (N=8/group). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. Significantly different from vehicle-treated animals (one-way ANOVA followed by Newman-Keuls multiple comparison test, **P*<0.05, ***P*<0.01).

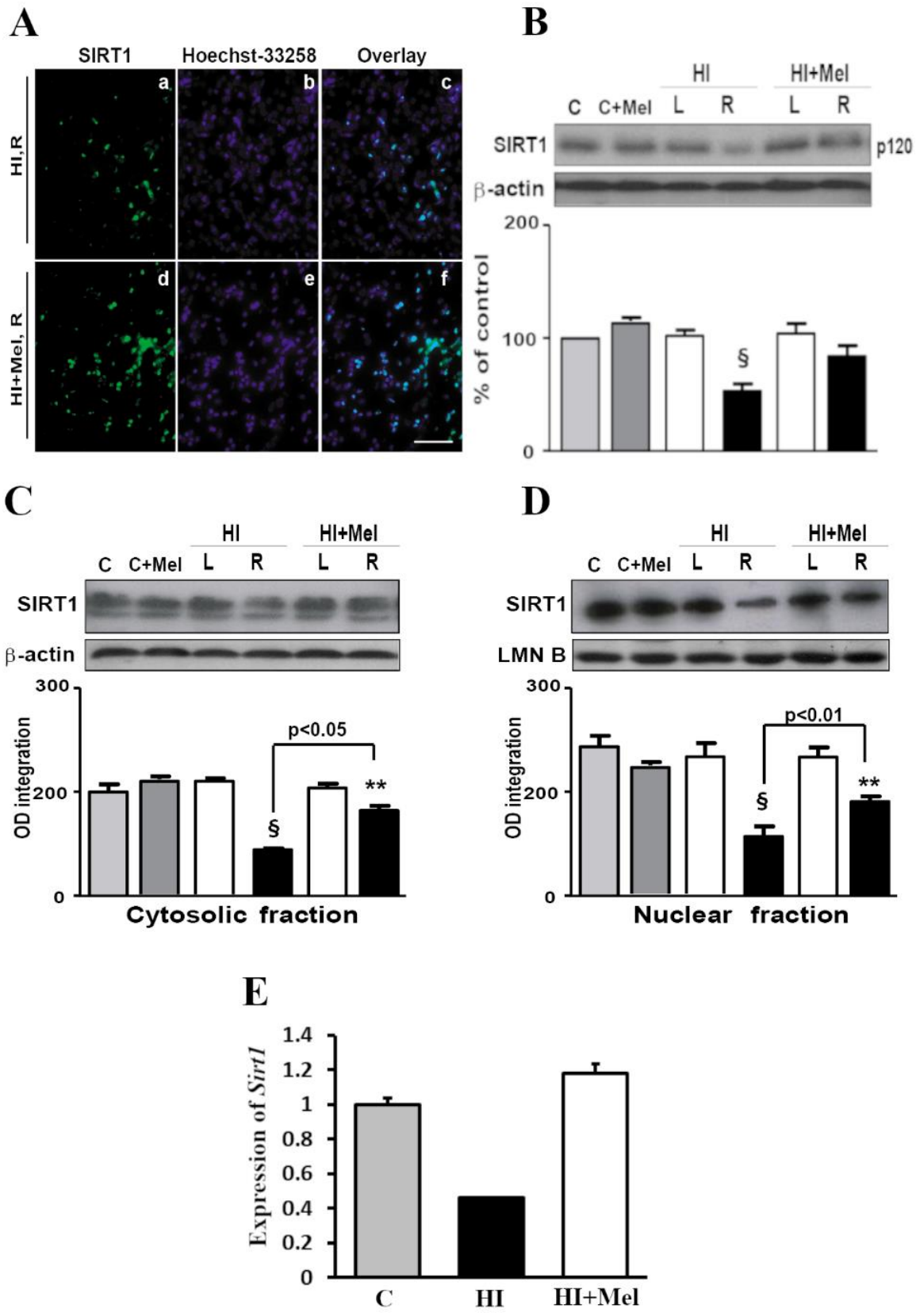

A
SIRT1
Hoechst-33258
Overlay
a
b
c
d
e
f
HI,R
HI+Mel, R
B
HI
HI+Mel
C
C+Mel
L
R
L
R
SIRT1
p120
β-actin
200
100
0
% of control
§
C
SIRT1
β-actin
300
200
0
OD integration
p<0.05
§
**
Cytosolic fraction
D
LMN B
p<0.01
Nuclear fraction
E
Expression of Sirt1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
HI
HI+Mel

FIGURE 3. Effect of neonatal hypoxia-ischemia and melatonin on SIRT1 expression. Photomicrographs (A) of experiments performed 1 h after HI showing cells expressing SIRT1 (green) in the lesioned (R) cerebral cortex of vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals. N=5/group. Scale bars, 100 μm. Representative Western blot and quantitative evaluation of SIRT1 expression in the whole cerebral cortex (B) and in the cytosolic (C) and the nuclear (D) fraction of the cerebral cortex of vehicle-treated (C) or melatonin-treated (C+Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals sacrificed 1 h after HI. β-actin or Lamin B (LMN B) was run as loading controls for the cytosolic and the nuclear fraction, respectively. Data are expressed as % of control or as OD integration and are the mean ± s.e.m. (N=8/group). Representative RT-PCR from cerebral cortex of vehicle-treated animals (C) and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals (E). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. Significantly different from vehicle-treated animals (one-way ANOVA followed by Newman-Keuls multiple comparison test, $^{**}P<0.01$, §$P<0.001$)

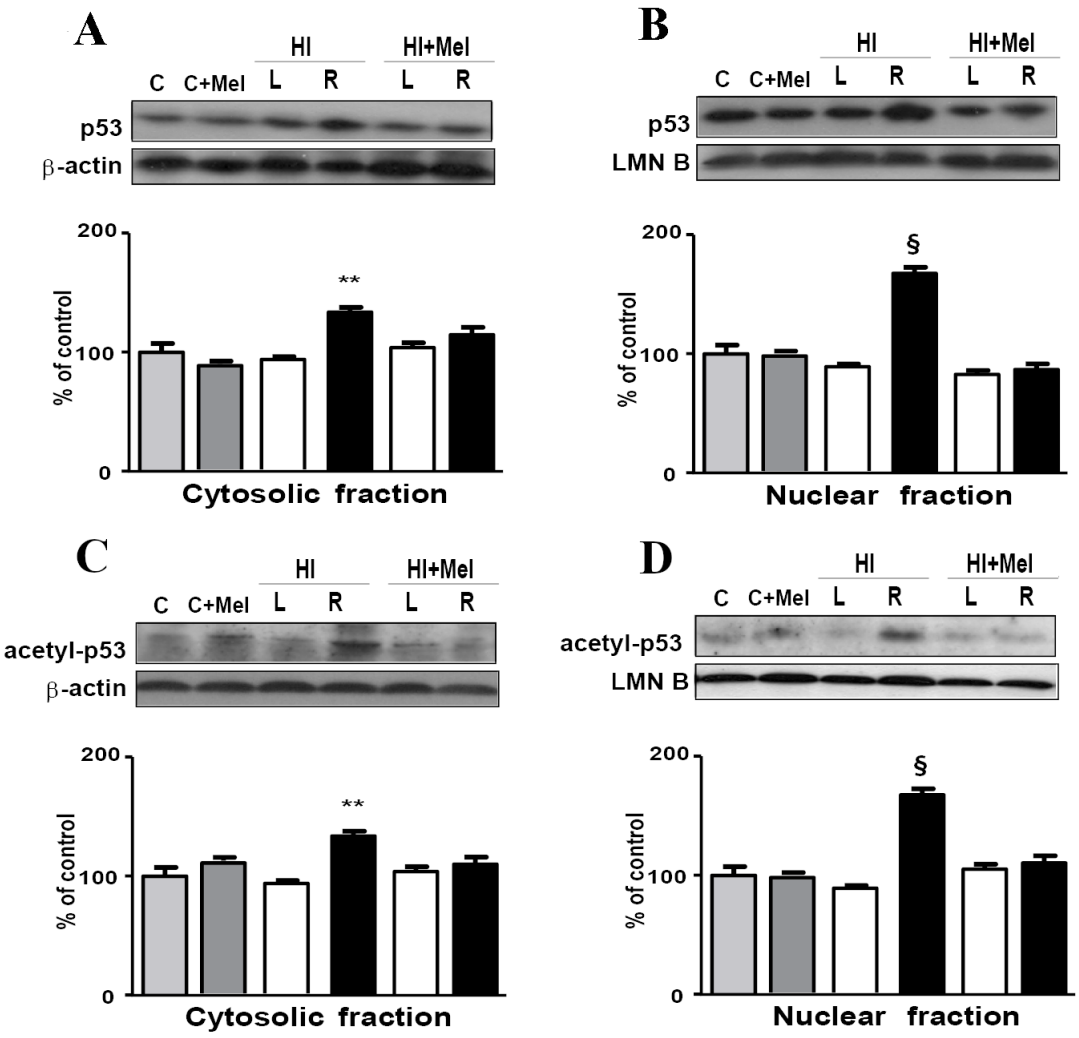


FIGURE 4. Effect of neonatal hypoxia-ischemia and melatonin on p53 expression. Representative Western blots and quantitative evaluation of p53 (A, B) and acetyl-p53 (C, D) expression in the cytosolic and the nuclear fraction of the cerebral cortex of vehicle-treated (C) or melatonin-treated (C+Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals sacrificed 1 h after HI. β-actin or Lamin B (LMN B) was run as loading controls for the cytosolic and the nuclear fraction, respectively. Data are expressed as % of control and are the mean ± s.e.m. (N=8/group). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. Significantly different from vehicle-treated animals (one-way ANOVA followed by Newman-Keuls multiple comparison test, ***P*<0.01, §*P*<0.001)

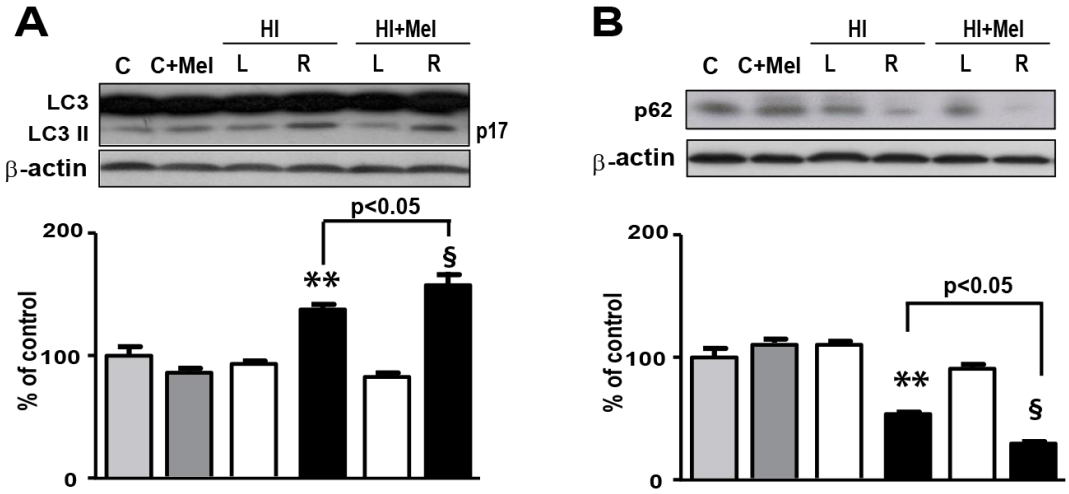


FIGURE 5. Effect of neonatal hypoxia-ischemia and melatonin on autophagy markers. Representative Western blots and quantitative evaluation of lipidated LC3 (LC3 II, A) and p62 (B) expression in the cerebral cortex of vehicle-treated (C) or melatonin-treated (C+Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals sacrificed 1 h after HI. β-actin was run as loading control. Data are expressed as % of control and are the mean ± s.e.m. (N=8/group). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. Significantly different from vehicle-treated animals (one-way ANOVA followed by Newman-Keuls multiple comparison test, ***P*<0.01, §*P*<0.001).

3 Elucidating the Functional Role of Endothelin-1 in the Developing Subventricular Zone

3.1.1 Neural Stem Cells within the Postnatal Subventricular Zone

Neural stem cells (NSCs) are the primary progenitor cells at different developmental stages that give rise to differentiated neurons and glial cells (astrocytes and oligodendrocytes) in the mammalian central nervous system (Kriegstein and Alvarez-Buylla, 2009). This multipotency is acquired during embryonic development and is maintained throughout life, contributing to brain plasticity. In the postnatal brain, there are two major neurogenic niches where NSCs reside: the subventricular zone (SVZ), lining the lateral ventricles, and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus (Kriegstein and Alvarez-Buylla, 2009; Zhao et al., 2006). NSCs in the postnatal SVZ display ultrastructural characteristics and markers of astroglial cells, including the expression of the astrocyte-specific glutamate transporter (GLAST), the brain lipid-binding protein (BLBP) and a variety of intermediate filament proteins, such as Nestin and Vimentin (Campbell and Gotz, 2002; Mori et al., 2005). These radial glia-like NSCs extend a basal process to terminate on blood vessels and an apical process with a primary cilium that contacts the cerebrospinal fluid and the ependymal cells lying on the surface of the ventricle, thus maintaining apical-basal polarity (Mirzadeh et al., 2008). In the postnatal brain, radial glia-like NSCs undergo symmetric cell divisions to either self-renew or to generate transient-amplifying cells that undergo cell division before generating young migrating neuronal progenitors, or neuroblasts (Doetsch et al., 1999). These neuroblasts migrate anteriorly as a network of tangentially oriented chains that converge at the anterior dorsal subregion of the SVZ to form the rostral migratory stream (RMS) (Doetsch and Alvarez-Buylla, 1996). Newly-generated neuroblasts migrate along the RMS to the olfactory bulb (OB) where they differentiate into mature local interneurons. In addition to neuroblasts, radial glia-like NSCs also generate intermediate $Olig2^{+}$ transit-amplifying cells (type C cells) that give rise to oligodendrocyte progenitor cells (OPCs) committed to the oligodendroglial lineage (Menn et al., 2006). Once specified, OPCs remain highly proliferative and motile, dividing as they migrate out of the SVZ throughout the CNS. Once in their final position, they exit the cell cycle and differentiate into mature myelinating oligodendrocytes (OLs) (Aguirre et al., 2007; Gonzalez-Perez et al., 2009).

3.1.2 Oligodendrocyte Development

OPCs are NG2-expressing proliferating cells that give rise to mature oligodendrocytes capable of myelinating the CNS. Myelin is a necessary prerequisite for saltatory conduction, the rapid propagation of the action potentials, and for metabolic support of axons. Therefore, the loss of myelin in demyelinating diseases such as Multiple Sclerosis (MS) has profound pathological consequences (Salzer, 2015). The process of CNS myelination by OLs is tightly regulated and involves dynamic cellular processes that occur throughout development and into adulthood.

In the mammalian embryo, OPCs populate different regions of the developing forebrain and mature during different time periods. Particularly, fate-mapping analyses have demonstrated three distinct waves of OPC generation (Kessaris et al., 2006). Following the initial period of neurogenesis, Sonic hedgehog (Shh) signaling drives the first phase of glial progenitor production in the medial ganglionic eminence at E12.5, under the transcriptional control of *Nkx2.1* (Tekki-Kessaris et al., 2001). These OPCs migrate tangentially and dorsally to colonize the entire forebrain (Kessaris et al., 2006). A few days later, a second wave of OPCs emerges from the lateral ganglionic eminence and is driven by the transcriptional control of *Gsh2*; those cells migrate dorsally to the cortex, dispersing throughout the forebrain in the process (Kessaris et al., 2006). A third and final wave of OPCs production occurs predominantly after birth, and projects radially from the dorsal SVZ and outer SVZ directly to the developing corpus callosum; these cells express the homeobox transcription factor *Emx1* (Kessaris et al., 2006). These dorsally originating OPCs migrate locally to populate the corpus callosum. During development, OPCs disperse uniformly throughout both the white and gray matter of the CNS and once they reach their final destination, many exit the cell cycle and mature into myelinating OLs (Hughes et al., 2013).

In the postnatal brain, the SVZ is the major source of new OPCs. $NG2^{+}$ cells display a slow turnover and maintain the ability to divide asymmetrically to self-renew and to produce OL, keeping a tight balance between proliferation, survival and differentiation (Polito and Reynolds, 2005). OPC proliferation and cell-cycle progression are strictly controlled by several molecular pathways and regulators.

Growth factors, in particular, regulate OL development, especially OPC migration and differentiation, and can also play a role in myelin maintenance (Baron et al., 2005; Miller, 2002).

Platelet-derived growth factor (PDGF) was one of the first characterized mitogens for OPCs. In the CNS, PDGF is synthesized during development from both astrocytes and neurons (Yeh et al., 1991) and its trophic activity is regulated by its receptor, PDGFRα, which is highly expressed by OPCs (Hart et al., 1989). PDGF promotes OPC survival and proliferation. In PDGFR-α null mice, the number of OPCs is dramatically reduced in the developing spinal cord, whereas its overexpression induces ectopic overproduction of OPCs (Calver et al., 1998).

Fibroblast growth factor (FGF) synergistically acts with PDGF as another crucial signal for OPC survival and proliferation. FGF upregulates the expression of PDGFR-α on OPCs, thus extending the proliferating phase during which OPCs or pre-OLs respond to PDGF (McKinnon et al., 1990). Furthermore, PDGF and FGF promote OPC migration *in vitro* (Gadea et al., 2009).

Similarly, Insulin-like growth factor-1 (IGF-1) promotes both OPC expansion and differentiation (Ye et al., 2002). Astrocytic IGF-1 promotes a significant increase in OL number and myelination *in vivo* (Zeger et al., 2007). IGF-1 acts synergistically with FGF-2 to coordinate OPC cell-cycle progression: specifically, the IGF-1/FGF-2 complex contributes to OPC proliferation by enhancing FGF-2 induction of cyclin D1, activation of G_1 cyclin–cyclin-dependent kinase (cdk) complexes and hyperphosphorylation of retinoblastoma protein (pRb) (Frederick and Wood, 2004). PDGF, FGF and IGF-1 have been shown to cooperate to promote OPC expansion (Goddard et al., 1999; Baron et al., 2000; Jiang et al., 2001). Once they bind their receptors, they activate the Erk1/2 or PI3K/Akt signaling pathways to trigger downstream effectors (Baron et al., 2000; Frederick et al., 2007). However, their OL-specific downstream targets are largely unknown.

In addition to growth factors, the Wnt (Wingless-type MMTV integration site family) and Notch signaling pathways also regulate OPC proliferation and differentiation. Wnt signaling pathway functions to promote self-renewal of neural stem and progenitor cells and is active prenatally in the dorsal spinal cord and subventricular zone of the CNS (Kalani et al., 2008). Recent studies have demonstrated that Wnt

signaling inhibits OL development: particularly, in rat OPC cultures, the addition of Wnt3a significantly decreased the number of OLs generated during in vitro differentiation (Feigenson et al., 2009). Likewise, Wang et al. (1998) found that when OPCs were co-cultured with cells expressing the Notch ligand Jagged1, over 40% of them failed to differentiate into oligodendrocytes, retaining a bipolar morphology and expression of OPC marker A2B5 (Wang et al., 1998).

Molecular and cellular processes controlling CNS developmental myelination often regulate CNS remyelination after injury; therefore, understanding the mechanisms underlying OPC proliferation and differentiation is essential for identifying signaling pathways and potential therapeutic targets involved in OL regeneration.

Recently, Endothelin-1 (ET-1) signaling emerged as a novel potential regulator of OL development. Previous studies performed in our laboratory found that ET-1 is an astrocyte-derived signal that regulates OPC migration and differentiation in purified OPC cultures (Gadea et al., 2009). These findings raise the question of whether ET-1 might affect OPC proliferation in the developing SVZ.

3.1.3 Endothelin-1 signaling pathway

The endothelins are a family of three 21 amino acid (aa) long peptides ET-1, ET-2 and ET-3, characterized by vasocontractile properties. ET-1 is the most abundant isoform and is primarily localized in the vascular endothelium, vascular smooth muscle cells, and in various epithelial tissues. All three endothelins are encoded by distinct genes and regulated at the level of mRNA transcription. The primary translation product of the *Edn1* gene is a 212-aa long protein called prepro-ET-1, which is processed by an endopeptidase to generate the 38-aa long big-ET-1. Big ET-1 is then cleaved by the endothelin-converting-enzyme (ECE) to generate the 21-aa active ET-1 peptide. Endothelin signaling is mediated by two distinct cell membrane ET receptors, Ednra and Ednrb, which are members of the seven transmembrane G protein-coupled rhodopsin superfamily. The binding of ET-1 to its receptors results in coupling of cell-specific G proteins that leads to the activation of a number of physiological functions.

ET-1 signaling has been extensively studied in the vascular system but less is known regarding its role in the brain. Endothelin-1 exerts a vasoconstricting role via activation of its receptors in the vascular smooth muscle cells, contributing to the

development of vascular diseases such as hypertension and atherosclerosis. In endothelial cells, ET-1 signaling promotes nitric oxide (NO) release, leading to vasodilation. Endothelin also controls water and sodium excretion and acid-base balance in the kidney under physiological conditions (Kohan, 2008), and promotes the development of glomerulosclerosis. Recently, ET-1 has been shown to play a role in neural crest cell development, participating in enteric neural crest cell migration and differentiation (Liu et al., 2018). Furthermore, mutation of the Ednrb gene is a major cause of Hirschsprung disease in mice and humans, a congenital digestive disease characterized by abnormal development of the enteric nervous system during embryogenesis (Heanue and Pachnis, 2007).

Recent experiments performed in our laboratory have shown that both ET-1 and its receptor Ednrb are highly expressed in the mouse postnatal SVZ, particularly in radial glia-like NSCs immunopositive for BLBP, S100β and GFAP markers (Adams et al., in preparation). Interestingly, ablating either ET-1 signaling or its receptor Ednrb in radial glia reduced proliferation of these cells, indicating that ET-1 promotes proliferation of radial glia (Adams et al., in preparation). In agreement with previous work (Gadea et al., 2009), we also found that Ednrb is expressed by OPCs in the postnatal SVZ. Ablation of ET-1 in the postnatal mouse SVZ or ablation of the receptor Ednrb specifically in OPCs both resulted in reduced proliferation of OPCs, suggesting that ET-1 regulates the balance between OPC proliferation and differentiation (Adams et al., in preparation). To test this hypothesis and to better understand the role of ET-1 in regulating OPC proliferation, in this study we assessed the effects of ET-1 overexpression in the mouse SVZ, using both *ex vivo* and *in vivo* models. Furthermore, we characterized the ET-1 expression pattern in the piglet postnatal SVZ, which more closely resembles the human SVZ.

Material and Methods

3.2.1 Animals

Mice

C57bl/6n (Jackson ID) mice were purchased from the Jackson Laboratory and were maintained in the animal facility of Children's National Medical Center. All animal procedures complied with the guidelines of the NIH and with the Children's Research Institute Institutional Animal Care and Use Committee (IACUC) guidelines.

Piglets

Yorkshire piglets (Archer Farms, Inc.) were used in this study. Piglets were housed in the research animal facility at Children's National Medical Center with a nutritionally balanced milk formula (OptiLac Baby Pig Milk Replacer, Hubbard) on a 12/12-hour light/dark cycle. All experiments were performed in compliance with the guidelines of the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals," and were approved by the Children's National Medical Center Animal Care and Use Committee.

3.2.2 Organotypic slice culture assay

Organotypic cultures were prepared as previously described (Humpel C., 2015). Briefly, brains from C57bl/6 mice (P9-P10) were dissected and glued (Super Glue Loctite) onto the chuck of an ice cooled vibratome Leica VT1200S. 270 µm thick coronal sections were cut and collected in chilled Neurobasal Medium enriched with N-2 Supplement (100X) and 10 µg/ml Gentamicin (all Gibco/ThermoFisher). The coronal slices were cultured on Millicell Cell Culture Inserts (Millipore, pore size 0.4 µm, diameter 30 mm) in media consisting of 50% MEM/HEPES, 25% HBSS, 25% horse serum, 2 mM $NaHCO_3$, 6.5 mg/ml glucose, 2mM glutamine (all Gibco/ThermoFisher), and 100 µg/ml primocin (InvivoGen). Organotypic slices were cultured for 96 hours. The media was replaced after 1 and 3 days in vitro, during which 100 nM Endothelin-1 (Tocris) was added to randomly selected groups of slices. Organotypic slices were fixed for 3 h at 4 °C in 4% paraformaldehyde (PFA) and then stored at 4 °C in 0.01% NaAz/PBS until use.

3.2.3 Molecular cloning

An ET-1 overexpression plasmid was generated by using the In-Fusion HD Cloning Kit (Takara Bio Company). Mouse cDNA from P15 whole brain was used as ET-1 template to amplify the ET-1 coding sequence using CloneAmp HiFi PCR Premix kit. Following PCR, the ET-1 coding sequence was inserted into the multiple cloning site of the pLVX-IRES-ZsGreen1 vector (Takara Bio Company). DNA sequencing was performed to confirm the correct ET-1 sequence.

3.2.4 Neonatal Electroporation

Postnatal day 1 mice were anesthetized by hypothermia. 2µl of plasmid (1µg/µl) were injected into the right lateral ventricular cavity using a 5µl Hamilton syringe to a depth of 2 mm. The site of injection was approximately equidistant from the lambdoid suture and eye and 2 mm lateral to the sagittal suture. Five 100 V electric pulses were applied (50 ms duration, 950 ms intervals), with the positive electrode positioned in the dorso-lateral region to direct the negatively charged DNA to the subventricular zone. After the pulses, the pups were placed on a thermal plate and when they had recovered, they were returned to their mother. Pups were then sacrificed after 48 hours, at postnatal day 3, for immunohistochemical analysis.

3.2.5 Piglet Tissue Harvest

Piglets were transarterially perfused through the common carotid artery. Transarterial perfusion with 2.0 liters of saline was followed by 2.0 liters of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4). Brains were removed and further post-fixed at 4 ºC in a 4% paraformaldehyde solution in 0.1 M PBS. Brains were then cut into smaller tissue blocks. Tissue blocks were cryoprotected in a 15% sucrose solution for 24 h, followed by a 48 h incubation in a 30% sucrose solution in 0.1 M PBS at 4ºC. All samples were embedded in O.C.T. compound, sliced with a cryostat at -20ºC, and stored at -80°C until immunohistochemical processing.

3.2.6 Immunohistochemistry

For mouse organotypic slices: fixed 270µm organotypic brain slices were blocked for 4h in PBS with 0.1% Triton X-100 (PBST) and 10% donkey serum. The slices were then incubated with specific primary antibody in PBST with 1% normal donkey serum (NDS) 2 days overnight at 4°C with orbital shaking. Following primary antibody

incubation, slices were washed in PBST four times for 30 min and incubated with species specific fluorescently-labeled secondary antibodies overnight. The day after, slices were washed with PBST three times for 30 min, incubated with DAPI for 30 min (Sigma-Aldrich) and were coverslipped with Fluoromont-G mounting media (SouthernBiotech).

For mouse electroporated tissue: 12µm coronal sections were rehydrated and heated in 10mM sodium citrate buffer (pH 8.5, 15' at 80 °C) for antigen retrieval immunohistochemistry. Tissue section were first stained with ET-1 antibody and then sequentially stained for other primary antibodies to reduce cross-reactivity. The ET-1 staining was amplified using the avidin biotinylated-HRP complex (ABC) method using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, sections were washed twice with PBS and then incubated for 1 hour at RT in a blocking solution containing 10% NDS in PBST. Sections were then incubated for 1 day overnight at 4°C with primary ET-1 antibody diluted in PBST containing 1% NDS. The next day, sections were rinsed in PBST (2 x 10 min) and incubated for 10 min with 0.3% hydrogen peroxide in PBS to quench endogenous peroxidase. Sections were washed in PBST (2 x 10 min) and incubated for 2 hours at RT in biotinylated goat anti-rabbit antibody in PBS (Jackson Immunoresearch), followed by 90 min in ABC solution (Vector Laboratories, prepared according to the manufacturer's instructions). Sections were then incubated with Tyramide-Alexa488 (Invitrogen) for 10 minutes at room temperature. Subsequently, sections were incubated overnight at 4°C with other primary antibodies. The next day, sections were washed in PBST and incubated with species-specific fluorescently-labeled secondary antibodies for 2h at room temperature. Sections were coverslipped with Fluoromont-G + DAPI mounting media (Invitrogen).

For piglet tissue: 20µm coronal sections were rehydrated and heated in 10mM sodium citrate buffer (pH 8.5, 15' at 80 °C) for antigen retrieval immunohistochemistry. Tissue sections were first stained with ET-1, Ednra, or Ednrb antibodies and then sequentially stained for other primary antibodies to reduce cross-reactivity. The ET-1, Ednra, and Ednrb staining was amplified using the avidin biotinylated-HRP complex (ABC) method using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, sections were washed twice with PBS and then incubated for 1 hour at RT in a blocking solution containing 1% BSA, 20% normal donkey serum (NDS), 0.3% Triton

X-100 in PBS. Sections were then incubated for 2 days overnight at 4°C with primary ET-1, Ednra, or Ednrb antibodies diluted in PBST containing 0.1% DNS. After 2 days, sections were rinsed in PBST (2 x 10 min) and incubated for 10 min with 0.3% hydrogen peroxide in PBS to quench endogenous peroxidase. Sections were washed in PBST (2 x 10 min) and incubated for 2 hours at RT in biotinylated goat anti-rabbit antibody in PBS (Jackson Immunoresearch), followed by 90 min in ABC solution (Vector Laboratories, prepared according to the manufacturer's instructions). Sections were then incubated with Tyramide-Alexa488 (Invitrogen) for 10 minutes at room temperature. Subsequently, sections were incubated overnight at 4°C with other primary antibodies. The next day, sections were washed in PBST and incubated with species-specific fluorescently-labeled secondary antibodies for 2h at room temperature. Sections were coverslipped with Fluoromont-G + DAPI mounting media (Invitrogen).

3.2.7 Bromodeoxyuridine (BrdU) labeling

To label proliferative cells, organotypic brain slices were incubated on day 3 in culture for 16 h with 10μM BrdU (Sigma-Aldrich). After fixation in 4% PFA, slices were incubated in 2N HCl for 30 min followed by washing in borate buffer (pH 8.5) for 30 min. A monoclonal rat-anti BrdU (Abcam, 1:250) and goat anti-rat Alexa-647 (Jackson Immunoresearch, 1:500) were used to visualize BrdU-labeled cells.

3.2.8 Antibodies

The following primary antibodies were used for staining mouse tissue: guinea pig anti-Olig2 (gift from Bennett Novitch), rabbit anti-NG2 (Millipore 1:200), rat anti-BrdU (Abcam 1:250), mouse anti-ET-1 (Meridan, 1:100), rabbit anti-ET-1 (Abcam, 1:500); rabbit anti-Ednra (Abcam, 1:500), rabbit anti-Ednrb (Abcam, 1:500), goat anti-Sox2 (Santa Cruz, 1:100), rabbit anit-Sox2 (Millipore, 1:100), chicken anti-GFAP (Abcam 1:500). The species-appropriate Alexa488, Alexa594, and Alexa647 secondary antibodies (Jackson Immunoresearch) were used.

3.2.9 Image Acquisition and Analysis

Stained tissue sections were imaged with a Leica SP5 confocal laser-scanning microscope. Images were random fields selected from anterior-dorsal SVZ areas. All images were processed and quantified using Fiji ImageJ software. Quantifications throughout the manuscript are the results of five independent experiments. For each

batch, eight ventricles were used and randomly assigned to the control or ET-1 group. Data are represented as mean ± s.e.m. (standard error of the mean). Statistical significance was defined as *$p \leq 0.001$. Student's test (Excel) was used to determine statistical significance for each group. GIMP software was used to make figures.

Results

3.3.1 Exogenous ET-1 increases the OPC population

Recent data obtained in our laboratory found that Ednrb, but not its ligand ET-1, is expressed by OPCs in the postnatal SVZ (Adams et al., in preparation). Interestingly, ablating either ET-1 throughout the SVZ or the receptor Ednrb specifically in the OPC population results in reduced proliferation of OPCs, suggesting that ET-1 might regulate OPC proliferation in the developing SVZ (Adams et al., in preparation).

To further investigate the role of ET-1 in OPCs development in the postnatal SVZ, we assessed the effects of ET-1 overexpression using a model of organotypic brain slices generated from P8-10 wild type mice. Organotypic brain slices have proven to be very useful in investigating cellular and molecular processes of the brain *in vitro*, because this system preserves the main architecture of the cells and the heterogeneity of cell population. Furthermore, the use of organotypic brain slices allowed us to easily test different ET-1 concentrations, thereby avoiding any side effects related to the vasoconstrictive effects of ET-1.

Slices were incubated with 100nM ET-1 for 72 hours and immunostained with OPC markers NG2 and Olig2 to evaluate changes in total number of OPCs. Figure 1 shows that, compared to control un-treated slices, ET-1 treatment strongly promoted an increase in the total number of $Olig2^+$ cells, which labels the OL lineage (50.90 ± 8.50 $Olig2^+$ cells for control group compared to 85.86 ± 9.34 $Olig2^+$ cells for ET1 treated group, $*p \leq 0.001$). ET-1 treatment also increased the number of $NG2^+$ cells, which represent the OPC population (15.68 ± 2.13 $NG2^+$ cells for control group compared to 33.65 ± 7.01 $NG2^+$ cells for ET-1 treated group, $*p \leq 0.001$).

Together, these results indicate that exogenous ET-1 induces an increase in OPCs within the early postnatal SVZ.

3.3.2 ET-1 promotes OPC proliferation

We then sought to investigate whether the increase in OPCs following ET-1 treatment was due to an increase in OPC proliferation. In order to identify proliferating OPCs, we carried out a 16 hour BrdU labeling of the brain slices, followed by double immunostaining with anti-BrdU, anti-Olig2, and anti-NG2 antibodies. Results revealed that exogenous ET-1 treatment increased the total number of $Olig2^+$ $BrdU^+$ OL lineage cells compare to control un-treated slices (16.35 ± 3.30 for control group compare to

37.64 ± 1.13 for ET1 treated group, *$p \leq 0.001$). Particularly, the total number of dividing OPCs ($NG2^+$ $BrdU^+$ cells) was increased 2.5 fold following ET-1 treatment, indicating an increase in the proliferative ratio of OPC cells after ET-1 treatment (8.64 ± 1.68 for control group compare to 25.13 ± 3.48 for ET1 treated group, *$p \leq 0.001$).

Taken together, these results suggest that a novel role of the ET-1 signaling pathway is to promote OPC proliferation in the developing postnatal SVZ.

3.3.3 Overexpression of ET-1 in the neonatal mouse SVZ

Organotypic brain slices have proven to be very useful in investigating cellular and molecular processes of the brain *in vitro*, because this system preserves the main cytoarchitecture and cellular heterogeneity. While this technique was indeed very helpful in understanding how ET-1 can modulate OPC development, the preparation of the slices also resulted in reactive astrogliosis. In order to test ET-1 overexpression in the uninjured, normally developing SVZ, we therefore decided to perform neonatal electroporation of ET-1 into the lateral ventricle of postnatal day P0/P1 pups to target the radial glia cell population and their progeny. First, we generated an ET-1 overexpression plasmid using the pLVX-IRES-ZsGreen1 vector that allows the expression of ET-1 and ZsGreen in any electroporated cells in the SVZ. DNA sequencing was performed to confirm the correct ET-1 sequence. Wild-type mice were then electroporated with the ET-1 plasmid or the control pLVX-IRES-ZsGreen1 plasmid into the right lateral ventricle. Pups were collected at postnatal day 3, two days post electroporation, and examined by immunostaining. Recombined GFP^+ cells were found along the edges of the ventricle, particularly in the dorsal (d) and lateral (l) side of the ventricle. As expected, brains transfected with the ET-1 plasmid displayed higher expression of ET-1 protein in the SVZ; those $ET\text{-}1^+$ cells were also immunopositive for Sox2 (a neural stem cell marker).

Together, these results demonstrate that we successfully overexpressed ET-1 in the neonatal mice SVZ using electroporation. This *in vivo* model is a great resource for future studies addressing the functional role of ET-1 signaling in the postnatal SVZ.

3.3.4 Characterization of ET-1 expression pattern in the Piglet SVZ

Currently, rodent models are widely used in biomedical research to better understand the mechanisms underlying cell proliferation and development, brain injury and regeneration. However, the human brain differs greatly from the rodent brain; therefore,

treatments developed with rodent models do not always translate successfully to humans (Herculano-Houzel, 2009). For example, it has been shown that the human brain expresses a much larger repertoire of genes than any other species and that the human brain cell composition and activity differs from the mouse counterpart, especially in the glia contents (Zhang et al., 2016). However, studying specific molecular and cellular mechanisms regulating brain plasticity often requires direct access to and manipulation of tissue, yet these types of studies cannot be performed in humans. Therefore, there is a need of animal models that more closely resemble humans.

The piglet is a powerful model organism in which to study human brain development because it possesses a highly evolved, gyrencephalic neocortex that more closely reflects the human one (Morton et al., 2017). Importantly, the porcine SVZ resembles the human counterpart, displaying similar cell composition and laminar organization (Morton et al., 2017). Within the porcine SVZ, four distinct regions are identified: (I) a single layer of ependymal cells lining the lateral ventricle, immune-positive for the neuroepithelial marker vimentin; (II) a dense, thick layer of DCX^+, $GFAP^+$ and $Sox2^+$ cells directly adjacent to the ependyma, named ventricular SVZ; (III) the parenchyma of the SVZ containing individual DCX^+, $GFAP^+$ and $Sox2^+$ cells and (IV) most laterally, a region containing large chains of DCX^+ cells named abventricular SVZ (Costine et al., 2015; Morton et al., 2017). Therefore, we choose to use a neonatal piglet model to assess the expression of ET-1 and its receptors in the porcine postnatal SVZ at P14. ET-1 protein and its receptors are highly expressed in the piglet SVZ, particular within layers I, II and III. Cells expressing ET-1 and the receptors Ednra and Ednrb were also immunopositive for radial glia-like NSC markers, GFAP and Sox2 (Fig. 4). Interestingly, the expression of the Ednrb receptor was higher than the Ednra receptor.

Therefore, the major signaling components of the ET-1 pathway are expressed in the porcine SVZ, indicating that ET-1 signaling in the postnatal SVZ is conserved across mammals and likely also present in the developing human SVZ. Furthermore, the piglet is as a suitable animal model for future studies investigating the role of ET-1 signaling in postnatal NSC physiology.

Discussion

Here, we identified the ET-1 signaling pathway as a novel regulator of OPC proliferation in the developing postnatal SVZ. Our *ex vivo* studies showed that 100nM ET-1 treatment is able to expand the OPC pool, specifically enhancing the overall proliferative ratio of $NG2^+$ cells. In order to test ET-1 overexpression in an *in vivo* model of the developing SVZ, we successfully overexpressed ET-1 in neonatal mice using electroporation. This *in vivo* model is a great resource for future studies that will investigate the functional role of ET-1 signaling in NSC and OPC development in the SVZ. Lastly, we characterized the expression pattern of the ET-1 pathway proteins in the piglet SVZ, confirming the high expression of ET-1 and its receptor Ednrb in the SVZ of a higher order animal model with a gyrencephalic brain.

Previous work from our laboratory first highlighted the importance of ET-1 in regulating the OPC population. Gadea et al. (2009) showed that ET-1 regulates OLs development by promoting migration and inhibiting differentiation (Gadea et al., 2009). Particularly, when treated with ET-1, purified OPC cultures displayed a more migratory and less mature phenotype, concomitant with a reduction in the number of differentiated OL cells immunopositive for O1 (a marker of mature OLs). Furthermore, an overall decrease in myelin basic protein (MBP) was also observed in the same conditions. These results suggest that ET-1 regulates early developmental stages in the OL lineage by maintaining OPCs in an undifferentiated, migratory state (Gadea et al., 2009). Interestingly, no changes in proliferation were observed when purified OPC cultures were treated with ET-1 (Gadea et al., 2009). Conversely, our results obtained in organotypic slices showed that 100nM of ET-1 promoted a rapid increase in OPC proliferation in the SVZ, specifically in the $NG2^+$ population. These differing results may be due to the different experimental models. Gadea et al. analyzed ET-1 signaling in dissociated single OPCs derived from the entire rat brain at embryonic day 20 (200nM ET-1 was used in this study). Cells within these cultures are potentially deprived of endogenous molecular signaling pathways and/or cellular interactions that may affect their proliferation/differentiation. Conversely, we assessed the proliferative capacity of ET-1 specifically within the SVZ using organotypic brain slices derived from P8-10 mouse brain. This system closely resembles the *in vivo* mouse SVZ because

it maintains the main cytoarchitecture, cell population heterogeneity and endogenous signaling pathways. Furthermore, ET-1's role in regulating OPC proliferation may be specific to the SVZ OPC population, and cultures derived from the entire brain may mask its proliferative role.

Interestingly, the mitogen activity of ET-1 has been previously described in other glial cell types. Berti and Mattera (2001) showed that ET-1 treatment in Schwann cells promoted proliferation and cell cycle progression by increasing the percentage of cells in S and G2/M phases, with a concomitant decrease in the percent of cells arrested in G0/G1. In another study from the Gallo laboratory, Gadea et al. found that cultured cortical astrocytes exposed to 100nM ET-1 displayed increased proliferation, as determined by a higher number of $Ki67^+$ and $GFAP^+$ cells (Gadea et al., 2008). Furthermore, when pure astrocyte cultures were treated with ET-1, there was an increase in BrdU incorporation and in the percentage of cyclin D1 and D3, which have a pivotal role in the G1/S phase transition (Koyama et al., 2004). Interestingly, these studies used the same concentration of ET-1 that we used in our study (100nM), indicating that ET-1 may exhibit different cellular responses depending on dosage. Overall, these findings are consistent with our results showing that ET-1 induced high levels of proliferation in the SVZ OPC population.

Cyclin D proteins are regulators of the G1/S phase transition and play an important role in cell cycle progression (Ortega et al., 2002). The regulation of cyclin D transcription is mediated by the transcription factors *c-fos* and *c-jun,* which are the major targets of mitogen-activated signal transduction pathways (Shaulian and Karin, 2001). *C-fos* and *c-jun* physically interact and form homodimers and heterodimers, resulting in the formation of the activating protein 1 (AP-1) complex (Shaulian and Karin, 2001). *C-fos* and *c-jun* recognize specific AP-1 binding sites on the promoter region of the cyclin's DNA sequence, thus inducing transcription of these genes (Shaulian and Karin, 2001). The stimulation of *c-fos* and *c-jun* activity is likely induced by the upstream mitogen activated protein kinase (MAPK) cascade. MAPK is a family of Ser/Thr protein kinases that convert a wide range of extracellular stimuli into cellular responses mediating mitosis, survival, apoptosis and differentiation. Conventional MAPK comprise the extracellular signal-activated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38

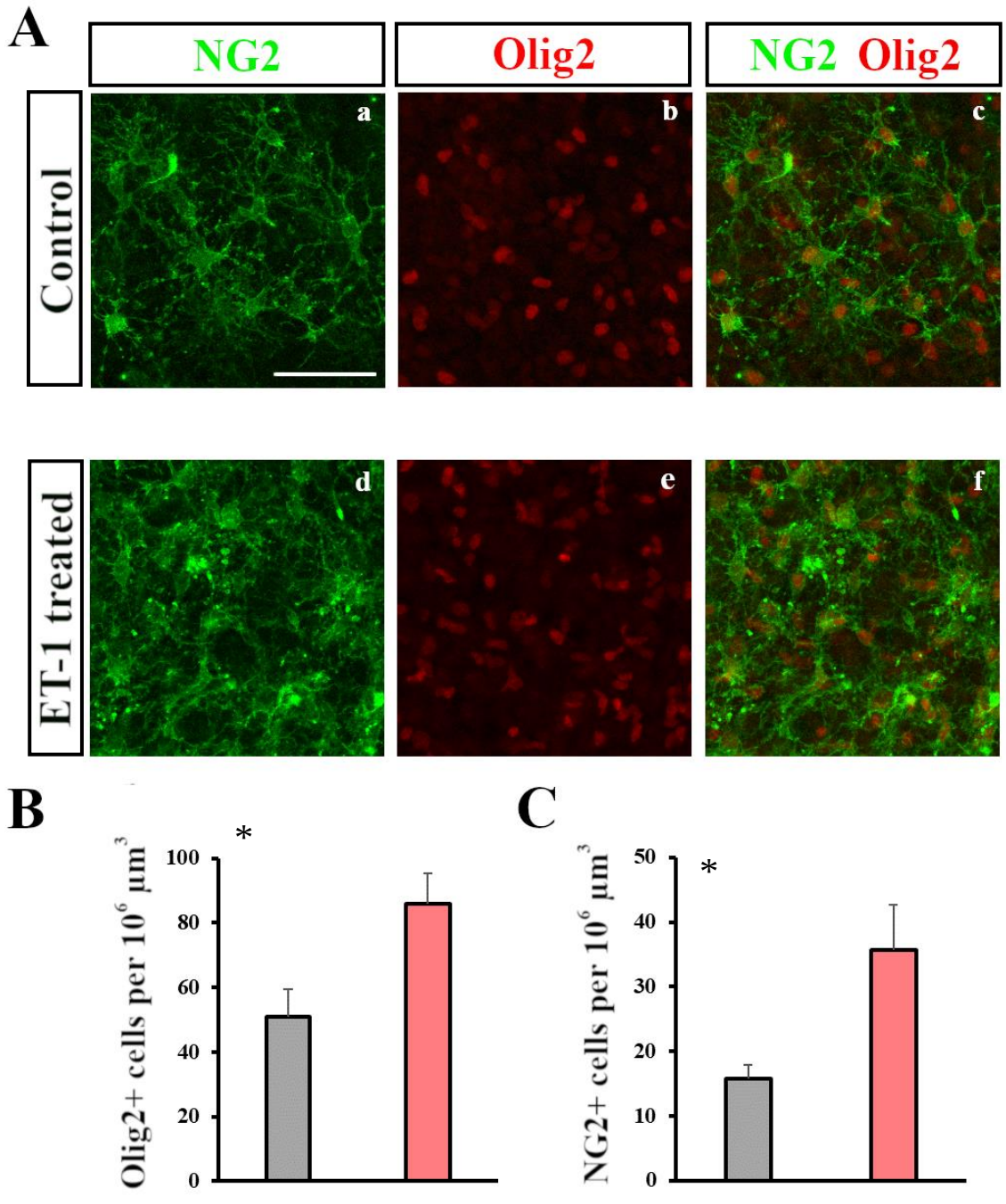
isoforms (α, β, γ, and δ), and ERK5 (Chen et al., 2001). Gadea et al. (2008) found that, in cultured cortical astrocytes, ET-1 activated both JNK and p38 pathways of the MAPK cascade, and induced *c-jun* expression at the mRNA level and protein levels. ET-1-induced *c-jun* phosphorylation also promoted a reactive phenotype in astrocytes by enhancing both proliferation and GFAP expression (Gadea et al., 2008). Interestingly, the effects of ET-1 in mediating *c-jun* activation and astrocytes proliferation were prevented by the Ednrb receptor antagonist, but not by the Ednra receptor antagonist. This suggests that the mitogen effects of ET-1 are selectively dependent on the Ednrb receptor. The regulation of *c-jun* transcription factor by ET-1 also involved the activation of a third MAPK signaling cascade, the ERK-dependent pathway. Previous studies reported that ET-1 activated the ERK pathway in astrocytes (Cazaubon et al., 1997) and caused phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in Schwann cells (Tabernero et al., 1998). Activation of the ERK signaling pathway promotes *c-fos* transcription by regulating two elements in the *c-fos* promoter, the cAMP response element (CRE) and the serum response element (SRE) (Karin et al., 1997). Two upstream transcription factors bind to CRE: CREB and the activating transcription factor-1 (ATF-1). Schinelli et al. (2001) found that in cortical astrocytes, ET-1 rapidly induced CREB and ATF1 by activating the ERK pathway (Schinelli et al., 2001). Particularly, these effects were specifically mediated by activation of Ednrb (Schinelli et al., 2001).

Therefore, it is likely that cell cycle regulators, such as cyclins D, might be downstream targets of the MAPK-dependent pathways activated by ET-1 to promote cell cycle progression and proliferation. Recently, the ability of ET-1 to activate the MAPK pathway by increasing ERK1/2 protein levels has been accounted to the activation of the EGF receptor (EGFR) (Harun-Or-Rashid et al., 2016). Muller cells – the major glial cells of the retina – highly express both ET-1 and its receptor Ednrb (Harun-Or-Rashid et al., 2016). The stimulation of the ET-1 receptor Ednrb in both chicken and human Muller cells promoted an increase in ERK1/2 phosphorylation via the transactivation of the EGFR. Blocking of the EGFR kinase reduced the activation of ERK1/2 induced by Ednrb stimulation thus confirming a role of EGFR in mediating the effects of ET-1 signaling in the activation of the MAPK pathway (Harun-Or-Rashid et al., 2016). NG2-expressing OPCs in the postnatal SVZ express high levels of EGFR and

its ligand EGF (Aguirre et al., 2005). Although these signaling pathways have been largely characterized in other types of glial cells (i.e. astrocytes and retinal glial cells), the same mechanisms may explain the effect of ET-1-induced proliferation in the OPC population within the SVZ.

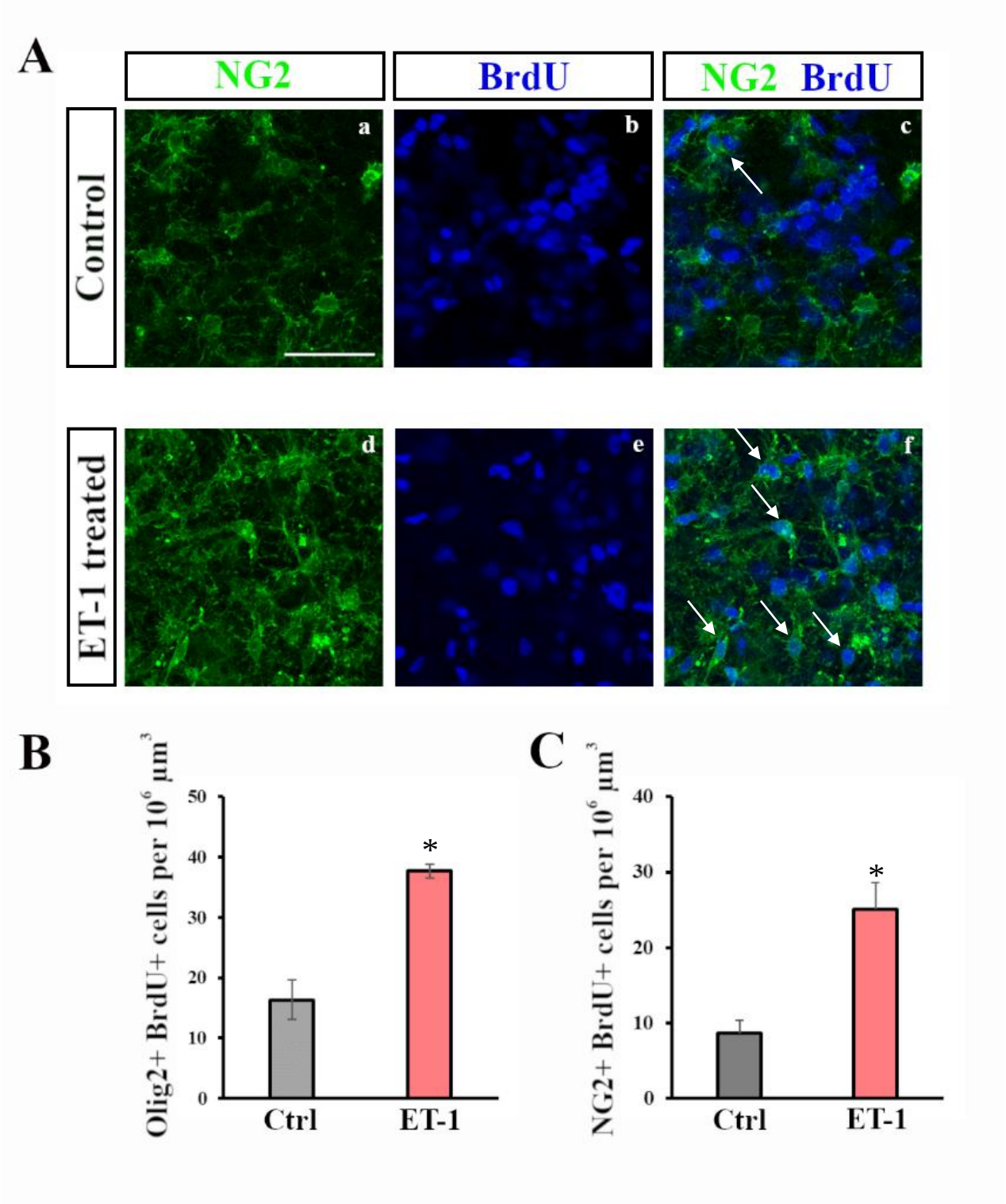
Processes controlling tissue generation during development often control its regeneration after injuries: therefore, ET-1 may be implicated also in regenerative processes that occur after brain injury or disease. Our lab recently identified ET-1 as a key regulator of OPC differentiation and regeneration following demyelination of the adult mouse corpus callosum (Hammond et al., 2014). Repairing of demyelinated plaques is normally carried out by endogenous OPCs that migrate to the lesion site, proliferate and differentiate into mature OLs to produce new myelin sheaths (Franklin and Ffrench-Constant, 2008). Hammond at al. (2014) found that ET-1 negatively regulated the differentiation of OPCs into OLs following demyelination, thus inhibiting the rate of remyelination (Hammond et al., 2014). Specifically, ET-1 induced upregulation of Jagged1 in reactive astrocytes, which in turn activated Notch signaling in OPCs to prevent differentiation and remyelination (Hammond et al., 2014). Importantly, ET-1 was also found to be highly upregulated by reactive astrocytes in demyelinated subcortical white matter regions in human MS tissue, indicating that this signaling mechanism may be conserved in human neurodegenerative diseases (Hammond et al., 2014). It remains unknown whether ET-1 is also upregulated in the SVZ following white matter demyelination. Interestingly, the production of new OPCs from the SVZ increases following demyelination (Aguirre et al., 2007); therefore, it is possible that ET-1 also regulates expansion of SVZ OPCs in the adult brain after injury.

In conclusion, this study reports a novel role for ET-1 signaling as a regulator of OPC proliferation in the developing SVZ. Understanding the mechanisms that control early developmental stages in the oligodendrocyte lineage is critically important to facilitate the development of pharmacological interventions in brain pathologies that involve the ET-1 system. By using our *in vivo* model of ET-1 overexpression, we further want to investigate the functional role of ET-1 signaling in the regulation of OPC migration and differentiation. Furthermore, future work will be aimed at addressing the importance of ET-1 signaling in the piglet SVZ by pharmacologically inhibiting the Ednra and Ednrb receptors.



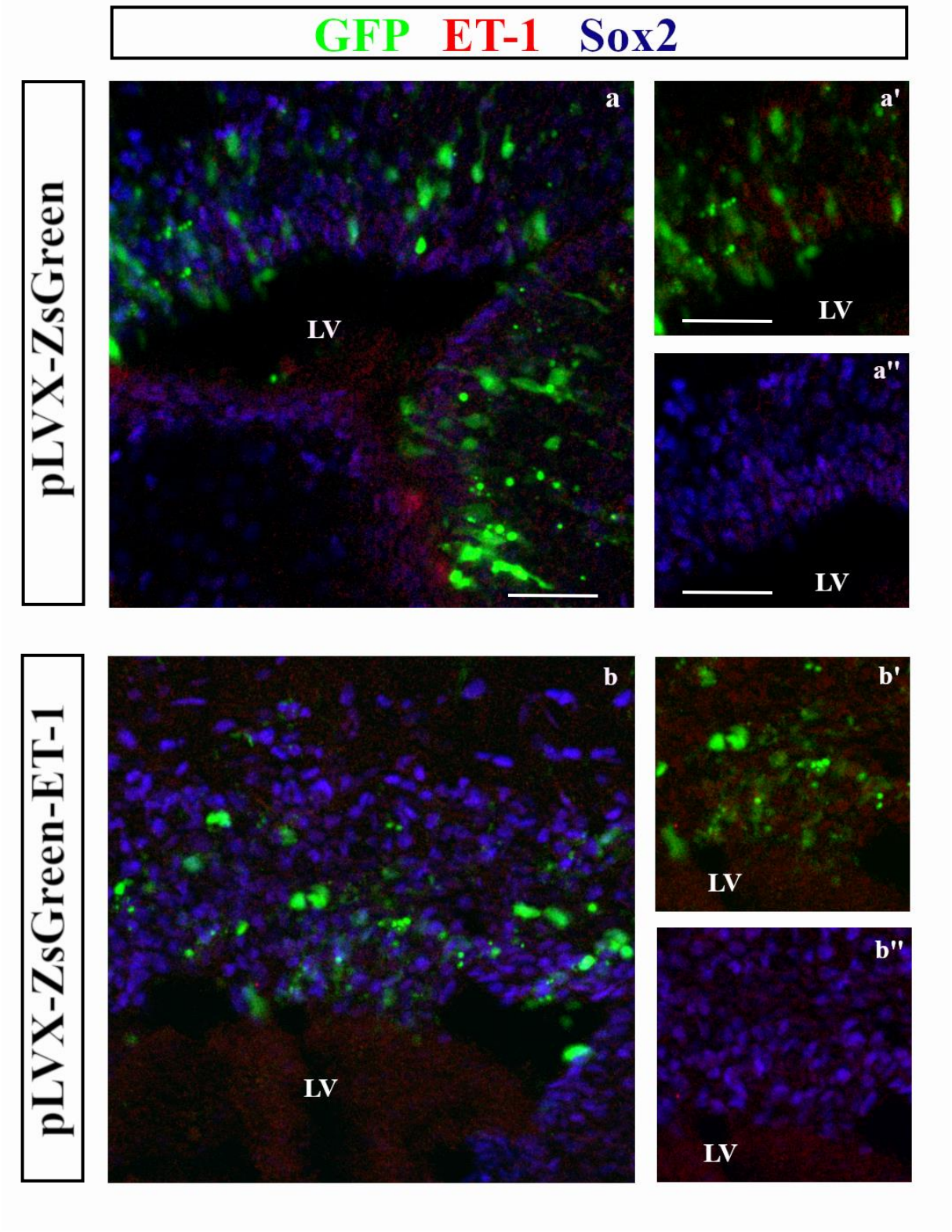
Exogenous ET-1 increases the OPC population. Confocal images of organotypic brain slices from P8/P10 wild type mice cultured for 72 hours in the presence or absence of 100nM ET-1 (A). The OPC population was assessed by immunostaining slices with antibodies against NG2 (a, d) and Olig2 (b, e). Double immunostaining of coronal brain slices showing increased number of $NG2^+$ cells in the ET-1 treated group (c, f). Scale bar, 50µm. Quantification of $Olig2^+$ (B) and $NG2^+$ (C) OPCs in the control

group and following ET-1 treatment. ET-1 significantly increased the total number of $Olig2^+$ and $NG2^+$ cells compared to the control group. Data are expressed as mean percentage ± s.e.m. of five independent experiments. Significantly different from control (Student's t-test,*$p<0.001$).



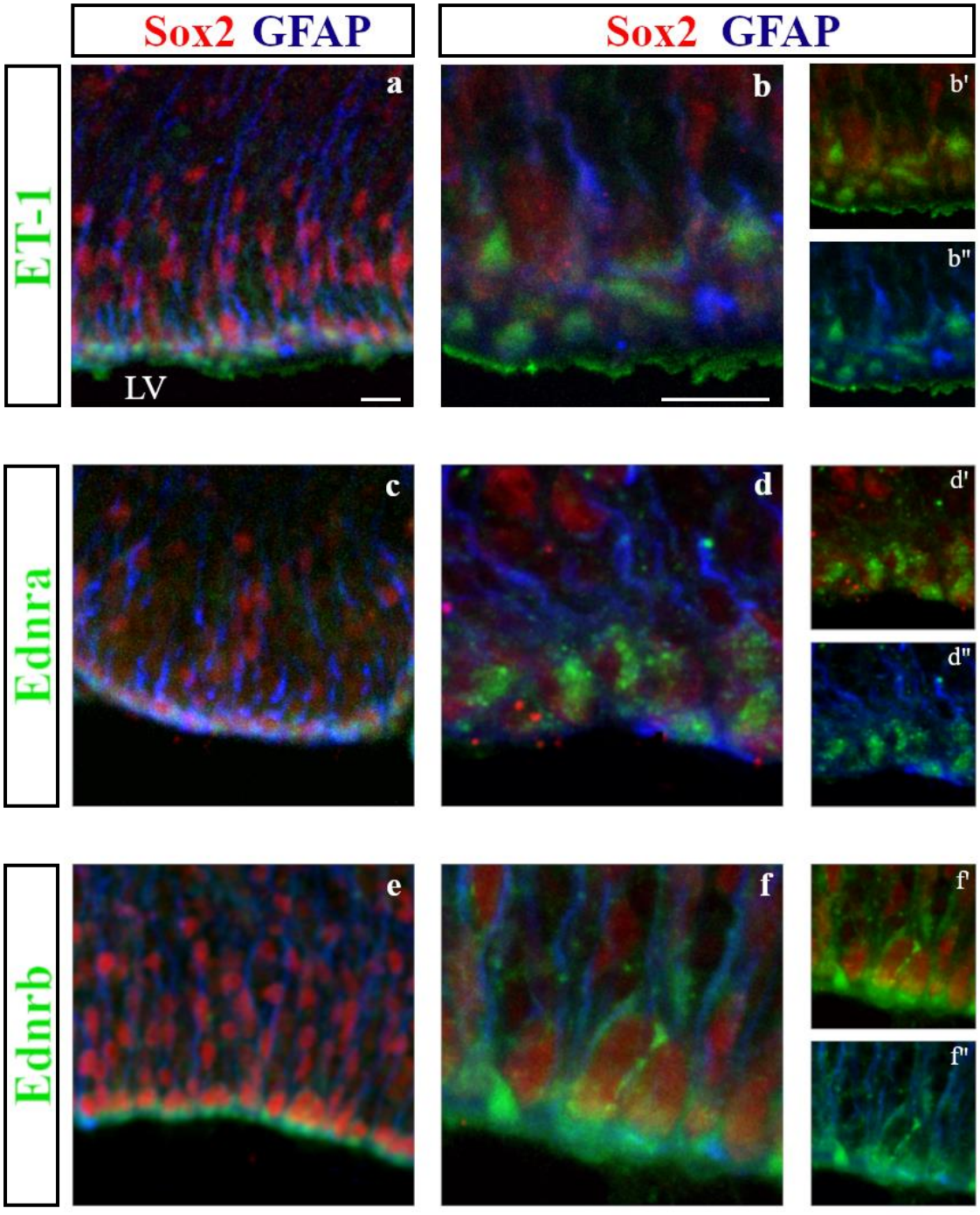
ET-1 promotes OPC proliferation. Confocal images of organotypic brain slices from P8/P10 wild type mice cultured for 72 hours in the presence or absence of 100nM ET-1 (A). Proliferation in the OPC population was assessed by immunostaining slices with antibodies against NG2 (a, d) and BrdU (b, e). Double immunostaining of coronal brain slices showing increased number of $BrdU^{+}/NG2^{+}$ cells in the ET-1 treated group (c, f).

The arrows point at examples of $NG2^+$ cells immunopositive for BrdU. Scale bar, 50μm. Quantification of $Olig2^+/BrdU^+$ cells (B) and $NG2^+/BrdU^+$ OPCs (C) in the control condition and following ET-1 treatment. ET-1 significantly increased the total number of $Olig2^+$ cells, specifically enhancing the proliferative ratio of $NG2^+$ OPCs compared to the control group. Data are expressed as mean percentage ± s.e.m. of five independent experiments. Significantly different from control (Student's t-test, *$p<0.001$).



Overexpression of ET-1 in the neonatal mouse SVZ. Immunostaining of GFP, ET-1, and Sox2 in the dorsal and lateral region of the ventricle in P3 mice after postnatal electroporation of pLVX-IRES-ZsGreen (control) or pLVX-ET-1-IRES-ZsGreen. The

high number of GFP^+ cells confirmed the efficiency of the electroporation. Few cells were immunopositive for ET-1 in the control condition while a higher amount was found in the ET-1 recombinant mice. Scale bar, 50μm.



Characterization of ET-1 expression pattern in the postnatal piglet SVZ. Immunostaining of ET-1, Sox2, and GFAP in the dorsal lateral region of the P14 piglet SVZ (a). Higher magnification image of the dashed white box in a (b). Smaller panels showing colocalization between ET-1 and Sox2 (b') and ET-1 and GFAP (b''). Immunostaining of Ednra, Sox2, and GFAP in the dorsal lateral region of the piglet

SVZ (c). Higher magnification image of the dashed white box in c (d). Smaller panels showing colocalization between Ednra and Sox2 (d') and Ednra and GFAP (d''). Immunostaining of Ednrb, Sox2, and GFAP in the dorsal lateral region of the piglet SVZ (e). Higher magnification image of the dashed white box in e (f). Smaller panels showing colocalization between Ednrb and Sox2 (f') and Ednrb and GFAP (f''). Scale bar,10μm.

4 Conclusions

Perinatal brain injury is the major established cause of neurological morbidity in term infants yet there are no approved pharmacological treatments able to improve outcomes. To guide strategies that could prevent the injury and/or lead to functional brain recovery it is important to gain more insight on the mechanisms underlying neurodegeneration, neuroprotection, and regeneration, keeping in mind that processes controlling tissue generation in the developing period often control its regeneration. Therefore, the identification of molecular signaling pathways that control central nervous system development are likely to provide critical insights into brain regeneration following injuries (Gallo and Deneen, 2014).

This thesis addressed two different aspects of this problem.

In the first part, we analyzed the neuroprotective effect of Melatonin following HI brain injury to understand the mechanisms underlying tissue protection and/or regeneration. The results highlighted the role of SIRT1 in modulating the early phase of the injury. Pharmacological doses of Melatonin administered five minutes after the end of the HI insult, indeed, strongly and rapidly modulated SIRT1 activity. Preserving SIRT1 expression resulted in decreased necrosis and apoptosis and increased autophagy. Overall, these results demonstrated that Melatonin is able to prevent the initiation of multiple signaling cascades that lead to severe brain damage and that SIRT1 can be a key player in this effect.

In the second part of this thesis, we analyzed the role of ET-1 in OPC development in the developing SVZ to understand if ET-1 can regulate the processes that drive physiological development and tissue generation. Our *ex vivo* studies showed that exogenous ET-1 treatment is able to expand the OPC pool, specifically increasing the overall proliferative ratio of $NG2^+$ cells. The expression pattern of the ET-1 pathway proteins were also characterized in the piglet SVZ, confirming the high expression of ET-1 and its receptor Ednrb in the SVZ of a higher order animal model with a gyrencephalic brain. We also successfully overexpressed ET-1 in the SVZ of neonatal mice using electroporation and this *in vivo* model of ET-1 overexpression will be useful for further studies addressing the functional role of this protein in NSC and OPC development in the SVZ.

In premature infants, hypoxic-ischemic damage is often associated with white matter brain injury due to maturation-dependent vulnerability of OL lineage cells during the perinatal period. Following HI, both the loss of OLs and failure of OPCs to differentiate induce increased SVZ progenitor cell proliferation (Jablonska et al., 2012). These regenerative processes are likely guided by the same molecular pathways that drive normal development. Whether ET-1 plays a role in the SVZ regenerative response after HI injury remains unknown, and a topic for future investigation. Interestingly, SIRT1 is highly expressed by OL lineage cells within the SVZ (Rafalski et al., 2013) and it might act together with ET-1 in the regulation of OPC development following HI brain injury. Therefore, further studies should be performed to address the role of SIRT1 and ET-1 in regenerative processes following brain injury.

5 Acknowledgments

Firstly, I would like to express my sincere gratitude to my advisors Prof. Walter Balduini and Vittorio Gallo for their continuous support during my PhD study and related research. I am grateful to Walter Balduini for giving me the opportunity to join his laboratory and for being a mentor during my first year of PhD. Thank you for understanding my needs and for supporting my decision of leaving the country to move to the United States.

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