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Melatonin modulates the Notch1 signaling pathway and Sirt3 in the hippocampus of hypoxic-ischemic neonatal rats

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The Notch1 signaling pathway plays a crucial role in the development of the central nervous system, governing pivotal functional activities in the brain, such as neurogenesis. Sirt3 is instrumental in managing mitochondrial homeostasis and is essential to cell survival. Dysregulation of these signaling pathways is implicated in the pathogenesis of a wide range of diseases, including neurodegenerative disorders such as stroke. We have previously shown that melatonin significantly improved the perinatal brain damage caused by hypoxia-ischemia (HI) through the activation of several protective mechanisms such as restoring mitochondria status and increasing the hippocampal cell proliferation. This study assessed whether melatonin affects the Notch1 signaling pathway and Sirt3 after neonatal HI. Results show that HI significantly increased Notch1 expression both in hippocampal neurons and glial cells as well as the expression of the key proteins of the pathway NICD, HES1, and c-Myc. Melatonin significantly prevented the Notch1 signaling pathway activation induced by HI, maintaining NICD and HES1 expression to control levels. In the same neurons, melatonin also prevents the Sirt3 depletion caused by HI. In summary, this study provides new insights into the effects of melatonin on the Notch1 signaling pathway and Sirt3 in in vivo neonatal brain ischemia. We suggest that the rapid modulation of the Notch1 signaling pathway and Sirt3 induced by melatonin may support neuronal survival during ischemia.

Keywords Notch, Sirtuin 3, Neonatal brain ischemia, Neuroprotection

Abbreviations

ANOVA	Analysis of variance
HI	Hypoxia-ischemia
PN	Postnatal
GFAP	Glial fibrillary acidic protein
MAP2	Microtubule-associated protein 2
Sirt3	Sirtuin 3
NICD	Notch1 intracellular domain
HES1	Hairy and enhancer of split 1

The Notch1 signaling pathway, which is remarkably preserved throughout evolution, plays a crucial role in the development of the central nervous system (CNS). During brain development, Notch signaling contributes to maintaining neural progenitor cells in an undifferentiated state, which can result in the inhibition of neurogenesis[1]. In the mature brain, instead, Notch signaling has an impact on synaptic plasticity, as well as learning and memory processes[2]. It is known that the Notch signaling pathway is instrumental in managing the functional activities of the brain, not only under normal physiological conditions but also in disease states such as ischemic stroke and Alzheimer's disease. It plays a key role in processes such as neuronal differentiation, glial formation, and microglia activation[3]. The Notch1 receptor protein, the core element of Notch1 signaling, is a single-pass transmembrane protein that remains inactive in the absence of ligand binding. Activation through ligand interaction triggers a series of proteolytic cleavages in Notch family receptors, leading to the release of the Notch1 intracellular domain (NICD). Once freed, the NICD enters the nucleus and stimulates the transcription of Notch1 target genes associated with cell-fate determination (HES1, HEY), proliferation (Myc), and cell growth

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arrest (p21). HES1 (Hairy-Enhancer of Split 1), in particular, plays a regulatory role in neuronal function and structure[4].

Sirtuin3 (Sirt3), a key member of the sirtuin gene family, is a histone deacetylase primarily found in the mitochondria that plays a crucial role in maintaining mitochondrial homeostasis[5]. Due to its involvement in various biological functions, Sirt3 plays a pivotal role in the development and progression of several diseases including neurodegenerative disorders such as stroke[6]. Specifically, Sirt3 is involved in a broad range of biological processes, such as aging, cell metabolism, inflammation, and apoptosis, which are key to stroke pathophysiology[7]. Furthermore, it was found that Sirt3 regulates the permeability of the human blood-brain barrier in response to ischemia[8], whereas its deficiency exacerbates ischemia-reperfusion injury[9]. The progression of brain damage typically involves a dysfunction of the mitochondria, leading to a variety of pathophysiological events, including inflammation, oxidative stress, and mitophagy[10]. Given the role of Sirt3 in maintaining mitochondrial homeostasis and metabolism, as well as reducing mitochondrial damage[5], investigating how Sirt3 operates could be instrumental in enhancing our understanding of brain injury development.

Recently reports highlighted a connection between the Notch signaling pathway and Sirt3 in several stress conditions. It was found that gastrodin regulated both the Notch1 signaling pathway and Sirt3 in activated microglia in neonatal rats subjected to cerebral hypoxic-ischemia (HI), as well as in activated BV-2 microglia[11]. In human gastric cancer cells, Sirt3 inhibited cell proliferation via the down-regulation of Notch1[12]. When the Notch1 signaling pathway was interfered with, it resulted in the inhibition of Sirt3 expression, suggesting that Sirt3 is a downstream gene of the Notch1 signaling pathway. Furthermore, Sirt3 has been found to promote autophagy in HK-2 human proximal tubular epithelial cells via the inhibition of Notch1/Hes1 signaling[13].

Melatonin (N-acetyl-5methoxytryptamine), a versatile and ubiquitous molecule well known as a potent indirect antioxidant and direct free radical scavenger[14], significantly improved the perinatal brain damage caused by HI[15,16] through activation of several protective mechanisms such as antioxidant, anti-inflammatory and antiapoptotic effects[17], delaying necrosis supporting autophagy, and reducing endoplasmic reticulum (ER) stress[18,19]. We recently found that treatment with melatonin immediately after neonatal HI modulated cell cycle dynamics and increased cell proliferation in the hippocampus[20]. Because of the role of the Notch1 pathway in neurogenesis, here we assessed its modulation by HI and melatonin. In addition, since the protective effects of melatonin are strictly interconnected to the restoration of the altered mitochondria status caused by HI[21] and there is a connection between the Notch signaling pathway and Sirt3 in stress conditions, we also assessed SIRT3 modulation.

Materials and methods

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 EU Directive 63/2010; Italian D.L. 26/14; research protocol authorization 582/2020-PR), were approved by the Animal Care Committee of the University of Urbino Carlo Bo, and reported in compliance with the ARRIVE guidelines. Pregnant Sprague-Dawley (Charles River) 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, pup rats were anesthetized with 5% isoflurane in O_2, and 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 3 h 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). Once the HI procedure was finished (and melatonin treatment or vehicle received), pups were returned to their dams until the experimental procedures were performed 1 h, 24–48 h after HI.

Drug administration

Melatonin (Sigma-Aldrich, Milan, Italy, M5250) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Milan, Italy, D5879) and diluted in normal saline solution to a final concentration of 5% DMSO (vehicle). The melatonin solution was injected intraperitoneally into pup rats 5 min after HI at the dose of 15 mg/kg. The first injection was performed 5 min after the ischemic-hypoxic procedure and repeated after 24 h and 48 h. Control animals received the same volume of the vehicle. We used this schedule of drug administration based on previous experiments that showed consistent and long-lasting neuroprotection with this dose and under these administration conditions[15,22,23].

Western blot analysis

Pups were anesthetized and euthanized by decapitation 1, 24–48 h after HI. Brains were rapidly removed, and hippocampal homogenates prepared as previously described[19]. The cytosolic, mitochondrial, and nuclear fractions were prepared from the hippocampus according to Nijboer et al.[24]. After mixing with sodium dodecyl sulfate gel-loading buffer and heating 4 min at 95 °C, samples (50 µg protein) were electrophoresed onto sodium dodecyl sulfate-polyacrylamide gel and proteins were transferred to a PVDF membrane. ColorBurst™ electrophoresis marker (3 µL/gel, Sigma, C1992) was used for qualitative molecular mass determinations and 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-Notch1 (1:1000, polyclonal; Cell Signaling Technology, #3608), anti-Cleaved Notch1 (NICD) (1:500, polyclonal; Cell Signaling Technology, #4147), anti-Hes1 (1:1000, polyclonal; Cell Signaling Technology, #11988), anti-c-Myc (1:1000, polyclonal; Cell Signaling Technology, #5605), anti-Sirt3 (1:1000, polyclonal; Cell Signaling Technology, #2627). Monoclonal antibodies against β-actin (1:4000, Santa Cruz Biotechnology, sc-8432) or against Hsp60 (1:1000, Santa Cruz Biotechnology, sc-13115) or a polyclonal antibody against Lamin B (1:1000,

Santa Cruz Biotechnology, sc-6216) were used as loading control and for data normalization. Densitometric analyses were performed using the NIH-Image J 1.45 software (https://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD, USA). Data were expressed as % of Control.

Immunohistochemistry

Pups were deeply anesthetized with 5% isoflurane in O_2 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 (Ino, 2003). 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-Notch1 and anti-NICD (1:1000 and 1:40 respectively, polyclonal; Cell Signaling Technology, #4147), anti-glial fibrillary acidic protein (GFAP, 1:300, monoclonal; Boehringer Mannheim GmbH, 813 369) or anti-neuron-specific nuclear protein (NeuN, 1:500; monoclonal; Millipore, MAB377), anti-microtubule associated protein 2 (MAP2, 1:500; rabbit, polyclonal; Millipore, AB5622). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, or Texas Red goat anti-mouse IgG, or Alexa Fluor goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology, sc-2359, sc-3797, sc-516248, respectively) were used to demonstrate immunofluorescence reactivity. The specificity of the reactions was evaluated in some slices by omitting the primary antibody from the incubation medium. Images were acquired on a Leica TCS SP5 II confocal microscope (Leica Microsystem, Wetzlar, Germany).

Data analysis

Image J 1.45 software (https://imagej.nih.gov/ij/) was used for all the quantitative image analysis. Statistical analyses were performed by one-way ANOVA using the GraphPad Prism 9.0 Computer program (GraphPad software, San Diego, CA, USA). The Bartlett's test was used to determine data homogeneity. 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

Cerebral hypoxia-ischemia increases Notch1 expression in neuronal and glial cells of neonatal hippocampus

To examine whether neonatal HI affects Notch1, we studied the hippocampal protein expression at different time points after injury. Western blot analysis revealed that Notch1 expression was significantly increased 1 h after HI compared to Ctrl condition, further increased after 24 h whereas returned to control levels after 48 h (Fig. 1A). To investigate the cell types in which Notch1 was expressed, double labelling immunohistochemical experiments were performed. Results showed that Notch1 was expressed in both neuronal and glial cells of ischemic hippocampus, as revealed by the strong co-localization of Notch1 with both the neuronal marker MAP2 and the astrocyte-specific marker GFAP (Fig. 1B).

Melatonin modulates Notch1 signaling pathway in ischemic neonatal hippocampus

Since Notch1 expression was already significantly affected 1 h after the HI insult, we decided to study the effect of melatonin on the Notch1 signaling pathway at this time because we were interested in the early effects of melatonin[19]. Western blot analyses confirmed that Notch1 hippocampal expression significantly increased in the early phase of ischemic brain damage development, i.e.1 h, and showed that melatonin treatment further increased Notch1 expression in the hippocampus of ischemic animals ($p \leq 0.05$ vs. Ctrl; Fig. 2A). To better analyze the effects of HI and melatonin on Notch1 signaling pathway, we analyzed the expression of NICD - Notch Intracellular Domain -, a key protein of the pathway, Hes1, a downstream effector of Notch1, and c-Myc, a Notch target gene[25]. Immunoblot analysis showed that NICD expression was significantly increased in the ischemic hippocampus 1 h after the injury ($p \leq 0.001$ vs. Ctrl; Fig. 2B); moreover, immunofluorescence results also demonstrated that NICD is significantly expressed in neurons of the ischemic hippocampus as revealed by the marked co-localization between NICD and NeuN in the hippocampal CA1 area (Fig. 2C). Melatonin, however, maintained NICD expression to control values (Fig. 2B). HES1 expression was significantly increased 1 h after HI ($p \leq 0.05$ vs. Ctrl) while melatonin administration maintained its expression to control levels (Fig. 3A). c-Myc expression was significantly reduced 1 h after HI ($p \leq 0.05$ vs. Ctrl) while melatonin restored its expression to values higher than the control group (Fig. 3B).

Melatonin preserves Sirt3 expression in neuronal cells of ischemic neonatal hippocampus

Sirt3, as reported in several studies, resided in mitochondria[26]; then, we firstly confirmed the deacetylase mitochondrial localization also in our experimental conditions. Immunoblot analysis performed in cytosolic, nuclear and mitochondrial fractions obtained from hippocampus of neonatal rats demonstrated that Sirt3 expression was not detected in the nucleus and cytosol (Fig. 4A,B), whereas it was highly expressed in the mitochondria (Fig. 4C). Compared to the control group, 1 h after HI, the Sirt3 expression was significantly reduced in the hippocampus of ischemic animals (Fig. 4C; $p \leq 0.001$). Melatonin did not affect Sirt3 expression in Ctrl animals nor in the contralateral hippocampus of ischemic animals, but completely restored its reduced expression in the lesioned hippocampus (Fig. 4C). Double-staining of Sirt3 with different antibodies against cell-type-specific antigens revealed a strong co-localization of Sirt3 and NeuN within the CA1 region of the hippocampus (Fig. 5A, panel f) and, in a lower amount, within the deep layers of the cerebral cortex of HI + Mel animals (Fig. 5A, panel c). Co-labeling with GFAP, instead, showed few Sirt3/GFAP-positive cells in the cerebral cortex (Fig. 5B, panel i), as well as in the hippocampus of ischemic animals treated with melatonin (Fig. 5B, panel n), indicating that melatonin mainly preserved Sirt3 expression in hippocampal neurons (Fig. 5A, panels d, e, f). Interestingly, the double-labeling immunohistochemical experiments reported in Fig. 5C also show a

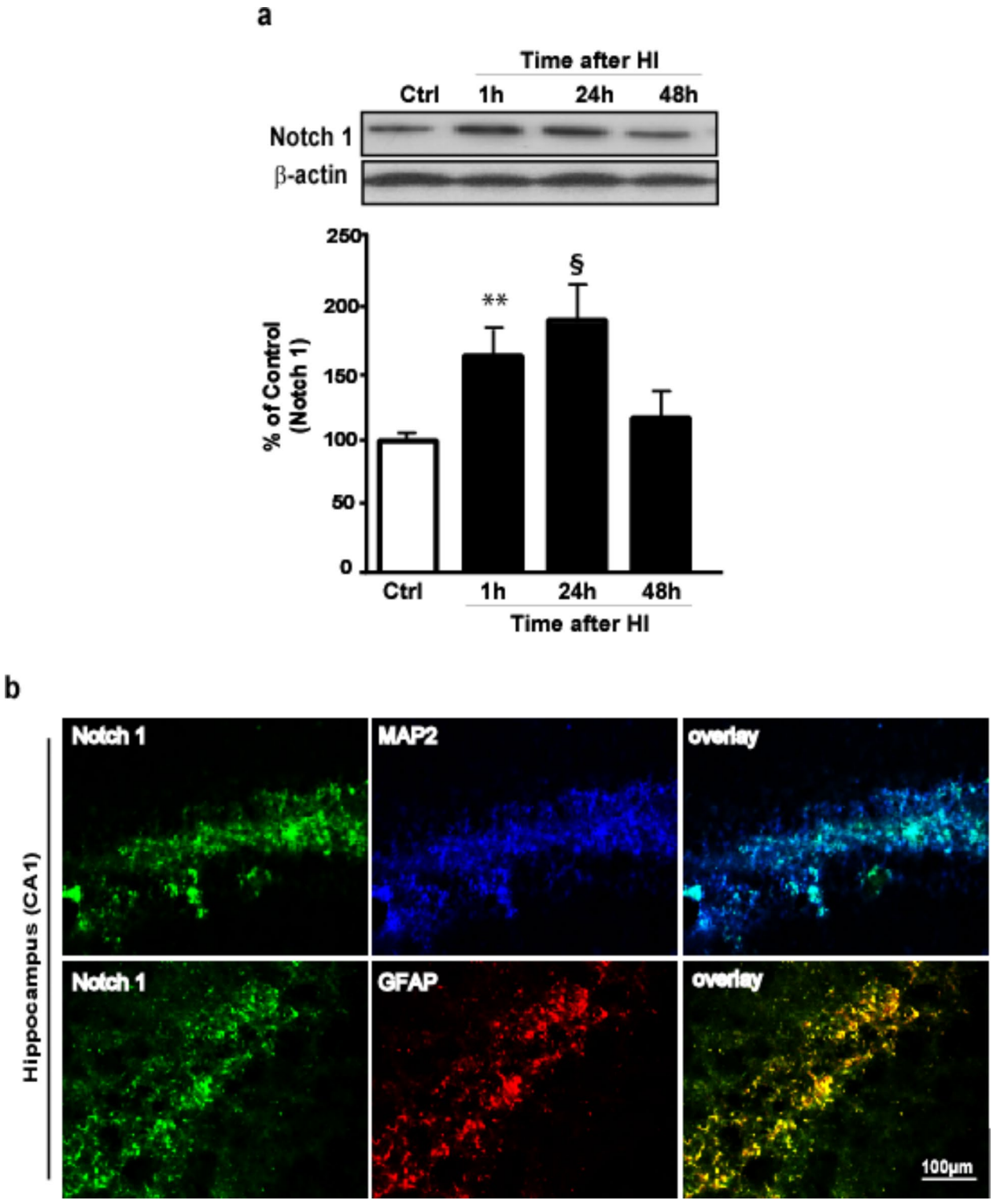

Fig. 1. Neonatal hypoxia-ischemia increases Notch expression in neuronal and glial cells of the hippocampus. (**a**) Representative blots and quantitative evaluation of Notch expression in the hippocampus of Control (Ctrl) and hypoxic ischemic animals (HI) sacrificed 1 h, 24 h and 48 h after HI. Data are expressed as % of Control (mean ± SE, $N = 5$/group). β-actin was run as internal standard. $^{**}p < 0.01$, $^{§}p < 0.001$ vs. Ctrl; One-way ANOVA followed by Newman–Keuls multiple comparison test. (**b**) Representative photomicrographs showing cells labelled with Notch (green), the glial marker GFAP (red) and the neuronal marker MAP2 (blue) in the injured hippocampus of ischemic animals sacrificed 1 h after HI. The overlay images show the co-localization of Notch and MAP2 (light blue) and of Neun and GFAP (yellow).

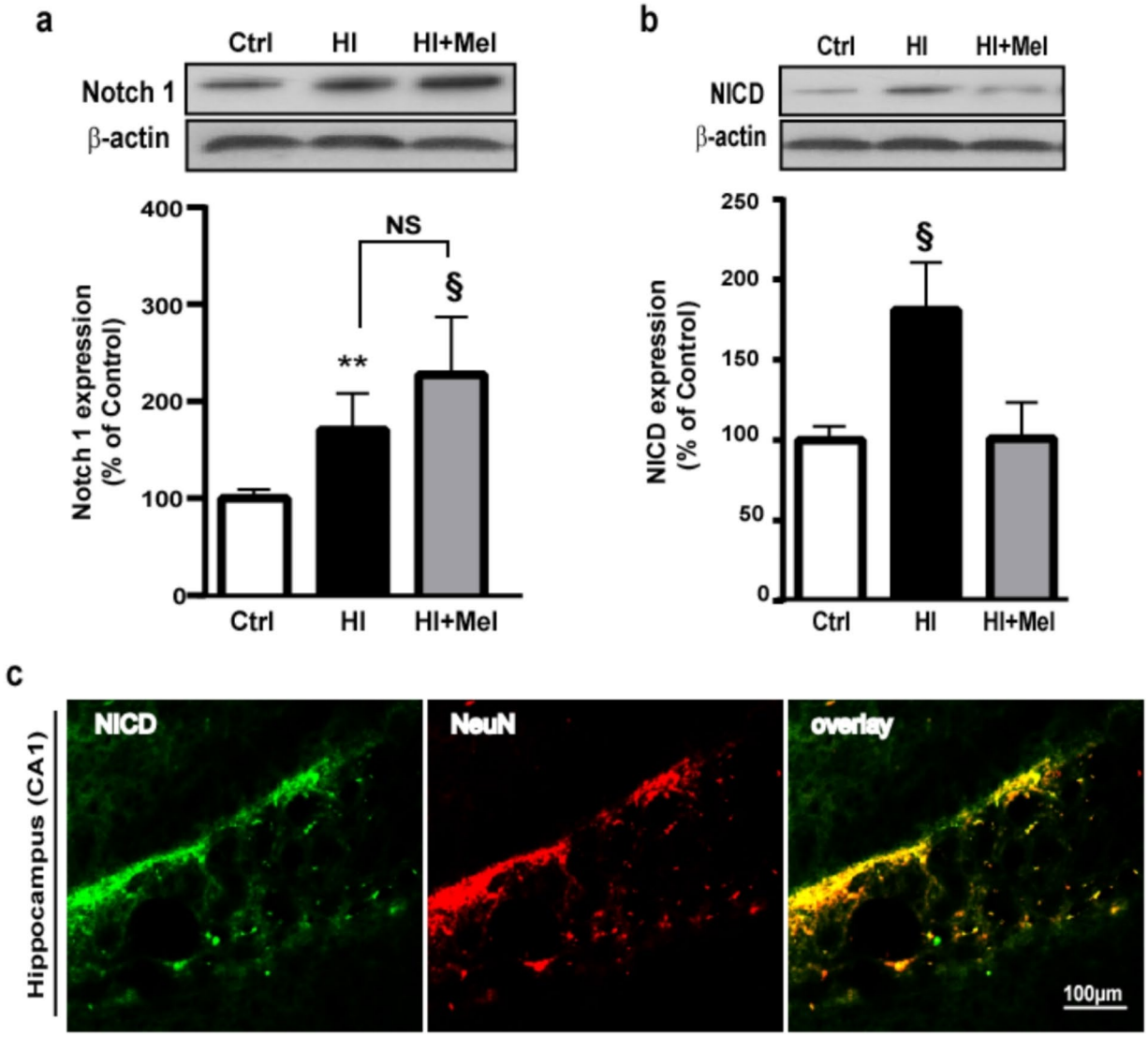

Fig. 2. Melatonin reduces the NICD expression induced by neonatal hypoxia-ischemia in hippocampal neurons. (**a**) Representative blots and quantitative evaluation of Notch 1 and Cleaved Notch1 (NICD; **b**) expression in the hippocampus of Control (Ctrl), hypoxic-ischemic (HI) and melatonin-treated hypoxic-ischemic (HI + Mel) animals sacrificed 1 h after HI. Data are expressed as % of Control (mean ± SE, $N = 5$/group). β-actin was run as internal standard. $^{**}p < 0.01$, $^{§}p < 0.001$ vs. Ctrl; NS, not significantly different; One-way ANOVA followed by Newman–Keuls multiple comparison test. (**c**) Representative photomicrographs showing cells labelled with NICD (green) and the neuronal marker NeuN (red) in the injured hippocampus of ischemic animals sacrificed 1 h after HI. The overlay shows the co-localization of NICD and NeuN (yellow).

clear colocalization between Sirt3 and Notch1, indicating that both proteins are expressed in the same neurons (Fig. 5C).

Discussion

The Notch signaling pathway is iteratively activated to regulate cell fates, cell proliferation, and cell death during the development process[27]. The pathway's crucial roles have been identified in maintaining the brain's physiological functions and neurodevelopmental disorders. Indeed, the Notch signaling pathway negatively affects both in vivo and in vitro hypoxic-ischemic brain damage[28]. Herein, we found increased Notch1 expression in the hippocampus of neonatal rats after HI in both neurons and glial cells. We also found that the Notch1 signaling pathway was activated since it increased the expression of both NICD, the active intracellular portion of Notch1, and HES1, a transcriptional modulator that determines cell fate decisions while reducing the expression of c-Myc, a target gene involved in cell proliferation. These findings are in line with the current understanding of

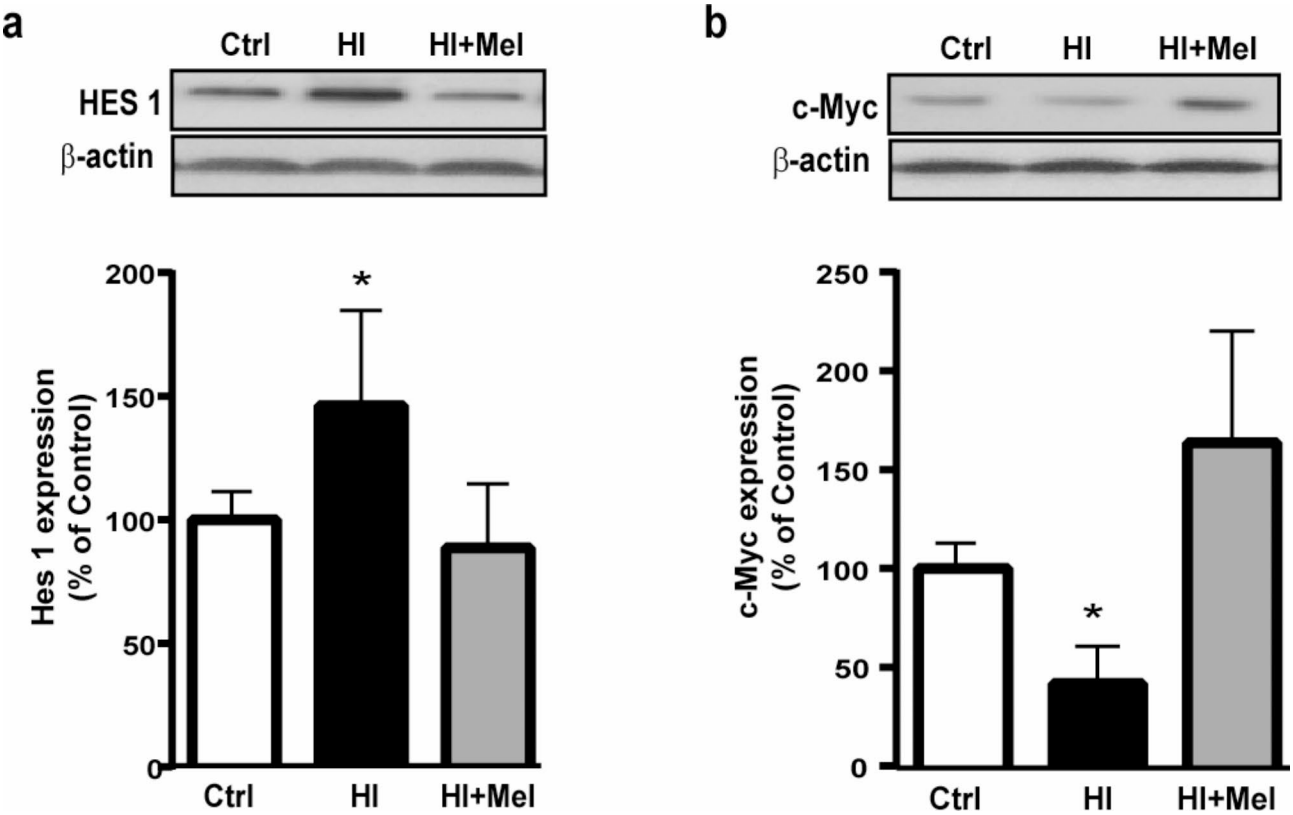

Fig. 3. Effects of neonatal hypoxia-ischemia and melatonin on HES1 and c-Myc expression. (**a**) Representative Western blots and quantitative evaluation of HES1 and c-Myc (**b**) expression in the hippocampus of Control (Ctrl), hypoxic-ischemic (HI) and melatonin-treated hypoxic-ischemic (HI + Mel) animals sacrificed 1 h after HI. Data are expressed as % of Control (mean ± SE, $N = 5$/group). β-actin was run as internal standard. $^{*}p < 0.05$ vs. Ctrl, One-way ANOVA followed by Newman–Keuls multiple comparison test.

the role of the Notch1 signaling pathway in neuronal damage in neurodevelopmental disorders[29,30]. For example, Arumugam and colleagues found that γ-secretase-mediated Notch signaling induced neuronal cell death in ischemic stroke and played a role in neuronal apoptosis[3]. In addition, Notch plays an important role in microglial activation and lymphocyte infiltration in stroke[31,32]. Melatonin has marked protective effects in neonatal brain ischemia (Supplemental Fig. 1). Here, we found that melatonin significantly prevented the Notch1 signaling pathway activation induced by HI, maintaining NICD and HES1 expression to control levels. However, despite the reduction of the Notch1 pathway activation after melatonin, Notch1 expression was increased. The reason for this apparent discrepancy is not clear. We speculate that this effect may be related to the multi-faceted nature of Notch1 signaling, which may exert different effects depending on the cellular context and the interaction with various signaling cascades that could modulate its activation[25]. Melatonin is known for its potent antioxidant and anti-inflammatory properties, which are conferred through several mechanisms, including activating antioxidant enzymes and inhibiting pro-inflammatory cytokines[33]. Also, it plays a role in neurogenesis and cell proliferation[34], and several key aspects, including the drive of neural stem cell activity, are modulated by Notch signaling[2]. Aligned with these effects are our recent findings showing that melatonin can modulate cell cycle dynamics and the proliferative potential of the surviving cells in the HI-injured area in the same animal model used herein[20]. The increased expression of c-Myc in ischemic animals treated with melatonin is in line with these effects of melatonin. However, the connection bridging cell cycle dynamics and proliferation by melatonin to the activation of the Notch1 pathway needs further investigation.

Sirtuins are histone deacetylases pivotal in various biological processes involved in stroke pathophysiology[35]. We previously found that HI reduced Sirt1 neuronal expression in the neonatal rat brain, which was preserved by melatonin treatment[18]. This study shows similar effects of HI and melatonin on Sirt3 expression. Indeed, the mitochondrial expression of Sirt3 was significantly reduced in the ischemic hippocampus one hour after injury and completely preserved in the hippocampus of ischemic animals treated with melatonin. Immunohistochemical experiments showed that Sirt3 was preserved mainly in ischemic neurons. These results suggest a protective role of melatonin in preserving mitochondrial functions in neurons in vivo, in keeping with our previous results after ischemic-like injury in hippocampal HT22 cells and in organotypic hippocampal cultures[21,36]. The role of Sirt3 in maintaining mitochondrial function, which is crucial for cell survival[37], is critical for neurons due to their high energy demand. Indeed, it was reported that Sirt3 deficiency aggravated neuronal cell apoptosis and neurological deficits in ischemic mice, which showed more severe blood-brain barrier disruption and inflammatory responses during the acute phase of ischemic stroke compared with wild-

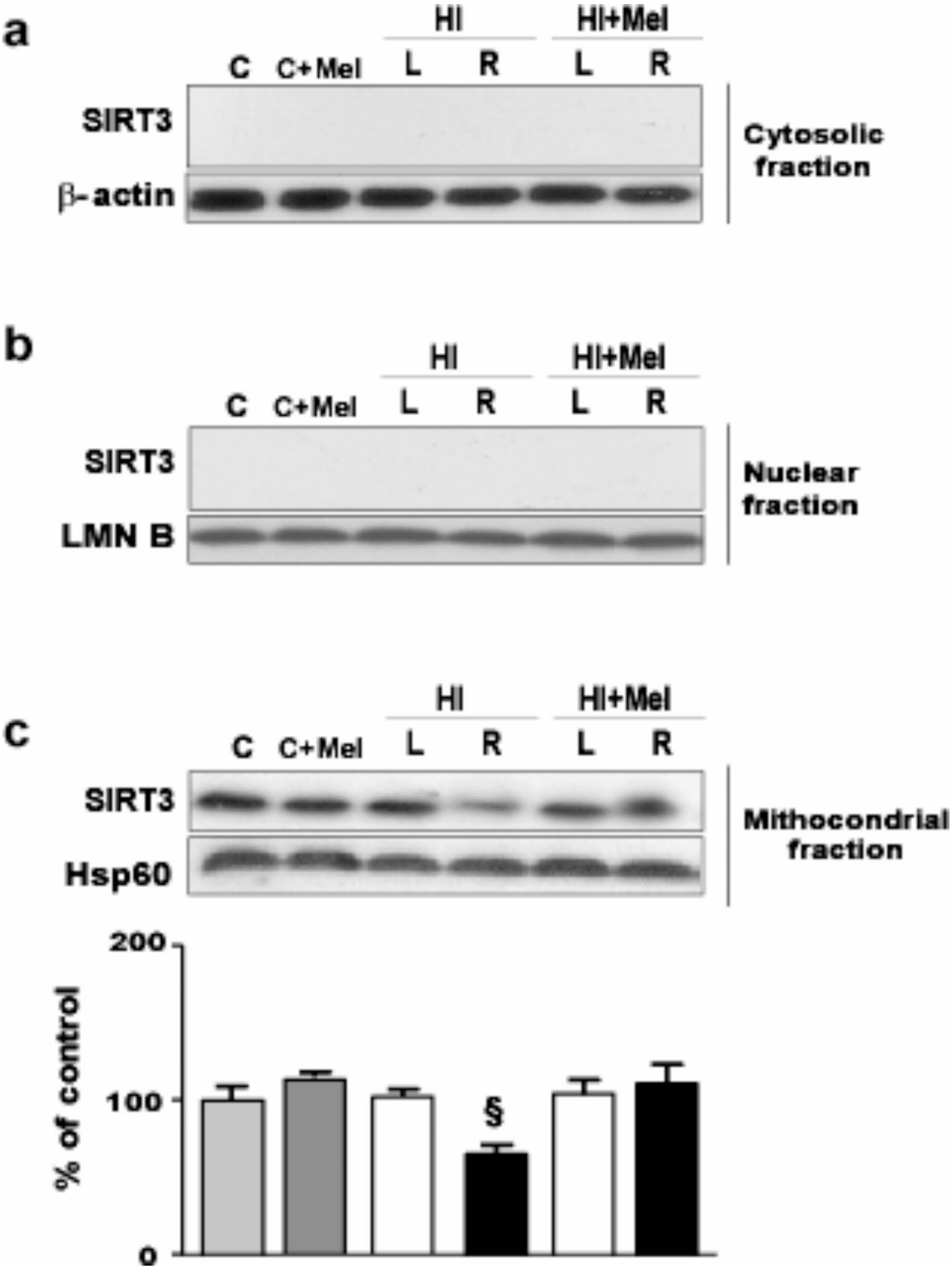

Fig. 4. Effects of neonatal hypoxia-ischemia and melatonin on SIRT3 expression. (**a**) Representative Western blots and quantitative evaluation of Sirt3 expression in the cytosolic, nuclear (**b**) and mitochondrial (**c**) fractions of the hippocampus of vehicle-treated (Ctrl) or melatonin-treated (Ctrl + Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI + Mel) ischemic animals sacrificed 1 h after HI. β-actin, lamin B (LMN B) and Hsp60 were run as loading controls for the cytosolic, nuclear, and mitochondrial fractions, respectively. Upper blots in (**a**) and (**b**) demonstrated that Sirt3 expression was not detected in the cytosolic (**a**) and nuclear (**b**) fractions of the hippocampus of neonatal rats. Data are expressed as % of Control (mean ± SE, $N = 5$/group). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. $^{§}p < 0.001$ vs. Control group, one-way ANOVA followed by Newman–Keuls multiple comparison test.

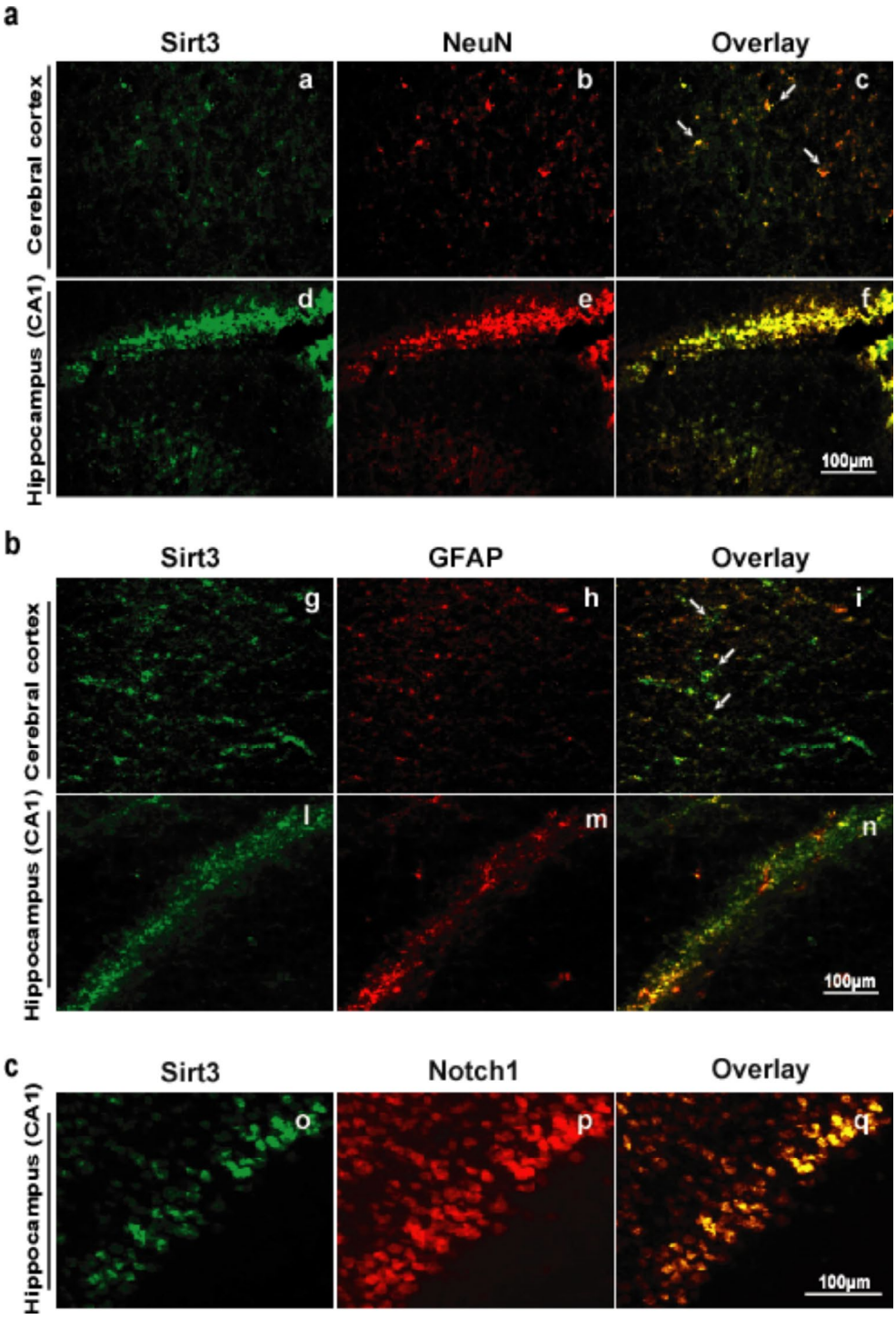

Fig. 5. Expression of Sirt3 in neuronal and glial cells of the ischemic hippocampus after melatonin treatment. (**a**) Representative photomicrographs of experiments performed 1 h after HI showing cells labeled with Sirt3 (panels a and d, green) and NeuN (panels **b** and **e**, red) in the injured cerebral cortex (panels a, b and c) and hippocampus (CA1 region) (panels **d**, **e** and **f**) of HI animals treated with melatonin (HI + Mel). The overlay (panels c and f) demonstrates co-localization of Sirt3 and NeuN (arrows, yellow). (**b**) Representative photomicrographs of experiments performed 1 h after HI showing cells labeled with Sirt3 (panels g and l, green) and GFAP (panels **h** and **m**, red) in the injured cerebral cortex (panels **g**, **h** and **i**) and hippocampus (CA1 region; panels **l**, **m** and **n**) of HI + Mel animals. The overlay (panels **i** and **n**) demonstrates co-localization of Sirt3 and GFAP (arrows, yellow). (**c**) Representative photomicrographs of experiments performed 1 h after HI showing cells labeled with Sirt3 (panel **o**, green) and Notch1 (panel **p**, red) in the injured hippocampus (CA1 region) of HI + Mel animals. The overlay (**q**) demonstrates co-localization of Sirt3 and Notch1 (yellow). $N = 5$/group.

type animals[38]. Interestingly, our results also showed that the melatonin-restoring effects on Sirt3 expression reduced by neonatal HI occurred in the same neurons that overexpressed Notch1 (Fig. 5C), suggesting a possible functional interaction between Sirt3 and Notch behind the melatonin protective effects. A limitation of the present study, however, is the lack of information on the relation linking these pathways to neuroprotection.

In summary, this study provides new insights into the effects of melatonin on the Notch1 signaling pathway and Sirt3 in in vivo neonatal brain ischemia. We suggest that the rapid modulation of the Notch1 signaling pathway and Sirt3 induced by melatonin may support neuronal survival during ischemia. Further studies are necessary to clarify the connections bridging these pathways to the neuroprotection.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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A.M. conceptualized and designed the study, performed experiments and data acquisition; W.B. critically reviewed the manuscript; S.C. performed experiments, and drafted and critically reviewed the manuscript. All authors approved the submitted version of the manuscript.

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Declarations

Competing interests
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