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Human–rat integrated microRNAs profiling identified a new neonatal cerebral hypoxic–ischemic pathway melatonin-sensitive

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Abstract

Neonatal encephalopathy (NE) is a pathological condition affecting long-term neurodevelopmental outcomes. Hypothermia is the only therapeutic option, but does not always improve outcomes; hence, researchers continue to hunt for pharmaceutical compounds. Melatonin treatment has benefitted neonates with hypoxic–ischemic (HI) brain injury. However, unlike animal models that enable the study of the brain and the pathophysiologic cascade, only blood is available from human subjects. Therefore, due to the unavailability of neonatal brain tissue, assumptions about the pathophysiology in pathways and cascades are made in human subjects with NE. We analyzed animal and human specimens to improve our understanding of the pathophysiology in human neonates. A neonate with NE who underwent hypothermia and enrolled in a melatonin pharmacokinetic study was compared to HI rats treated/untreated with melatonin. MicroRNA (miRNA) analyses provided profiles of the neonate's plasma, rat plasma, and rat brain cortexes. We compared these profiles through a bioinformatics tool, identifying Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways common to HI brain injury and melatonin treatment. After evaluating the resulting pathways and the literature, to validate the method, the key proteins expressed in HI brain injury were investigated using cerebral cortexes. The upregulated miRNAs in human neonate and rat plasma helped identify two KEGG pathways, glioma and long-term potentiation, common to HI injury and melatonin treatment. A unified neonatal cerebral melatonin-sensitive HI pathway was designed and

Michael D. Weiss and Silvia Carloni are joint first authors.

validated by assessing the expression of protein kinase Cα (PKCα), phospho (p)-Akt, and p-ERK proteins in rat brain cortexes. PKCα increased in HI-injured rats and further increased with melatonin. p-Akt and p-ERK returned phosphorylated to their basal level with melatonin treatment after HI injury. The bioinformatics analyses validated by key protein expression identified pathways common to HI brain injury and melatonin treatment. This approach helped complete pathways in neonates with NE by integrating information from animal models of HI brain injury.

KEYWORDS
hypothermia, hypoxic–ischemia, melatonin, miRNA, neonatal brain injury, neonatal encephalopathy

1 | INTRODUCTION

Neonatal encephalopathy (NE) is a pathological condition that involves a temporary impairment in brain perfusion. In neonates, NE represents a serious injury to the brain that can lead to life-threatening disabilities or death. The only known treatment that clinically provides beneficial effects is therapeutic hypothermia. Nevertheless, the low effectiveness of hypothermia is a driving force for the urgent development of adjunct pharmaceutical agents.[1] Melatonin, a hormone well known for its involvement in the circadian rhythm, was tested in preclinical trials for perinatal NE and demonstrated neuroprotective effects by pleiotropic and immunomodulatory mechanisms.[2–4] Moreover, our group was the first to define the pharmacokinetic profile of 0.5 mg/kg melatonin given orally over 4 h. This dosage was given to five neonates with NE undergoing therapeutic hypothermia. The highest plasma concentration occurred between 3 and 12 h after the completion of the infusion, which was a longer half-life to compare animals and human adults.[1] Supported by these results, our group wanted to study the pathologic cascades modulated by melatonin in a neonate with NE undergoing hypothermia and compare the results with an animal model of hypoxia–ischemia (HI). To accomplish our goal, microRNA (miRNA) detection in plasma samples was used as the key indicator because it verified the contribution of miRNA dysregulation in several pathophysiologic conditions. In this study, we present an innovative approach to integrate the lacking pathways modulated in human neonates with NE by using an HI animal model. Plasma biological material was the unifying trait between humans and rats. Using the miRNet bioinformatics tool (https://www.mirnet.ca/), the KEGG pathways modulated by the miRNAs analyzed in both human neonates and rats were retrieved.

2 | MATERIALS AND METHODS

2.1 | Human subject enrollment

The study was designed and conducted in accordance with the Declaration of Helsinki and approved by The University of Florida Institutional Review Board (Clinical Trials.gov Identifier: NCT02621944). Before enrollment in the study, written informed consent was obtained from the parents of five neonates admitted to the Neonatal Intensive Care Unit at the University of Florida, Gainesville, USA. The neonate considered in this study (M001) was enrolled within 24 h of birth and had already been considered in our previous study where entry criteria for hypothermia are given.[1]

2.2 | Human subject blood sampling

Plasma was sampled at 0, 12, and 48 h from an infusion of melatonin for analysis. Due to the small total blood volume of a neonate, only 2.5 ml of blood was obtained at each sampling time point.

The analysis of plasma melatonin concentration was calculated at the different time points using the following equation:

$$C(t) = \begin{cases} C(t) = A(1 - \exp(-k_{e1}t)), & t \le t_{\text{inf}} + t_{\text{abs}}, \\ C(t) = A(\exp(-k_{e1}(t - t_{\text{inf}} - t_{\text{abs}}) - \exp(-k_{e1}t)), & t > t_{\text{inf}} + t_{\text{abs}}. \end{cases}$$

2.3 | Animals, cerebral HI, and drug treatment

All surgical and experimental procedures were carried out in accordance with the Italian regulation for the care

and use of laboratory animals (according to the EU Directive 2010/63/EU) and were approved by the Animal Care Committee of the University of Urbino Carlo Bo. Pregnant Sprague–Dawley rats were housed in individual cages where body temperature was maintained at 37°C. The day of delivery was considered Day 0. Neonate rats from different litters were randomized, normalized to 10 pups per litter, and kept in a regular light/dark cycle (lights on 8 a.m.–8 p.m.). On postnatal Day 7, pup rats underwent unilateral ligation of the right common carotid artery via a midline neck incision after anesthesia with 5% isoflurane in O_2. After artery ligation, the wound was sutured, and the animals were allowed to recover for 3 h under a heating lamp. Pups were placed in airtight jars and exposed for 2.5 h to a humidified nitrogen–oxygen mixture (92% and 8%, respectively) delivered at 5–6 L/min (HI). Melatonin (M5250; Sigma Chemical) was dissolved in dimethyl sulfoxide (DMSO; D5879; Sigma Chemical) and diluted in saline solution to a final concentration of 5% DMSO (vehicle). The melatonin solution was injected intraperitoneally to pup rats at a single dose of 15 mg/kg 5 min after the HI procedure (HI + MEL, $n = 2$). The HI-injured animals (HI, $n = 2$) and sham-operated controls (Ctrl, $n = 2$) received a corresponding volume of vehicles.

2.4 | Plasma and tissue collection

Pup rats were anesthetized and euthanized by decapitation 1 h after HI, 55 min after the administration of melatonin. Blood and brain samples were rapidly collected. The brain was removed and the cerebral cortex was sonicated in lysis buffer using an Ultrasonic Liquid Processor XL Sonicator (Heat System Ultrasonic Inc.). Homogenates were centrifuged for 5 min at 18 500 *g* (4°C) and the supernatants were aspirated and stored at −80°C until used.

2.5 | Total RNA extraction from neonate

Total RNA with miRNAs were extracted from the plasma by using an automated Maxwell RSC extractor and Maxwell® RSC miRNA Tissue Kit (Promega AS1460). Four hundred microliters of plasma were mixed with 200 µl of 1-thioglycerol/homogenization solution; 200 µl of lysis buffer and 20 µl of proteinase K were added to samples, mixed, and incubated for 15 min, before transferring all samples to a Maxwell cartridge for automated extraction. miRNA libraries were prepared starting from 100 ng of cortex RNA or from 5 µl of plasma RNA, in accordance with QIAseq® miRNA Library Kit (Qiagen) indications.

2.6 | Total RNA extraction from rat samples

Total RNA of the rat plasma and cerebral cortex samples was extracted using a Total RNA Purification Kit (Norgen). For the cerebral cortex RNA extraction, 200 mg of tissue were homogenized in 400 µl of buffer. Plasma total RNA extraction was performed on 200 µl of the sample. The concentration of RNA was determined using NanoDrop ND 1000 (Thermo Scientific). The RNA samples were stored at −80°C until use.

2.7 | microRNA profiling through small RNA-seq

After RNA quality and integrity check, miRNA-sequencing libraries were prepared using a QIAseq miRNA Library Kit (Qiagen). Sequencing of miRNA was carried out according to the Illumina pipeline on a NextSeq 500 Instrument (Illumina) using the NextSeq High Output Kit v2 (75 cycles) (Illumina). Obtained sequences were uploaded to the Gene Globe Data Analysis Center (https://geneglobe.qiagen.com/us/analyze) to perform: adapters and low-quality base trimming, unique molecular index sequence identification, and insert sequence alignment to miRBase V21 database. Raw miRNA counts were further normalized using the DeSeq2 package for R, low abundance features were filtered out, and differential miRNA expression analysis was performed with Agilent Gene spring GX software (Agilent Technologies).[5]

Differentially expressed miRNAs were selected to have at least a 1.5-fold expression difference between their geometrical mean in the two groups of interest.

2.8 | Bioinformatics analyses

The web-based miRNet tool (https://www.mirnet.ca) has been used to enable statistical analysis and functional interpretation of experimentally verified data from miRNA studies.[6] We used this tool to investigate the pathways significantly modulated ($p < .05$) by next-generation sequencing up- and downregulated miRNA analyses.

A simple string identifier SID1.0 tool[7],[p.1] was used to identify common miRNAs and pathways. miRNet analyses were combined with the bioinformatics tool KEGG PATHWAY Database (https://www.genome.jp/kegg/pathway.html) and only the KEGG pathways

modulated from the same miRNAs in both humans and rats were considered. The graphical representations and all the information about the pathways found were acquired from the KEGG PATHWAY Database.[8]

2.9 | Western blot analysis

Cerebral homogenates (50 μg) were electrophoresed onto sodium dodecyl sulfate gel and proteins were transferred to a PVDF membrane. Blots were blocked and probed with the following primary antibodies: anti-glial fibrillary acidic protein (GFAP) (1:2000, monoclonal; Boehringer Mannheim GmbH), anti-PKCα (1:1000, monoclonal; sc-8393; Santa Cruz Biotechnology), anti-phospho (p)-AKT (1:1000, polyclonal; #4060; Cell Signaling Technology), anti-AKT (1:1000, monoclonal; sc-5298; Santa Cruz Biotechnology), anti-ERK1/2 (1:1000, monoclonal; sc-514302; Santa Cruz Biotechnology), and anti-p-ERK1 (1:1000, monoclonal; sc-7383; Santa Cruz Biotechnology). β-Actin monoclonal antibody (1:4000; sc-8432; Santa Cruz Biotechnology) was used as control. Image J 1.45 software (https://imagej.nih.gov/ij/) was used for quantitative blot analyses.

3 | RESULTS

3.1 | Neonatal subject characteristics

Patient M001 was outborn at an estimated gestational age of 39 weeks. Delivery occurred by C-section due to nonreassuring fetal heart tones during labor. At delivery, a placental abruption was noted. Apgar scores were 1/1/1/3 at 1, 5, 10, and 15 min, respectively. The patient required cardiopulmonary resuscitation for 3min and was intubated at 5 min. The cord blood gas demonstrated pH 6.895, $PaCO_2$ 73 mmHg, $PaO_2 < 16$ mmHg, HCO_3 14 mEq/L, and a base excess-19 with a lactic acid of 16.6 mmol/L. The initial Sarnat score was Stage II. The neonate was intubated for 2 days, including during the melatonin infusion. The melatonin infusion was started at 8 h. A magnetic resonance imaging (MR)I obtained on Day 4 did not have evidence of HI injury. Small foci of hemorrhage were present in both cerebellar hemispheres. The MRI also showed a small-volume subdural hematoma posteriorly on the right. The subject was discharged on Day 11.

The melatonin was infused enterally (0.5 mg/kg). The infusion started 1 h after the neonates reached the target temperature of 33.5°C (hypothermia).

The estimated melatonin pharmacokinetic parameters in M001 patient with HI encephalopathy (HIE) was as follows: individual maximal serum concentration (C_{max}) 0.19 and individual mean time to maximal concentration (T_{max}) 7–8 h.

We collected plasma at 0, 12, and 48 h after infusion and found that with a 4 h–0.5 mg/kg infusion, melatonin plasma concentrations increased after 10 h and decreased after 52 h.

3.2 | microRNA profiling

One neonate with NE diagnosis (M001) out of the five treated with melatonin[1] had sufficient and qualitatively acceptable plasmatic material to be analyzed in this study for the miRNA profiling. Total RNA from the plasma from all five neonates with NE enrolled in the melatonin study was analyzed, but only one of the five neonates was under the limit of accurate quality control by regular methods.[9] In a similar manner, HI rat models were treated with melatonin, and plasma samples and brain cortexes were collected for miRNA profiling to gain insight into the molecular mechanisms involved in the neuroprotective effects of melatonin treatment. A schematic overview of the research approach is illustrated in Figure 1.

3.3 | Plasma microRNAs modulation in a human neonate treated with melatonin

miRNAs modulated by melatonin treatment were analyzed at 0, 12, and 48 h from infusion. In this manner,

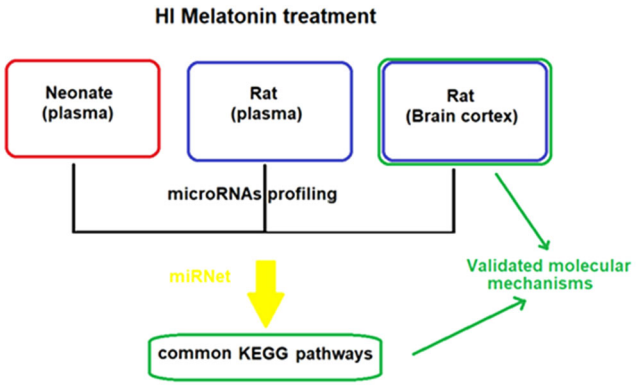

FIGURE 1 Schematic research approach. Three different biological samples were used to perform microRNA (miRNA) profiling analyses to identify the mechanisms involved in the beneficial effect of hypoxia–ischemia treatment with melatonin. Plasma was profiled from neonates and rats, while the brain cortex was from rats only. The common Kyoto Encyclopedia of Genes and Genomes pathways identified through miRNet were used to validate in rat brain cortex the molecular mechanisms involved in melatonin treatment during HI.

independently from hypothermia treatment, only the miRNAs modulated by melatonin were considered. Time points were chosen based on the melatonin pharmacokinetic profile described in reference[1] because at 12 and 48 h plasma has the highest melatonin bioavailability (0.19 and 0.17 μg/ml, respectively). As shown in Supporting Information: Table S1, up- and downregulated miRNAs were considered, and most of the miRNAs have a similar modulation trend after 12 and 48 h from melatonin infusion (in bold in Supporting Information: Table S1 and Figure S1). This modulation trend suggests that miRNA modulation is not dependent on the presence of melatonin in plasma and the beneficial effects are maintained even after 48 h.

Common miRNAs were also investigated for their biological functions, revealing that among similar modulation trend miRNAs, those upregulated are mainly involved in cell cycle, cell death, and inflammation (Supporting Information: Figure S1A), while those downregulated are related to other functions (liver-linked) (Supporting Information: Figure S1B).

On the other hand, miRNAs with a different modulation, both up- and downregulated, seem to be implicated in cell death and inflammatory processes (Supporting Information: Figure S2A,B).

3.4 | Plasma- and cortex-modulated miRNAs in rats treated with melatonin

miRNAs in rat plasma and brain cortex were evaluated by small RNA-seq technique. miRNAs significantly modulated were considered in HI-injured rats (HI), and HI-injured and melatonin-treated rats (HI + MEL) and were compared to the control group (Supporting Information: Table S2). To consider the experimental variability that may occur in two different litters, two series of independent experiments were performed, and only miRNAs with the same modulation in both litters were selected.

3.5 | Pathways modulated by upregulated miRNAs common to the neonate and rat plasma

Even if identified with the same code number, miRNAs in human and rats may have different gene regulation properties. Hence, the investigation was performed only on the common KEGG pathways that miRNet associates with both human and rat miRNA modulation. Using this approach, we were able, for the first time, to associate melatonin activity between HI rats and a neonate with NE. This association of species from plasma samples gave us the possibility to use brain cortex tissue to validate the mechanisms involved in the common pathways identified. The pathways associated with downregulated genes were excluded because no hits were found in the HI + MEL rat plasma group and making comparisons were impossible. Conversely, two KEGG pathways in the melatonin-treated neonate with NE and rats were obtained (gray in Supporting Information: Table S3), namely, glioma and long-term potentiation (Supporting Information: Figures S3 and S4). Both pathways are tightly connected to other pathways, including the calcium signaling, MAPK signaling, cell cycle, mTOR signaling, and ErbB signaling pathways (yellow in Supporting Information: Table S3).

3.6 | Pathways modulated by upregulated miRNAs common to neonate plasma, rat plasma, and rat brain cortex

Rat brain cortex was the only available biological material for molecular mechanism analyses. For this reason, the pathways identified in melatonin-treated HI rat brain cortex were compared with those highly modulated from Supporting Information: Table S3 (common to three of four groups). Eight correspondences were found: acute myeloid leukemia, bladder cancer, cell cycle, chronic myeloid leukemia, colorectal cancer, endometrial cancer, thyroid cancer, and Wnt signaling pathway (bold in Supporting Information: Tables S3 and S4).

3.7 | Validation of the identified KEGG pathways in cerebral cortexes of neonatal rats after HI

A unified or neonatal cerebral HI (NCHI) pathway showing the common KEGG pathways identified from bioinformatics analyses of human and rat samples was found (Figure 2).

To validate these results, we analyzed the expression of key proteins in the cerebral cortex of Ctrl, HI, and HI + MEL animals 1 h after the insult. First, GFAP expression was evaluated to support the experimental model of HI and melatonin treatment as previously reported.[11] HI significantly increased GFAP expression in HI animals analyzed (Figure 3A). Melatonin markedly increased the HI-induced GFAP expression in the early phase of brain damage development, that is, 1 h after HI (Figure 3B). PKCα followed a similar pattern. The immunoblot analysis of the Akt signaling pathway

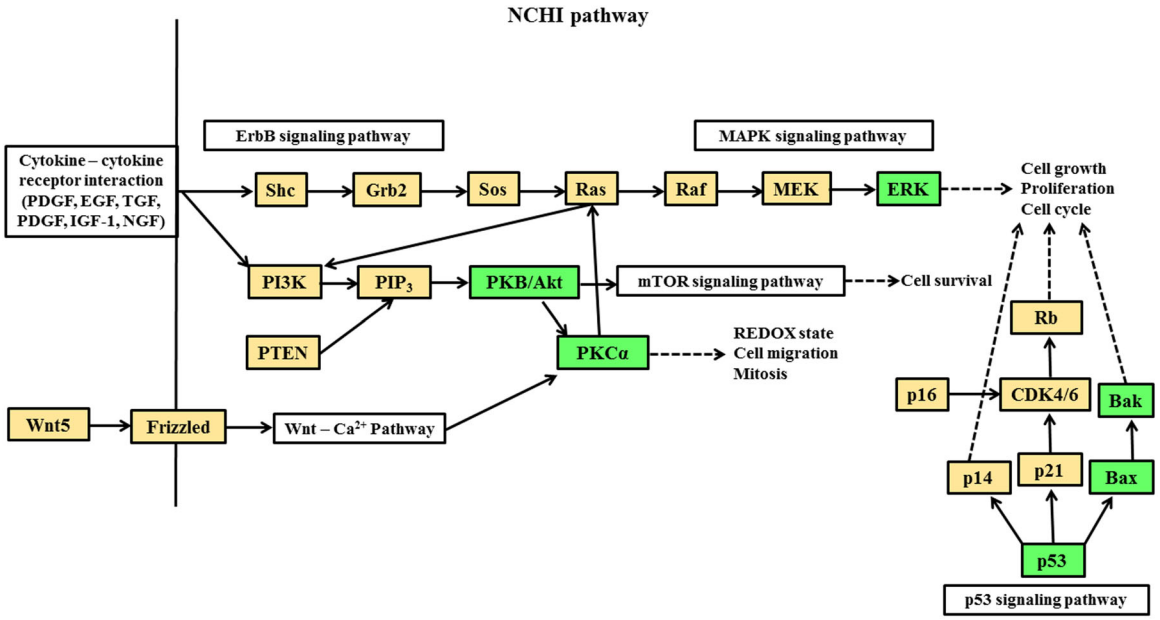

FIGURE 2 Unified pathway of the common Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways—the neonatal cerebral hypoxic–ischemic (NCHI) pathway. The unified pathway, also called the NCHI pathway, was elaborated from the interchange of information between the KEGG pathways resulting from the bioinformatic analysis of the upregulated microRNAs (miRNAs) in a neonate with neonatal encephalopathy (NE) and in hypoxic–ischemic (HI) rats both treated with melatonin. Herein, the main actors (in yellow and green) involved in HI brain injury mechanisms and positively modulated by melatonin signaling pathways like those related to the calcium signaling pathway, MAPK, the cell cycle, mTOR, and ErbB, even if not in common between NE neonate and HI rats treated with melatonin (Supporting Information: Table S3), are tightly connected to, and sometimes part of, the glioma and long-term potentiation KEGG pathways. In green are highlighted the effectors whose expression levels were evaluated by our group in this manuscript and in our previous work.[10] PKCα, activated by calcium-dependent mechanisms and also modulated by PKB/Akt checkpoint, regulates the downstream redox and antiapoptotic state, the cell migration, and the mitosis. Akt is directly involved in the mTOR cascade, and hence in cell survival, its signaling is also strictly controlled by PTEN and Ras, a key factor in both Akt and ERK cascade and at the same time is modulated by PKCα. ERK, as part of the MAPK signaling pathway, is strongly related to downstream results such as cell growth, proliferation, and cell cycle. The latter is influenced by the p53 signaling pathway, in particular by Bax and Bak, death promoters: melatonin can reduce Bax translocation to the mitochondria resulting in an antiapoptotic outcome.

revealed that HI reduced Akt phosphorylation that, on the contrary, was enhanced in melatonin-treated HI animals. The level of Akt protein expression did not change. The ERK1/2 signaling pathway was also in common (Figure 2). We found similar basal expression of ERK1/2 in all rat samples and a significant increased expression of p-ERK1 1 h after HI injury.

4 | DISCUSSION

NE diagnosis at early stages is fundamental for the development of therapeutic strategies. Unfortunately, the therapeutic options available are restricted to hypothermia, which is the standard of care for moderate and severe HIE. Melatonin, allopurinol, topiramate, erythropoietin, *N*-acetylcysteine, $MgSO_4$, and xenon are being evaluated in preclinical and clinical trials to determine the beneficial effects in neonates with NE undergoing hypothermia. These trials also seek to better understand the mechanisms of action of each agent.[12] In our study, we investigated the pathways in HI brain injury and the pathways affected by melatonin treatment. Melatonin was selected because several studies showed it to be neuroprotective in NE subjects in the short and long term, either as a single treatment or in combination with therapeutic hypothermia.[1] Literature clarifies important differences in the events occurring in adult ischemic stroke and neonatal HI, but for ethical and health considerations, sufficient and qualitative neonatal samples for analysis are rare compared with adult subjects. This rarity is the major limitation in NE studies and identifies the reason for a lack of information in this field. Our work is important because of the parallel analyses performed on plasma samples of HI rat models and a human neonate with NE. This exquisite opportunity allowed for extremely accurate miRNA profiling and identification of the pathways involved in NE.[13] miRNA

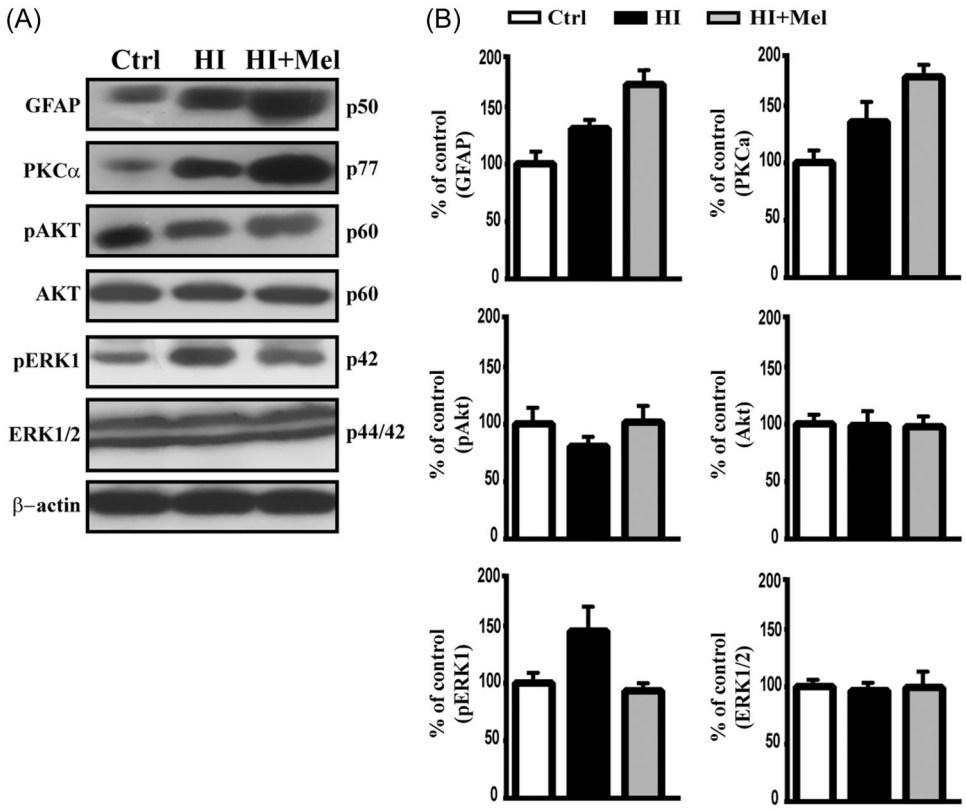

FIGURE 3 Expression of key proteins involved in Kyoto Encyclopedia of Genes and Genomes pathways identified. Representative Western blot (A) and quantitative evaluation (B) of glial fibrillary acidic protein, PKCa, phospho (p)-Akt, Akt, ERK1/2, and p-ERK1 from control (Ctrl), hypoxic-ischemic (HI), and ischemic melatonin-treated (HI+ MEL) rats killed 1 h after HI. β-Actin was used as the loading control. To avoid experimental variability that may occur in two different litters, two series of independent experiments were performed ($n = 12$ animals).

profiles were obtained from the analysis of plasma and brain cortexes of HI-injured rat models not/treated with melatonin and from 0-, 12-, and 48-h plasma of a neonate with NE treated with melatonin. The modulation of neonate miRNAs at 12 and 48 h was compared to 0 h of melatonin treatment to avoid the influence of hypothermia. Originally, the neonate with NE was recruited for a pharmacokinetic analysis of melatonin with four additional neonates, but only plasma from one neonate was quantitatively and qualitatively acceptable for the analyses. From the comparison of human and rat upregulated miRNA profiles, two KEGG pathways were identified as being partially involved in HI brain injury and directly affected by melatonin treatment: the glioma and long-term potentiation pathways. By comparing the pathways and investigating the literature, a unified pathway was developed, the NCHI pathway (Figure 2). The results obtained from the western blots (i) validated the bioinformatics analyses, (ii) were confirmed with literature about neonatal HI, and (iii) were essential for the elaboration of a more detailed schema of the factors that play a fundamental role in the pathophysiologic pathways of HI and those pathways affected by melatonin. Method validation included the evaluation of the modulation of specific NCHI pathway components, while GFAP was necessary to assess the fairness of the experimental model. One hour after HI brain injury, GFAP levels increased in the HI animal models compared to the controls. The GFAP levels increased by a greater amount in those treated with melatonin. These results are consistent with previous works in which the role of astrocytes underwent substantial revision. The related release of GFAP is considered as prosurvival factor linked to neuronal support in early phases of HI injury.[11] PKCα, which is normally activated through Ca^{2+}-dependent mechanisms as reported in the NCHI pathway, showed a similar trend, increasing in HI brain injury and increasing even more in the presence of melatonin treatment. This upregulation is supported by the well-known melatonin downstream antiapoptotic and reactive oxygen species-accumulation reduction effects and from the analyses performed in other neuropathological conditions but never in neonatal NE.[14–16] These studies that indicated a PKCα activation

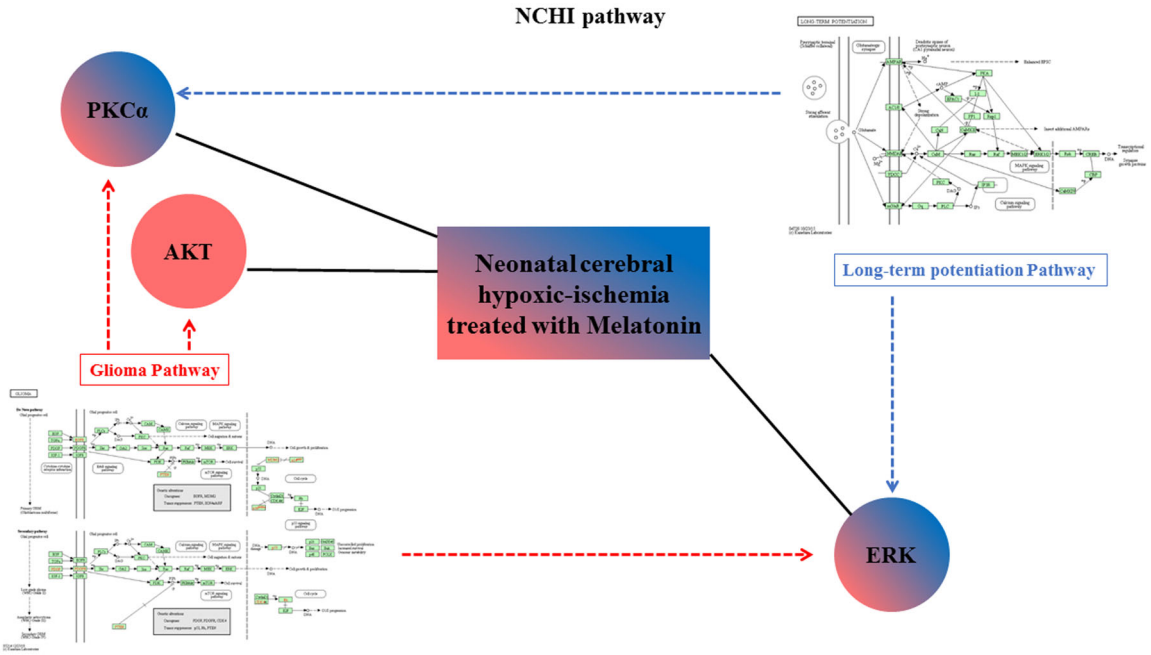

FIGURE 4 Neonatal cerebral hypoxic–ischemic (NCHI) pathway. The neonatal cerebral hypoxic–ischemic (NCHI) pathway is the result of the intersection of the two Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways derived from the bioinformatics analyses of the upregulated microRNAs (miRNAs) found in the plasma of the neonate with neonatal encephalopathy (NE) and in the plasma of hypoxic–ischemic (HI) rats after treatment with melatonin. The two KEGG pathways are glioma (in red) and long-term potentiation (in blue). PKCα, Akt, and ERK were selected and investigated through evaluation of their expression levels in the rat brain cortex to validate the new pathway elaborated. This work represents a unique integration of information between different species where lacks are present and there are great difficulties in obtaining reliable data.

by melatonin make us suspect a similar behavior in neonatal NE and could explain the positive outcomes in terms of oxidative stress and apoptosis, although further experiments must be done. Moreover, in our pathway, PKCα seems also associated with cell migration and mitosis, a novel observation for neonatal NE. Akt and ERK1/2 protein expression were similar among Ctrl, HI and HI + MEL rats, while the phosphorylated forms were altered in HI models and restored to their basal concentration by melatonin treatment. ERK1/2, together with MAPK-signaling pathways, mediate several functions, including cell growth, proliferation, and survival.[17] In cerebral ischemic injury, melatonin demonstrated a neuroprotective effect by precisely decreasing ERK1/2 phosphorylation and preventing an uncontrolled proliferation, possibly in favor of neural stem cell differentiation. In this context, our experiments have confirmed the NCHI pathway, which has given rise to further studies of upstream targets such as Ras, Raf, and MEK, and Shc, Grb2, and Sos, which have never been explored in neonatal NE influenced by melatonin.[18–21] PI3K/Akt, followed by the activation of the mTOR pathway, is considered a survival pathway. It mediates mitochondrial functions in cases of tissue O_2 level compromise by modulating apoptosis and autophagy. Additionally, p-Akt is strongly associated with DNA damage.[21,22] In our study, melatonin activated Akt phosphorylation and restored basal concentrations after HI brain injury. This activation and restoration were previously reported in the literature. Therefore, we propose that melatonin is neuroprotective by preventing DNA fragmentation and thereby promoting neuronal survival in NE. Melatonin may also act through other pathways that are involved, for example, p53, which is restored to control levels by melatonin, BAX protein, whose levels decreased after neonatal HI-induced brain injury (as seen in our previous investigations[10]), and p21 whose phosphorylation is reduced in HI mice.[21,23] p53/p21 are also important cell cycle regulators that, forming an axis with p16/cyclin-dependent kinases 4 and 6/retinoblastoma protein/p14, were investigated to determine the effects of melatonin on neuropathological conditions. Only a few works have described their behavior in HI brain injury, let alone in neonates with NE.[24–26] Those pathways are activated by growth factors like PDGF, IGF-1, NGF, EGF, TGF, and their respective receptors, but the fine-tuning occurs at different levels. Even if not directly connected, some factors can crucially modulate other pathways, making a dense network of regulation. For example, p-ERK can translocate in the nucleus and activate

transcription factors, promoting cell cycle progression. PTEN is able to inactivate PIP_3, produced by PI3K and thus inhibit the PI3K/Akt pathway.[24] We also speculate that melatonin benefits are caused by this complex interconnection that could create a virtuous circle.

Of note, the existence of several melatonin receptors, among them, frizzled, which is stabilized in its β-catenin by Wnt, emerged as being directly involved in HI brain injury and also in tumors like melanoma.[24]

In conclusion, the intersection of the pathways found in the HI rat model and a human neonate with NE, both treated with melatonin, offered the possibility to compare not only the mechanisms involved in rat and neonate plasma and then validated through the rat brain cortex but also to integrate some of the pathways from rats to neonates, for whom minimal information exists. Furthermore, our results identify pathways that could be investigated in neonates with NE in the future. Most of the actors found have never been explored for HI injury or in human neonatal NE (Figure 4).

This work represents the starting point for the investigation of other effectors and intermediates involved in NE found in the NCHI pathway supporting that, when biological samples are limited, different species may be considered to make more robust limited scientific data.

AUTHOR CONTRIBUTIONS

Michael D. Weiss: Conceptualization; supervision; interpretation of data; writing—original draft; visualization; writing—original draft; writing—review and editing. **Silvia Carloni**: Conceptualization; methodology; validation; visualization; interpretation of data; writing—original draft. **Tania Vanzolini**: Investigation; visualization; interpretation of data; writing—review and editing; writing—original draft. **Sofia Coppari**: Formal analysis (bioinformatics analyses); methodology; visualization; writing—original draft. **Walter Balduini**: Supervision; visualization; interpretation of data; writing—original draft. **Giuseppe Buonocore**: Conceptualization; data curation; investigation; writing—original draft. **Mariangela Longini**: Investigation; data curation; writing—original draft. **Serafina Perrone**: Investigation; data curation; writing—original draft. **Livia Sura**: Methodology; data curation; writing—original draft. **Atefeh Mohammadi**: Methodology; data curation; writing—original draft. **Marco Bruno Luigi Rocchi**: Data curation; formal analysis (statistical analyses); writing—original draft. **Massimo Negrini**: Methodology; data curation; visualization; review and editing. **Davide Melandri**: Supervision; interpretation of data; writing—review and editing. **Maria Cristina Albertini**: Conceptualization; data curation; formal analysis (bioinformatics analyses); funding acquisition; investigation; interpretation of data; project administration; writing—original draft; and writing—review and editing. All the authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information: Material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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