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FACULTAD DE CIENCIAS QUÍMICAS Y FARMACÉUTICAS

VULNERABILITY TO RECURRENT METABOLIC INSULTS FOLLOWING PERINATAL ASPHYXIA IN BASAL GANGLIA OF RAT: EFFECT OF NEONATAL TREATMENT WITH NICOTINAMIDE

Tesis presentada a la Universidad de Chile para optar al grado de Doctor en Bioquímica por:

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DEDICATION:

In the hope that this work may in some way contribute to a better understanding of Perinatal Asphyxia, this is dedicated to all the people who believed in me along this journey. To my family and friends for being always my biggest supporters. And to my thesis directors, whose kindness and patience guided me through the roads of science.

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I. ABSTRACT

Perinatal Asphyxia (PA) implies the interruption of oxygen supply, causing energy failure, leading to cell dysfunction and ultimately cell death, affecting in particular neurocircuitries of basal ganglia. The long-term effects occurring in brain after PA worsen the ability of the CNS to cope with stressors occurring along life.

Dopaminergic neurons of substantia nigra have been reported to be very sensitive to PA, and vulnerable to any conditions implying oxidative stress, although it has not been studied yet how dopaminergic neurons cope with postnatal stressors once exposed to an initial event of neonatal hypoxia and how that affects basal ganglia neurocircuitry.

The enhanced vulnerability of basal ganglia following PA was investigated with a protocol combining *in vivo* and *in vitro* experiments. Asphyxia exposed (AS) and caesarean-delivered control (CS) rat neonates were used at P2-3 for preparing triple (substantia nigra, neostriatum and neocortex) or SN-mono organotypic cultures. At DIV 18, cultures were exposed to different concentrations of H_2O_2 (0.25-45mM), added to the culture medium for 18h. After a 48h recovery period, the cultures were either assessed for cell viability (LIVE/DEAD® Viability/Cytotoxicity assay), or formalin fixed for neurochemical phenotype by confocal microscopy. Energy metabolism (ADP/ATP ratio), oxidative stress (GSH/GSSG), tissue reducing capacity, and lactate dehydrogenase (LDH)-release assays were applied to homogenates or supernatants of parallel culture series.

In CS cultures the number of dying cells was similar in substantia nigra, neostriatum and neocortex, but it was several times increased in all regions of AS cultures evaluated under the same conditions. A H_2O_2 challenge led to a concentration-dependent increase in cell death, more prominent in CS than in AS cultures. The cell phenotype of dying/alive cells was investigated in formalin fixed cultures exposed to 0 or 1mM of H_2O_2, co-labelling for DAPI, TUNEL, MAP-2, GFAP, and tyrosine hydroxylase, also evaluating the effect of a single dose of nicotinamide (0.8 mmol/kg, i.p. injected in 100 µL, 60 min after delivery). PA produced a significant increase in the number of DAPI/TUNEL cells/mm^3, in substantia nigra and neostriatum. 1 mM of H_2O_2 increased the number of DAPI/TUNEL cells in all regions of CS and AS cultures.

The effect of PA on cellular phenotypes was more prominent in substantia nigra, where the number of MAP-2/TH positive cells/mm^3 was decreased in AS compared to CS cultures, also by 1 mM of H_2O_2, both in CS and AS cultures. Neuronal apoptosis was increased, both in CS (> 2-fold) and AS (> 3 fold) cultures. The disruptive effect of 1 mM of H_2O_2 was stronger in cultures from AS than that from CS neonates on TH positive cells (decreased by ~70% versus ~30%), while the effect on MAP-2 positive cells was similar in both groups (decreased by ~60% versus ~50%). 1 mM of H_2O_2 led to a strong increase in the number of TUNEL-positive neurons, both in CS and AS groups. In neocortex, similar MAP-2 positive apoptosis was observed.

The ADP/ATP ratio was increased in AS culture homogenates compared to CS, and increased by 1 mM of H_2O_2, both in CS and AS cultures. The GSH/GSSG ratio was decreased in AS compared to CS cultures, meanwhile 1 mM of H_2O_2 decreased that ratio in both groups.

AS samples showed a decreased total reducing capacity, and high LDH levels compared to CS. Similar effects were observed comparing mono and triple organotypic cultures, suggesting that the vulnerability of basal ganglia neurocircuitries after PA relies mainly, but not exclusively on substantia nigra.

The present results demonstrate that PA induces long-term changes in metabolic pathways, implying energy failure and oxidative stress, priming cell vulnerability to both neuronal and astroglial phenotypes, affecting the coping capacity of neurons and astroglia against further postnatal challenges. The observed effects were region dependent, the substantia nigra being more prone to cell death than other regions.

Nicotinamide administration *in vivo* prevented the deleterious effects observed after PA and after a recurrent metabolic insult *in vitro.*

II. INTRODUCTION

Perinatal asphyxia (PA) implies deregulation of gas exchange, resulting in hypoxemia, hypercapnia and metabolic acidosis in vital organs, including the brain (Low 1997). Despite important advances in perinatal care, PA remains a severe condition, with high prevalence worldwide (2 to 10/1000 live births), leading to neonatal death or long-lasting developmental deficits, including neuropsychiatric dysfunctions (Douglas-Escobar and Weiss 2013).

The interruption and sudden oxygenation cause energy failure, triggering biochemical cascades leading to cell dysfunction and ultimately to cell death, affecting neurocircuitries of basal ganglia (Klawitter et al. 2007) and hippocampus (Morales et al. 2008; Neira-Peña et al. 2015). The long-term effects observed after PA also imply metabolic and neuronal network alterations, impairing the ability of the CNS to cope with stressors occurring along life (Marriot et al. 2017).

Dopaminergic neurons of *substantia nigra* have been reported to be very sensitive to hypoxic conditions and oxidative stress, dying by apoptotic caspase-dependent and/or independent mechanisms (Andersson et al. 1995; Dell'Anna et al. 1997; Morales et al. 2008; Edwards et al. 2008), while the number of nNOS neurons has been shown to be increased in mesencephalic tissue after PA (Klawitter et al, 2007). The reasons explaining why dopaminergic neurons are susceptible to metabolic insults have not been clarified yet, although it has been reported that dopamine neurons are characterised by high-energy requirements, and chemical instability at physiological pH. In fact, the regional vulnerability to the effects of PA can be associated to permanent deficits in cell metabolism, mitochondrial function, activation of intrinsic apoptotic pathways (Johnston and Hoon, 2000), oxidative stress (Mondal et al. 2010) or to recurrent postnatal insults (Marriot et al. 2016).

Hypoxia and generation of reactive oxygen (ROS) and nitrosylated (RNS) species lead to nuclear translocation of the oxygen sensor HIF-1α, stimulating the expression of multiple genes to cope with low oxygen tension (Ke & Costa 2006). Indeed, HIF-1α is associated to genes controlling cell metabolism and

mitochondrial function, down-regulating the citric acid cycle and enhancing anaerobic glycolysis, diminishing the metabolic capacity of the cells, disrupting the ADP/ATP ratio, affecting synaptogenesis and astrocyte function (Vangeison et al. 2008). HIF-1α translocation stimulates pro-apoptotic genes, including Nix, Noxa, Bnip3, and Apoptosis Inducing Factor (AIF), genes from the Bcl-2 family (Bruick 2000, Sowter et al 2001), but also the expression of sentinel proteins such as Poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 signalling occurs via the synthesis of poly (ADP-ribose) chains, recognized by DNA-repairing enzymes, such as DNA ligase III (Leppard et al. 2003). The generation of ADP-ribose monomers requires NAD^+, which is the reason why PARP-1 over activation further depletes NAD^+ stores, resulting in progressive ATP depletion (Berger, 1985; Hong et al, 2004; Berger et al. 2005).

There is a crosstalk between PARP-1 and HIF-1α (Martin-Oliva et al, 2006). Under hypoxic conditions and/or oxidative stress, PARP-1 modulates HIF-1α activity, preventing its degradation by the proteasome pathway (Gonzalez-Flores et al, 2014). Under energy crisis, extracellular glutamate levels accumulate, stimulating extra synaptic NMDA receptors, resulting in increased NO production, impairing mitochondrial function and enhancing oxidative stress by formation of peroxynitrite. NO can directly decrease mitochondrial membrane potentials, enhancing the liberation of pro-apoptotic proteins (Moncada et al. 2006) and the expression and activation of several pro-oxidant enzymes in brain tissue, such as NADPH oxidase and neuronal nitric oxide synthase (nNOS) (Hwang et al. 2002). Studies using models of oxidative stress have shown that pharmacological inhibition of PARP-1 attenuates nNOS activation, suggesting a mechanism of neurotoxicity, since inhibitors of PARP isoforms significantly reduce cell death induced by oxidative conditions (Du et al. 2003).

Studies by our group showed that PA increases PARP-1 activity, triggering a pro-inflammatory signalling cascade leading to apoptotic-like cell death in mesencephalon of neonate rats. A single dose of the NAD^+ precursor/PARP-1 inhibitor nicotinamide (0.8 mmol/kg, i.p.),1 h post-delivery, prevented the effect of PA on PARP-1 activity and apoptotic cell death in mesencephalon (Allende-Castro et al. 2013; Neira-Peña et al. 2015a,b).

Nicotinamide treatment following PA promotes neuroprotection *in vivo*, showing region-dependent effects. While the mesencephalon is prone to activation by inflammatory cascades, that effect is, however, prevented by nicotinamide. Thus, pharmacological inhibition of PARP-1 can restore metabolic functions including efficient energy production, preventing ROS generation, as well as cell death cascades.

Energy metabolism and mitochondrial homeostasis change along postnatal development (Mattson et al. 2007), existing a tight control of mitochondrial function involving several redox status sensing proteins, oxygen availability and ADP/ATP ratio, among others (Pérez-Pinzon et al. 1999). Mitochondrial fusion and fission are essential for mitochondrial quality control. Changes in mitochondrial morphology and function can affect the response to ischemia-reperfusion injury in models of oxidative stress with cardiac myocytes (Liu et al. 2009). Apoptosis and necrosis are enhanced after sustained hypoxic conditions, however, necrosis has been described as a mechanism of cell death independent from mitochondrial dysfunction, contributing to neuronal loss by apoptosis-independent pathways. Nevertheless, mitochondrial homeostasis is critical for maintaining NAD^+ levels, preventing AIF release and PARP-1 over activation (Neira-Peña et al. 2013).

Using models of global postnatal brain hypoxia/ischemia *in vivo*, lactate dehydrogenase (LDH) release was reported to be an early marker of cytotoxicity during hypoxic ischemic encephalopathy in serum and cerebrospinal fluid (Hongyan et al. 2015). The effects of recurrent metabolic insults following PA on LDH release have not been previously studied using the present model of PA, although it has been proposed that neuronal maturity and cellular phenotypes (Rice et al. 1981) play a pivotal role in the activation of cytotoxicity (Gao et al. 1999).

Although substantia nigra has been reported to be a vulnerable brain region to PA *in vivo* and *in vitro*, there is no information about its coping capacity under recurrent metabolic insults or whether *in vitro* mimicking of basal ganglia neurocircuitries is useful for assessing resistance or susceptibility of dopamine

neurons, which can ultimately influence the manner dopaminergic systems respond to metabolic challenges.

This thesis attempted to characterise how PA primes cell viability and vulnerability to a second metabolic insult *in vitro,* using organotypic cultures, and whether a pharmacological treatment with nicotinamide *in vivo* can protect to the effect of a secondary insult *in vitro.*

The phenotype of cultured cells affected by PA was assayed with immunocytochemistry in formalin fixed cultures, labelling for DAPI (nuclear staining), TUNEL (apoptosis), MAP-2 (neuronal phenotype), GFAP (astroglial phenotype), and tyrosine hydroxylase (TH, dopamine phenotype), also evaluating the effect of a single dose of nicotinamide (0.8 mmol/kg, i.p.) or saline (100 µL, i.p.), 60 min after delivery to asphyxia-exposed and the corresponding caesarean-delivered controls. Energy metabolism was evaluated by the ADP/ATP ratio, and oxidative stress by measuring the GSH/GSSG ratio and reducing capacity of the tissue by a modified ferric reducing/antioxidant power assay in culture homogenates.

To address necrosis, a commercial LDH release assay was applied to supernatants of all experimental groups to determine necrotic cytotoxicity as a marker of cell loss.

Thus, we proposed that PA diminishes the coping capacity of basal ganglia to recurrent metabolic insults, impairing cell metabolism and phenotype-specific cell death in CNS. Nicotinamide was used as a pharmacological PARP-1 inhibitor, protecting from the long-term effect of PA, further investigating the interaction between the PA outcome and the coping capacity of basal ganglia *in vitro*.

III. HYPOTHESIS

The vulnerability of basal ganglia to recurrent metabolic insults following perinatal asphyxia implies a long-term impairment of cell metabolism that is prevented by the administration of nicotinamide.

IV. MAIN OBJECTIVE

To determine whether systemic nicotinamide treatment *in vivo* after perinatal asphyxia affects the vulnerability of cells following a recurrent metabolic challenge in organotypic cultures.

V. SPECIFIC OBJECTIVES

1. To study the effect of perinatal asphyxia and pharmacological treatment with nicotinamide on cell survival after a second metabolic insult using organotypic cultures of basal ganglia by fluorescent probes, monitoring neuronal and astroglial markers by immunocytochemistry.
    1.1. To analyse the effect of a recurrent metabolic insult on cell viability in brain cultures from asphyxia exposed versus control neonates (*ex vivo*) using fluorescent probes.
    1.2. To analyse the effect of nicotinamide on cell viability following a recurrent metabolic challenge in organotypic cultures from nicotinamide– and saline–treated animals exposed to perinatal asphyxia and the corresponding controls using fluorescent probes.
    1.3. To determine if nicotinamide treatment *in vivo* after perinatal asphyxia affects the vulnerability of basal ganglia cells following a recurrent metabolic challenge in organotypic cultures by monitoring neuronal and astroglial markers.

2. To study the effect of perinatal asphyxia and pharmacological treatment with nicotinamide on the metabolic state after perinatal asphyxia, and after a second metabolic challenge in organotypic cultures of basal ganglia, assessing cell metabolism by ADP/ATP ratio; oxidative stress by measuring GSH/GSSG ratio, and antioxidant power capacity by luminescent and colorimetric probes and cytotoxicity by a LDH release assay using an enzymatic assay.
    2.1. To monitor energy metabolism by ADP/ATP ratio in cultures derived from brain tissue exposed to perinatal asphyxia after a metabolic challenge and nicotinamide treatment using luminescent probes.
    2.2. To determine oxidative stress conditions by measuring GSH/GSSG ratio in organotypic cultures derived from asphyxia-exposed rats treated with nicotinamide, and exposed to a recurrent insult using colorimetric probes.
    2.3. To analyse the antioxidant capacity of cultures from asphyxia-exposed rats and thereafter exposed to a recurrent metabolic insult using colorimetric probes.
    2.4. To analyse cell necrosis in tissue exposed to perinatal asphyxia, and to a recurrent metabolic insult by LDH release assay using an enzymatic assay.

3. To study the effect of perinatal asphyxia and pharmacological treatment with nicotinamide on the vulnerability of substantia nigra to a recurrent metabolic challenge using organotypic mono-cultures *in vitro.*

VI. MATERIALS AND METHODS

Animals

Wistar albino rats from the animal station of the *Molecular & Clinical Pharmacology Programme, ICBM*, Faculty of Medicine, University of Chile, Santiago, Chile, were used along the experiments. The animals were kept in a temperature- and humidity-controlled environment with a 12/12 h light/dark cycle and fed *ad libitum,* when not used for the experiments, monitoring permanently the wellbeing of the animals by qualified personnel.

Ethic Statement

All procedures were conducted in accordance with the animal care and use protocol established by a Local Ethics Committee for experimentation with laboratory animals at the Medical Faculty, University of Chile (Protocol CBA# 0447 FMUCH) and by the ad-hoc commission of the Chilean Council for Science and Technology Research, (CONICYT), endorsing the principles of laboratory animal care (NIH; N° 86-23; revised 1985).

Perinatal asphyxia

Pregnant rats within the last day of gestation (G22) were euthanized by neck dislocation and hysterectomised. One or two pups per rat dam were removed immediately and used as non-asphyxiated caesarean-delivered controls (CS). The remaining foetuses-containing uterine horns were immersed in a water bath at 37 °C (Figure 1) for 21 minutes (asphyxia-exposed rats, AS). Following asphyxia, the uterine horns were incised and the pups excised, stimulated to breathe and after an approximately 60 min observation period on a warming pad, evaluated with an Apgar scale for rats, according to Dell'Anna et al. (1997).

Nicotinamide treatment

Nicotinamide (0.8 mmol/kg, i.p.) or saline (sterile 0.9% NaCl, i.p.) was administered in a volume of 100 µL 1 h after delivery (measured as the time the foetuses were excised from the uterine horns). Vehicle groups were injected with 100 µL of a 0.9% NaCl solution. The neonates were then transferred to a surrogate mother pending for the organotypic culture procedures.

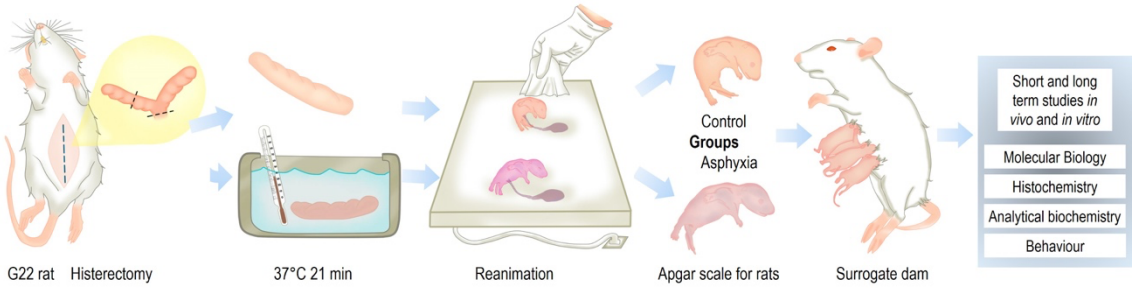

Figure 1. Representation of the Model of perinatal asphyxia. Pregnant Wistar rats (gestation day 22) were anaesthetised, euthanized by neck dislocation and hysterectomised. Three or four pups were removed immediately, corresponding to caesarean-delivered controls (CS), and remaining foetuses were immersed in a water bath at 37°C for 21 min (AS). Following asphyxia, pups were removed, stimulated to breathe and after 45 min were evaluated following the Apgar scale for rats. Then, the neonates were given to surrogate dams, waiting for experimental procedures. (Marriot et al., 2016)

Organotypic Culture

Different series of rat neonates of both sexes were used for preparing organotypic cultures, 2 to 3 days after birth according to Klawitter et al. (2007). The brain was rapidly removed under sterile conditions and stored in a Petri dish containing Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Life Technologies AB, Täby, Sweden). Coronal sections (300 µm) were cut by a microslicer (DTK-2000, Dosaka CO, Japan; 300-µm thick) and stored in cold DMEM. Samples from substantia nigra (SN), neostriatum (Str) and neocortex (Cx) were dissected out and placed on a coverslip (NuncThermanox Coverslips; Nunc, Naperville, IL, USA) containing a spread layer of chicken plasma (25 µL), coagulated by bovine thrombin (20 µL/350 µL DMEM, Sigma, St. Louis, MO, USA) (the standard thrombin solution contained 1000 NIH units in 750 µL of H_2O). The coverslips were then transferred into sterile Nunc flat CT-tubes containing 750 µL of culture medium, and grown at 35 °C, and 10% CO_2 in a cell incubator using a roller device exposing the cultures to gaseous or water phases every minute (Klawitter et al. 2007). At DIV 3, the medium was changed to a serum-free medium (Neurobasal-A medium supplemented with B27 2%, GIBCO BRL; glucose 5 mM; L-glutamine 2.5 mM, Sigma). The medium was changed every 3–4 days.

Metabolic Challenge (Recurrent Metabolic Insult: H_2O_2)

At DIV 18, cultures were treated with different concentrations of H_2O_2 (0.25–45 mM, diluted in Neurobasal-A culture media) for 18 h at 35 °C, to thereafter be returned to the standard condition, pending further analysis.

In vitro monitoring and cell viability

The progression of the cultures was periodically monitored with an inverted microscope equipped with Hoffmann optics (Nikon T100), taking representative pictures (Figure 2 A-B). Some cultures were analysed for cell viability at DIV 20-21 by the LIVE/DEAD® Viability/Citotoxicity kit L3224 (Molecular Probes, Eugene, OR, USA), using ethidium-homodimer (EthD-1) and calcein-acetoxymethyl ester (AM) for labelling dead and alive cells, respectively. Alive cells are distinguished by a green fluorescence produced by the action of intracellular esterases cleaving the AM group, retaining the calcein dye within the alive cells. Dying cells are identified by a red fluorescence produced by EthD-1

entering the cells with damaged membranes and binding to nucleic acids. For quantification, six representative images of ~0.02 mm^3 per culture were counted, according to Klawitter et al. (2007).

Immunohistochemistry and TUNEL Assay

Antibodies. Primary antibodies: (i) anti-MAP2 (chicken, ABCAM, ab, 5392; 1:750 incubated in blocking solution containing 2% bovine serum albumin (BSA); 4% normal goat serum, and 0.1% Triton X-100); (ii) anti-GFAP (mouse, Sigma, G3893, 1:500), and (iii) anti-TH (sheep, ABCAM ab113, 1:1000). Secondary antibodies: (i) anti-mouse-FITC (goat, Jackson Laboratories, 1:200); (ii) anti-ChickenAlexa594 (goat, Jackson Laboratories, 1:200); (iii) anti-rabbit-Alexa488 (donkey, Jackson Laboratories, 1:200), and (iv) anti-sheep-Alexa 647 (donkey, Jackson Laboratories, 1:300 in 1X PBS with 0.1% Triton X-100).

Formalin-fixed organotypic cultures were rinsed with 0.1 M PBS and treated with blocking solutions (normal goat serum 6.5% or horse serum, bovine serum albumin, BSA (2%), and 0.3% Triton X100 in PBS, depending on the protocol and primary antibody species), and then incubated with primary antibodies against cell phenotype (MAP-2, GFAP or TH). The respective primary antibodies were diluted in 0.1 M PBS with 0.3% Triton X-100, added and incubated overnight at 4 °C in darkness. Samples were rinsed with PBS and incubated with the correspondent secondary antibody and counterstained with 4,6 diamino-2-phenylindol (DAPI; Invitrogen 0.02 M; 0.0125 mg/mL; for nuclear labelling) for 60 min. Mounted samples were first examined with an epifluorescence microscope and then by confocal microscope.

TUNEL. Formalin-fixed organotypic cultures were stained with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, #90418) and processed as follows: formalin fixed organotypic cultures were rinsed three times with PBS and permeabilized with a solution containing 50% ethanol, 50% acetic acid, for 5 min at 20 °C and treated for endogenous peroxidase quenching, with 3% H_2O_2 in methanol for 10 min. Then, the cultures were washed with a commercial solution provided by the manufacturer of the TUNEL assay to incubate with TdT Enzyme during 80 min at 37 °C (Millipore). The reaction was stopped with Stop Wash Buffer and then incubated with a Ready-to-Use anti-digoxigenin HRP-conjugated

antibody. After washing, the cultures were incubated with 488 or 594 conjugated tyramide, depending upon available secondary antibodies, at a dilution of 1:100 in 0.1 M PBS, as substrate for HRP enzyme, during 10 min at room temperature.

Energy Metabolism Assessment

ADP/ATP determination

The ADP/ATP ratio was measured in culture homogenates by a fluorescent assay. Cells from entire triple cultures were homogenized with a tissue grind pestle in a MAK-135A (Sigma-Aldrich, St. Louis, MO, USA) buffer, to be then analysed in a 96-well Grainer plate using a ADP/ATP ratio assay kit (Sigma MAK 135), in a microplate reader (BioTek Synergy HT Microplate Reader). Data is expressed as relative fluorescence units (RFU).

GSH/GSSG Ratio Assay

Triple and mono- organotypic cultures were homogenized in phosphate buffer 0.1 M pH 7.5, supplemented with 5 mM EDTA disodium salt and protease inhibitor cocktail (1 mM sodium orthovanadate, 1mM PMSF, 5 mM EDTA, 1 mM EGTA, 10 mM NaF and protease inhibitor cocktail, Calbiochem set III). Samples were centrifuged at 14,000 rpm for 10 minutes at 4ºC. Supernatant was collected and deproteinated with metaphosphoric acid (5%) and 10% trichloroacetic acid. Total reduced glutathione (GSH) was measured directly in the supernatant, while oxidized glutathione (GSSG) 2- vinylpiridine was used for derivatization, followed by neutralization of the derivatized supernatant with triethanolamine. After the reaction, the supernatant was treated with DTNB (5,5′-dithiobis-2-nitrobenzoic acid), NADPH and glutathione reductase (Sigma, G3664). The TNB (5-thio-2-nitrobenzoic acid) resulting from the reaction was measured by a kinetic method at 412 nm in a Multi-Mode Microplate Reader (Synergy HT Biotek Instruments, Inc).

Potassium Ferricyanide Reducing Power Assay.

Reducing power was determined in cultures homogenized with 0.1 M PBS (pH 7.3). Samples were centrifuged at 10,000 rpm for 10 min and 50 µL of the supernatant was added to 300 µL of 0.1 M PBS and 300 µL of 1% potassium ferricyanide. The mixture was incubated for 25 min at 50 °C. Then, 300 µL of 10% of trichloroacetic acid was added and the mixture was incubated on ice for 5 min, to be centrifuged at 14,000 rpm for 10 min. Supernatants were obtained again and 300 µL of distilled H_2O and 70 µL of 0.1% ferric chloride was added, incubated for 3 min and absorbance measured in 200 µL samples at 700 nm. Data was normalized by total protein concentration for each sample. Protein concentration for the potassium ferricyanide reducing power assay was determined by the bicinchoninic acid (BCA) method (Pierce, BCA™ Protein Assay kit). BCA reacts with complexes between copper ions and peptide bonds to produce a purple-end product. Reagent A (sodium BCA 1%; sodium carbonate 2%; sodium tartrate 0.16%; NaOH 0.4% and sodium bicarbonate 0.95%, pH 11.25) and reagent B (cupric sulphate X5 H2O 4%) were mixed. Two hundred microliters of the A and B mix and 10 µL of every sample were incubated at 37 °C for 30 min. The samples were read at 562 nm. A standard curve of absorbance versus micrograms for BSA protein was prepared and the protein concentration of every sample was calculated.

Quantification of Proteins

The total protein concentration was determined using a commercially available bicinchoninic acid (BCA) assay kit from Pierce (Thermo Fisher Scientific, Rockford, IL USA). Absorbance was measured at 562 nm in a Multi-Mode Microplate Reader (Synergy HT Biotek Instruments, Inc., Winooski, VT, USA).

LDH cytotoxicity Assay

LDH release was measured using entire culture supernatants from triple and mono organotypic cultures, using a colorimetric assay (LDH Cytotoxicity Assay Kit, Thermo Scientific™) to quantitatively measure lactate dehydrogenase (LDH) released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis (Koh & Choi, 1987; Chan et al. 2013). After incubation at room temperature for 30 minutes, the reaction was stopped and LDH activity

determined by spectrophotometric absorbance at 490 nm.

Image Processing and Stereological Analysis

The following parameters were quantified, number of: (i) MAP-2$^+$ cells/mm^3; (ii) GFAP$^+$ cells/mm^3; (iii) MAP-2 $^+$ TH$^+$ cells/mm^3; (iv) TUNEL $^+$ MAP-2$^+$ cells/mm^3; (v) TUNEL$^+$ GFAP$^+$ cells/mm^3, and (vi) TUNEL$^+$ MAP-2 $^+$ TH$^+$ cells/mm^3, counterstaining for DAPI (nuclear staining) in all cases. Microphotographs (5-6 per zone, 8 stacks for each case) were taken from each zone of the triple organotypic cultures (SN, Str and Cx) in the field of a confocal-inverted Olympus-fv10i microscope with a 60× objective lens. The area inspected was 0.04 mm^2. The thickness (Z-axis) was measured for each case. The number of positive stained cells for each marker was expressed as cells/mm^3, in cultures from CS and AS, using ImageJ software, confirmed by proper DAPI labelling.

Statistical analysis

All results are expressed as means ± standard error of the means (SEM). Effects were evaluated by ANOVA unbalanced factors followed by Benjamini-Hochberg correction as a post hoc test for multiple comparisons. For statistically significant differences, the probability of error was set up to less than 5%. Data analysis was performed with a XLSTAT software, version 2018. 20.1.49878 (ADDINSOFT SARL, Paris, France) and GraphPad Prism Software version 7.00 for Macintosh, GraphPad Software, La Jolla California USA.

VII. RESULTS

Table 1 shows the outcome of PA induced by 21 min water immersion of foetuses containing uterine horns (AS) removed by a caesarean section from ready to delivery rat dams. The physiological status was evaluated by an Apgar scale applied 40 min after delivery (time for the uterine excision), comparing AS versus caesarean-delivered control (CS) rat pups. Following 21 min of PA, the rate of survival was ~60%. Surviving pups showed a long-term decreased respiratory frequency (supported by forced gasping), decreased vocalization, cyanotic skin, akinesia and rigidity, indicating a severe insult.

Table 1. Apgar evaluation

Apgar scale for evaluating the consequences of perinatal asphyxia 40 min after utherine excision (counted as the time of delivery); *n,* number of pups; *m,* number of dams. (*) Gasping forced reflex inspirations. In control rats gasping is absent after 1 min from delivery. (**) 4 indicates coordinated and spontaneous movements, 0 indicates no movement.

Apgar evaluation of neonate rats		
Parameters	**Caesarean-delivered pups (0 min asphyxia) (*n* = 15; *m* = 15)**	**21 min asphyxia (*n* = 15; *m* = 15)**
Body weight (g)	6.29 ± 0.087	6.19 ± 0.063
Survival	100%	60%
Respiratory frequency (events/min)	72.3 ± 1.90	32.5 ± 2.2
Presence of gasping (yes; %) (*)	0 %	41.74 ± 11.93 %
Skin color		
Pink-Blue	100 %	0%
Blue-Pink-Blue	0%	100%
Presence of vocalizations (yes, %)	100%	33.63% ± 10.24
Spontaneous Movements (4,3,2,1,0) (**)	4	0

Effect of perinatal asphyxia and nicotinamide on a recurrent metabolic insult performed in triple organotypic cultures.

Triple organotypic cultures (SN, Str, Cx from CS (n = 30) and AS (n = 30) neonates) were continuously monitored, assessing attachment and development on the coverslip (Fig. 1, pictures taken at DIV 0, 5, 11 and 18 with a Nikon T100 digital camera on the stage of an Inverted Microscope). At DIV 1, the thickness of organotypic cultures was 300 µm, decreasing to ~45 µm at DIV 20-22, also measured on the stage of a confocal microscope (Olympus FVIOi) (Z-axis). The tissue became fused and integrated as previously reported (Klawitter et al. 2007). No differences were observed in coverslip adhesion or development between organotypic cultures from CS or AS **(Fig 2 a,b)**.

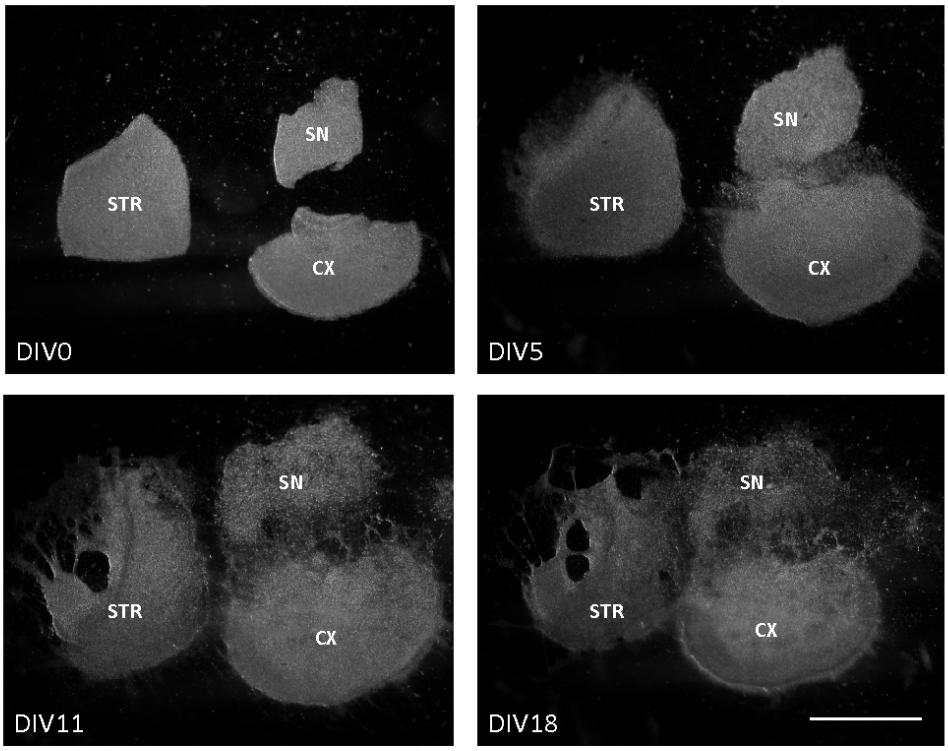

Figure 2A. Triple organotypic culture following. Monitoring triple organotypic cultures from substantia nigra (SN), neostriatum (STR) and neocortex (CX). The progression of cultures is shown at DIV 0, 5, 11 and 18. Observe that the thickness of the tissue decreases over time (scale bar: 1000µm).

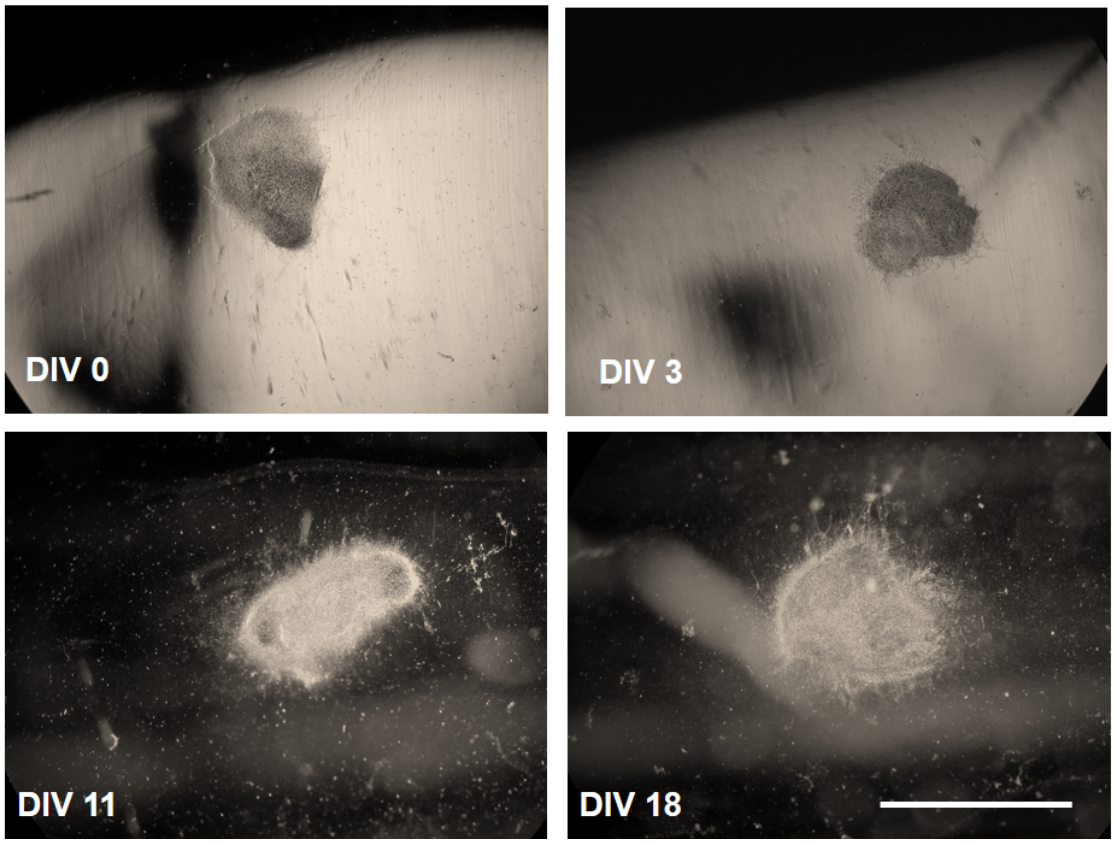

Figure 2B. Single organotypic mono-culture of Substantia nigra following. Monitoring triple organotypic cultures from substantia nigra (SN). The progression of cultures is shown at DIV 0, 3, 11 and 18. Observe that the thickness of the tissue decreases over time (scale bar: 1000µm).

At DIV 18, cultures were subjected to a recurrent insult, consisting of a high concentration of H_2O_2 (0.25–45 mM), added to the culture medium for 18 h. After a 48-h recovery period, the cultures were assessed for cell viability (alive and dead cells, LIVE/Dead Viability/Cytotoxicity® kit) or formalin fixed for assessing cell phenotype. In parallel experimental series, ADP/ATP and GSSG/GSH ratio, as well as reducing/antioxidant power were assessed. **Table 2 A-B** shows the effect of PA and H_2O_2 (0.25–45 mM) on the number of EthD-1$^+$ cells/mm^3, quantified in SN, Str and Cx of organotypic cultures from control (CS) and asphyxia-exposed (AS) neonates. In cultures from CS animals treated with 0 mM H_2O_2, the number of dying cells was similar in SN, Str and Cx (2054-2980 EthD-1-positive cells/mm^3), but it was several times increased in cultures from AS animals (7264-9789 EthD-1$^+$ cells/mm^3).

Table 2A,B. Effect of perinatal asphyxia and recurrent insult (A). Protection by nicotinamide (B). Triple organotypic cultures (substantia nigra, neostriatum, neocortex) from asphyxia-exposed and control neonates were exposed to a high concentration of H_20_2, evaluating changes in cell viability (Ethidium-homodimer positive cells/mm^3) (approximately 8 samples per brain region), assessed at 100X with an inverted Nikon T100 microscope at DIV 18.

Table 2A: Effect of different concentrations of H_20_2 (0.25-45 mM) added to the culture medium for 18h. *Effect of PA* (**[a]P<0.05**; CS versus AS); *effect of H_20_2* (**[b]P<0.05**; H_20_2 treated versus the corresponding control (CS or AS; H_2O_2 0mM). Data expressed as the mean ± SEM.

Experimental groups	**Effect of perinatal asphyxia and recurrent (H_20_2) insult**: Brain regions		
Caesarean-delivered (CS)	**Substantia nigra**	**Neostriatum**	**Neocortex**
H_2O_2 0mM; n=6	2980±252	2156±236	2054±1 99.8
H_2O_2 0.25mM; n=6	**13900±647 (>4X)[b]**	**13186±859 (>5X)[b]**	**17047±1057 (>7X)[b]**
H_2O_2 0.5mM; n=6	**15629±675 (>5X)[b]**	**13427±614 (>5X)[b]**	**17129±990 (>7X)[b]**
H_2O_2 1mM; n=6	**18037±889 (>6X)[b]**	**15763±714 (>6X)[b]**	**17723±785 (>7X)[b]**
H_2O_2 45mM; n=4	**116688±2940 (>35X)[b]**	**65053±2390 (>25X)[b]**	**83298±2164 (>35X)[b]**
Asphyxia-exposed (AS)			
H_2O_2 0mM; n=6	**9537±382 (>3X)[a]**	**9789±637 (>4X)[a]**	**7264±435 (>3X)[a]**
H_2O_2 0.25mM; n=6	9800±890	7190±416	9903±582 (>1.3X)
H_2O_2 0.5mM; n=6	**12153±508 (>1.2X)[b]**	9314±485	**10755±882 (>1.4X)[b]**
H_2O_2 1mM; n=6	**27313±1505 (>3X)[b]**	**21914±1185 (>2X)[b]**	**20794±1122 (>2X)[b]**
H_2O_2 45mM; n=4	**137698±2252 (>10X)[b]**	**144969±4385 (>15X)[b]**	**141269±3269 (>19X)[b]**

Table 2B: Effect of saline (Sal, 100 µl, i.p.), or nicotinamide (NAM, 0.8 mmol/kg, i.p.) 1h after delivery on the effect of 0 mM or 1 mM of H_20_2 ([a]**P<0.05**; CS+Sal versus AS+Sal); ([b]**P<0.05**, CS+Sal+0mM H_20_2 versus CS+Sal+1mM H_20_2, or AS+Sal+0mM H_20_2 versus AS+Sal+1mM H_20_2); ([c]**P<0.05**, CS+Sal+0mM H_20_2 versus CS+NAM+0mM H_20_2; CS+Sal+1mM H_20_2 versus CS+NAM+1mM H_20_2; AS+Sal+0mM H_20_2 versus AS+NAM+0mM H_20_2; AS+Sal+1mM H_20_2 versus AS+NAM+1mM H_20_2). Data expressed as the mean ± SEM.

Experimental groups	**Effect of nicotinamide** Brain regions		
Caesarean-delivered (CS)	**Substantia nigra**	**Neostriatum**	**Neocortex**
CS+Sal+0mM H_2O_2; n=6	2807±173	2856±172	2173±190
CS+NAM+0mM H_2O_2; n=6	**1913±190 (by 32%)[c]**	2632±183	**1360±130 (by 38%)[c]**
CS+Sal+1mM H_2O_2; n=6	**16059±419 (>5X)[b]**	**13701±415 (>4X)[b]**	**17833±615 (>8X)[b]**
CS+NAM+1mM H_2O_2; n=6	**11606±364 (by 28%)[c]**	**9640±314 (by 30%)[c]**	**11962±294 (by 33%)[c]**
Asphyxia-exposed (AS)			
AS+Sal+0mM H_2O_2; n=6	**7057±270 (>2-fold)[a]**	**9320±351 (>3-fold)[a]**	**8100±296 (>3-fold)[a]**
AS+NAM+0mM H_2O_2; n=6	**4327±267 (by 39%)[c]**	8486±344	6047±277 **(by 26%)[c]**
AS+Sal+1mM H_2O_2; n=6	**27325±449.4 (>3X)[b]**	**23617±729 (>2X)[b]**	**21142±567 (>2X)[b]**
AS+NAM+1mM H_2O_2; n=6	**14542±1454 (by 47%)[c]**	20362±345	**14585±350 (by 31%)[c]**

Under the basal condition (0 mM H_2O_2) cell death was significantly decreased in all regions of cultures from nicotinamide-treated animals (CN or AN, *c*= P<0.05), compared to the corresponding vehicle groups (CSal or ASal). The neuroprotective effect induced by nicotinamide administration *in vivo* was different depending upon the analysed region. Substantia nigra showed the largest decrease in cell death after nicotinamide treatment. In most cases, nicotinamide-treated samples showed fewer dead cells/mm^3 when compared to the corresponding controls (CSal or ASal), following 0 mM or 1 mM of H_2O_2 **(Table 2B; Figure 3 A-D).**

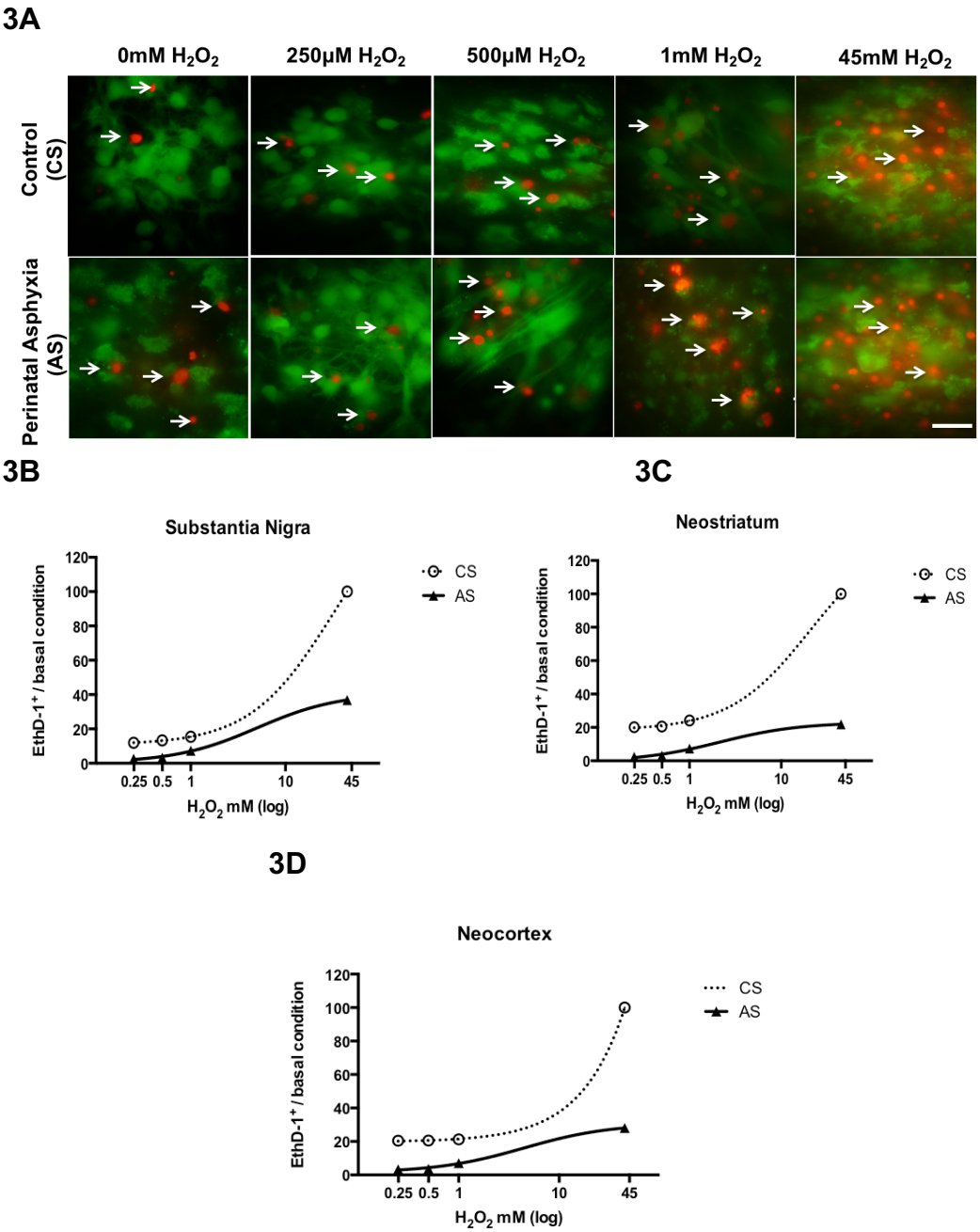

Figure 3. Viability test of substantia nigra, neostriatum and neocortex of organotypic cultures from control and asphyxia-exposed rats: effect of H_2O_2 treatment. At DIV 21-22, cell viability was evaluated in triple organotypic cultures. (A) Representative microphotographs of substantia nigra, showing alive (Calcein-AM$^+$, green fluorescence) and dead (EThD-1$^+$, red fluorescence, arrows) cells in cultures from control and asphyxia-exposed rats, treated with increasing concentrations of H_2O_2 (0, 0.25, 0.5, 1.0 and 45 mM), added directly into the culture medium for 18h and a 48h recovery period. *Scale bar:* 50µm. (B-D) Quantification of EThD-1$^+$ cells/mm^3 in (B) substantia nigra, (C) neo-striatum and (D) neocortex from control (dashed lines; N=6) and asphyxia–exposed (black lines; N=6) rats. Significant differences between CS and AS were only observed under 1 mM H_2O_2 treatment. $p<0.05$, F-ANOVA followed by a post-hoc Fischer LSD test.

A H_2O_2 challenge led to a concentration-dependent increase in cell death, in cultures from both CS and AS animals. A significant increase in cell death was already observed at 0.25 mM of H_2O_2 in CS cultures (>4-fold), but in AS cultures, a significant increase was only observed after 0.5 mM of H_2O_2 (>1.2-fold). At the highest tested concentration (45 mM of H_2O_2), cell death increased several times in all cultures, the effect was still more prominent in CS than in AS cultures (Figs. 2 and 3). Larger than 45 mM of H_2O_2 resulted in lack of viability in all cases.

The effect of nicotinamide on PA and on a recurrent insult was evaluated following 1 mM of H_2O_2 only. **Table 3** shows the effect of saline (Sal) or nicotinamide (NAM) on the number of EthD-1+ cells/mm as above. Pre-treatment with nicotinamide decreased the number of dying cells in SN and Cx from both control and asphyxia-exposed animals (by ≈30%), treated with 0 mM H_2O_2. No effect was observed in Str. One mM of H_2O_2 increased cell death in CS (>four- to eight-fold) and AS (>three-fold) cultures, decreased by nicotinamide in all regions, although the effect on Str cell viability was minor.

Effect of perinatal asphyxia and nicotinamide on neuronal (MAP-2, TH) and astroglial (GFAP) cell phenotypes following a recurrent metabolic insult.

To study the phenotype of cells responding to PA and to a recurrent metabolic insult, mono and triple cultures were formalin fixed and treated for immunofluorescence and confocal microscopy. *In situ* DNA nick-end labelling (TUNEL) was performed to identify cells undergoing apoptosis, treated also for neuronal (MAP-2), astroglial (GFAP) and dopamine (tyrosine hydroxylase, TH) phenotype. TH was only evaluated in mesencephalon, the region where dopamine cell bodies are localized. At the end of the immunofluorescence protocol, the samples were labelled with DAPI, for identifying nuclei, making possible to quantify individual cells **(Table 3, Figure 4).**

Morphological evaluation of nuclei was carried out as a control for positive TUNEL stain. Since TUNEL-positive apoptotic bodies have different size in degenerated cultures (Elliot et al. 2007), the morphology of all TUNEL-positive nuclei was analysed using a filter for the wavelength of DAPI stain (Figure 4).

A total of 8 samples per zone was obtained by confocal microscopy. The inspected area was 0.04 mm^2 for each stack. The thickness (Z-axis) was measured for each case. The number of TUNEL positive cells in samples from CS and AS was counted using ImageJ software, confirmed by proper DAPI labelling. TUNEL positive cells were counted manually, when immunoreactivity overlapped at three levels through a section (Z-step 1 μm). An investigator blinded to the treatment made the respective quantification for all parameters.

The data is expressed as DAPI /$TUNEL^+$ cells/mm^3. Similar density of nuclei was observed in fields of the same region (SN, STR and CX), regardless of PA or H_2O_2 treatment (CS, CSal, CN, versus AS, ASal, AN).

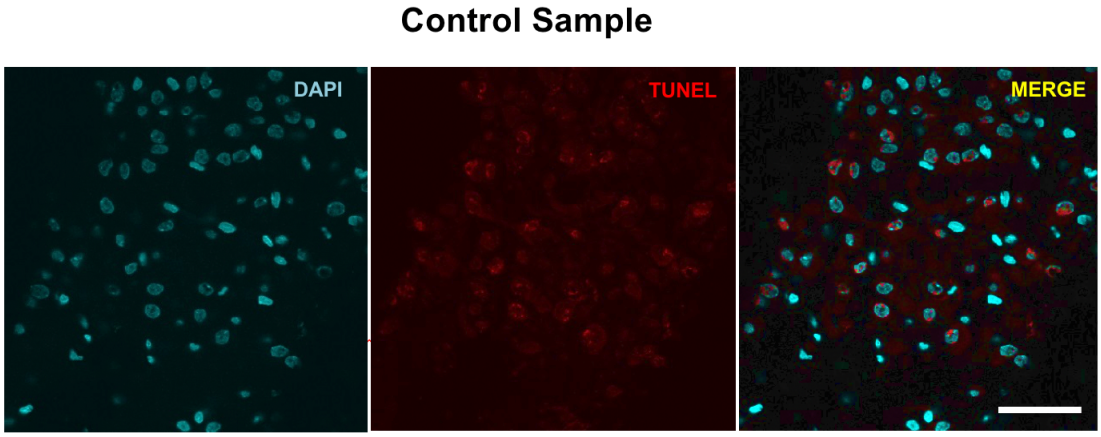

Figure 4. DAPI positive nuclei (DAPI$^+$) and apoptotic nuclei (TUNEL$^+$/DAPI$^+$) in a control sample from Cx.
Nuclei (DAPI$^+$, light blue) are observed in left pannel. Apoptotic nuclear bodies (TUNEL$^+$, red) are observed in middle pannel and the co-localisation of TUNEL$^+$/DAPI$^+$, used as a morphological requirement for quantification is shown in the right pannel. *Scale bar:* 50µm.

Table 3: Quantification of DAPI/TUNEL positive cells evaluated in triple organotypic cultures. Number of DAPI and DAPI/TUNEL$^+$ cells per mm^3 (6-8 samples per brain region, evaluated at 60X with an Olympus Confocal Microscope fv.10i). Multivariate analysis of variance (MANOVA) was performed. Two-way F-ANOVA indicated a significant effect of PA ($F_{(7,64)}$ = 44.818, ^{a}P<0,001), 1 mM H_2O_2 ($F_{(5,59)}$ = 28.43, ^{b}P<0,001), and Nicotinamide ($F_{(6,59)}$ = 39.477, ^{c}P<0,001), ns= non-significant.

Experimental groups	**Number of DAPI and DAPI/TUNEL$^+$ (cells/mm^3)**					
	Substantia nigra		**Neostriatum**		**Neocortex**	
Caesarean-delivered (CS)	**DAPI /mm^3**	**DAPI /TUNEL /mm^3**	**DAPI /mm^3**	**DAPI /TUNEL /mm^3**	**DAPI /mm^3**	**DAPI /TUNEL /mm^3**
CS +0mM H_2O_2; n= 6	95763±2099.08	11863±418.16	123303±2458.81	11266±416.96	105793±2504.57	7921±234.82
CS+N+0mM H_2O_2; n= 6	97693±2377.48	7610±342.96	124463±1925.86	11371 ±493.49	85996±6712.59	4446±448.38
CS +1mM H_2O_2; n= 6	81232±1127.53	**31907±623.95 (>2.7 X)^b**	114809±1704.56	**19233±735.57 (>1.7 X)^b**	100436±1268.95	**12925±635.35 (1.63 X)^b**
CS+N+1mM H_2O_2; n= 6	94780±2223.72	**21022±997.17 (by 34%)^c**	123625±2314.56	**15352**±779.15 **(by21%)^c**	100155±2842.47	**9740 ±465.97 (by 25%)^c**
Asphyxia-exposed (AS)						
AS +0mM H_2O_2; n= 6	85425±1607.56	**14819 ±500.31 (>1.24 X)^a**	132868±1953.38	12748±666.29 (1.13 X)	102924±1451.32	**9140 ±492.94 (by 17%)^a**
AS+N+0mM H_2O_2; n= 6	84802±1339.44	11896±518.19	124463±1925.89	12025 ±460.42	103292±2249.46	7956 ±446.07
AS+1mM H_2O_2; n= 6	70282±1293.48	**39815±1005.24 (>2.68 X)^b**	104719±1926.85	**20480±998.15 (>1.6 X)^b**	105389±1184.47	**13475±648.57 (>2.1 X)^b**
AS+N+1mM H_2O_2; n= 6	76068±1543.49	**32532 ±925.85 (by 18%)^c**	132223±2455.91	**18246±776.71 (by 14%)^c**	100314±1819.71	**10173±514.49 (by 25%)^c**

*Substantia nigra (**Table 4**)*

The TUNEL assay showed that PA increased cell death in SN *in vitro*, similarly to that observed by the Viability Eth-D1 probe, *in vitro*.

Analysis of MAP-2 revealed a significant decrease in the number of neurons in SN of triple cultures from AS, compared to that from CS neonates (Figure 5). Approximately 20% of MAP-2 positive neurons were also positive to TH, both in cultures from CS and AS animals. As the number of DAPI/TUNEL$^+$ neurons increased in AS samples, TH positive neurons decreased by approximately 50% in SN of AS animals (**Table 4, c.f.9**).

1 mM of H_2O_2 led to a significant decrease in the number of both MAP-2 and TH-positive cells, being the effect particularly prominent in cultures from AS animals (Figure 7). The effect of H_2O_2 was also observed when co-labelling for MAP-2/TUNEL (increased >2-fold). Interestingly, 1 mM of H_2O_2 treatment had an effect in SN of CS cultures only, but not in SN of AS samples. The effect of 1 mM H_2O_2 was largely prevented by nicotinamide, both in cultures from asphyxia-exposed (AN) and control (CN) animals. No protection by nicotinamide was observed on the number of GFAP-positive cells, but the number of GFAP/TUNEL positive cells was strongly increased by 1 mM of H_2O_2 (≈3 fold), in cultures from both CS and AS animals (Figure 6).

Table 4: **Effect of perinatal asphyxia on vulnerability to a recurrent metabolic insult evaluated in SN organotypic cultures: protection by nicotinamide (NAM).** Number of positive cells for neuronal (MAP-2, TH) and astroglia (GFAP) markers per mm^3 (6-8 samples per brain region). Multivariate analysis of variance (MANOVA) was performed. Two-way F-ANOVA indicated a significant effect of PA ($F_{(7,31)}$ = 18.922, [a]P<0,001), 1 mM H_2O_2 ($F_{(3,18)}$ = 21.155, [b]P<0,001), and NAM ($F_{(4,14)}$ = 22.32, [c]P<0,001), ns= non-significant.

Experimental groups	**Substantia nigra**					
Caesarean delivered (CS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**TUNEL$^+$ Neuronal and astrocyte double labelled cell/mm^3**		
	MAP2 /mm^3	**GFAP /mm^3**	**MAP2+ TH /mm^3**	**MAP2+ /mm^3**	**GFAP+ /mm^3**	**MAP2+TH+ /mm^3**
CS+0mM H_2O_2; n=6	38811±846.83	60972±1110	10617±347.38	4416±270.09	7863±389.70	1251±139.53
CS+N+ 0mM H_2O_2; n=6	43974±889.93	58343±792.6	10825±308.69	3257±213.29	5710±370.52	816±130.17
CS+1mM H_2O_2; n=6	**20607±575.65 (by 47%)[b]**	60795±633	**7240±309.39 (by 32%)[b]**	**8603±266.35 (>1.9)[b]**	**23863±724.38 (>3 X)[b]**	**3810±271.04 (>3 X)[b]**
CS+N+ 1mM H_2O_2; n=6	**32444±902.82 (>1.5 X)[c]**	52491±3707.3	8745±372.79	**5723±302.56 (by 35%)[c]**	**11790±929.26 (by 50%)[c]**	**1402±146.99 (by 67%)[c]**
Asphyxia-exposed (AS)				**+TUNEL**		
AS+0mM H_2O_2; n=6	**30821±558.86 (by 21%)[a]**	58898±1155.5	**5663±293.11 (by 47%)[a]**	**2494±237.16 (by 43%)[a]**	**11137±338.48 (>1.4 X)[a]**	1484±173.57
AS+N+ 0mM H_2O_2; n=6	33860±912.55	52830±1180.51	7348±352.36	3739±319.57	6232±245.89	1038±123.49
AS+1mM H_2O_2; n=6	**12858±466.80 (by 58%)[b]**	63031±2017.36	**1741±207.64 (by 69%)[b]**	**7372±382.18 (>2.9 X)[b]**	**28525±718.51 (>2.5 X)[b]**	1680±193.90
AS+N+ 1mM H_2O_2; n=6	**16680±771.65 (>1.3 X)[c]**	56214±966.8	**4476±392.19 (>2.5 X)[c]**	6246±375.59	30166±747.36	1431±207.78

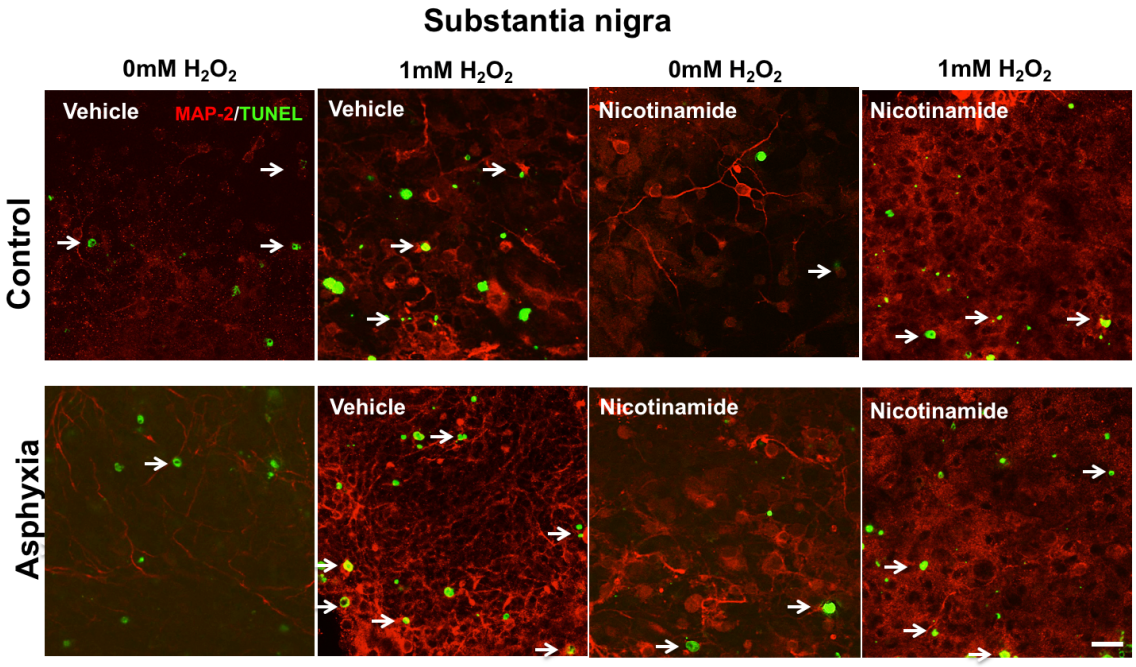

Figure 5. Neurons (MAP-2$^+$) and neuronal apoptosis (TUNEL$^+$/MAP-2$^+$) in substantia nigra of triple organotypic cultures from control and asphyxia-exposed rats after 0 or 1 mM H_2O_2 treatment: effect of nicotinamide.
Representative microphotographs showing TUNEL$^+$ (green) and MAP-2$^+$ (red)-cells in substantia nigra from triple organotypic cultures of control and asphyxia-exposed rats. Arrows show TUNEL/MAP-2 double labelling, indicating neuronal apoptosis. *Scale bar:* 20µm.

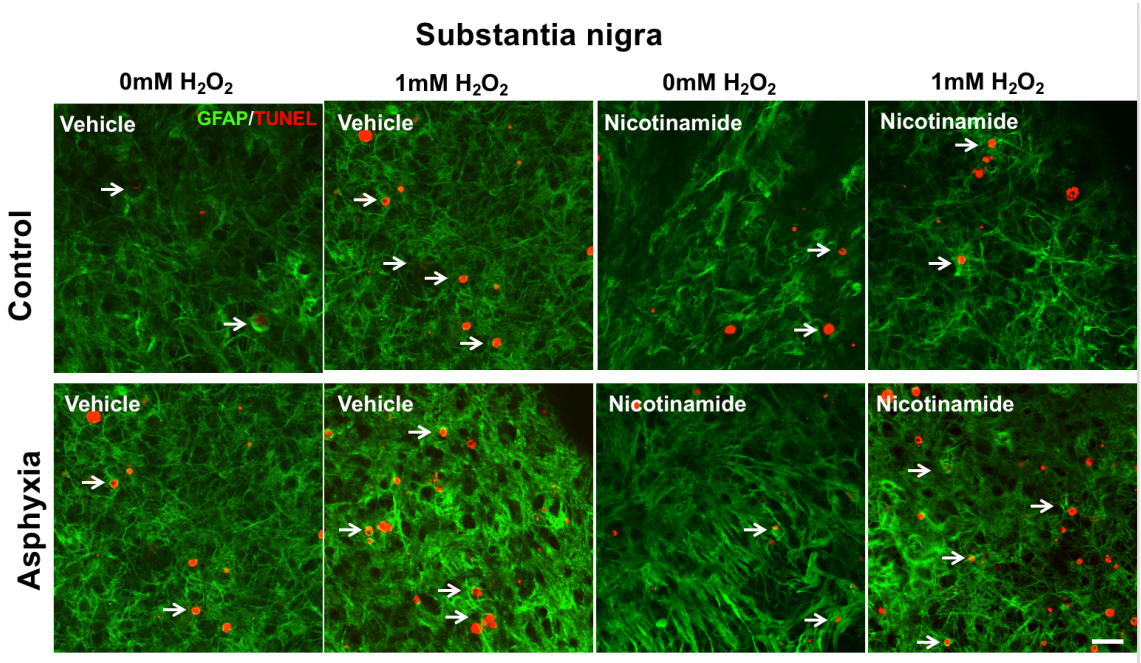

Figure 6. Glial cells (GFAP$^+$) and astroglial apoptosis (TUNEL$^+$/GFAP$^+$) in substantia nigra of triple organotypic cultures from control and asphyxia-exposed rats after 0 or 1 mM H_2O_2 treatment: effect of nicotinamide. Representative microphotographs obtained by confocal microscopy showing TUNEL$^+$ (red) and GFAP$^+$ (green)- cells in substantia nigra from triple organotypic cultures of control and asphyxia-exposed rats. Arrows show TUNEL/GFAP double labelling, indicating astroglial apoptosis. *Scale bar:* 20µm.

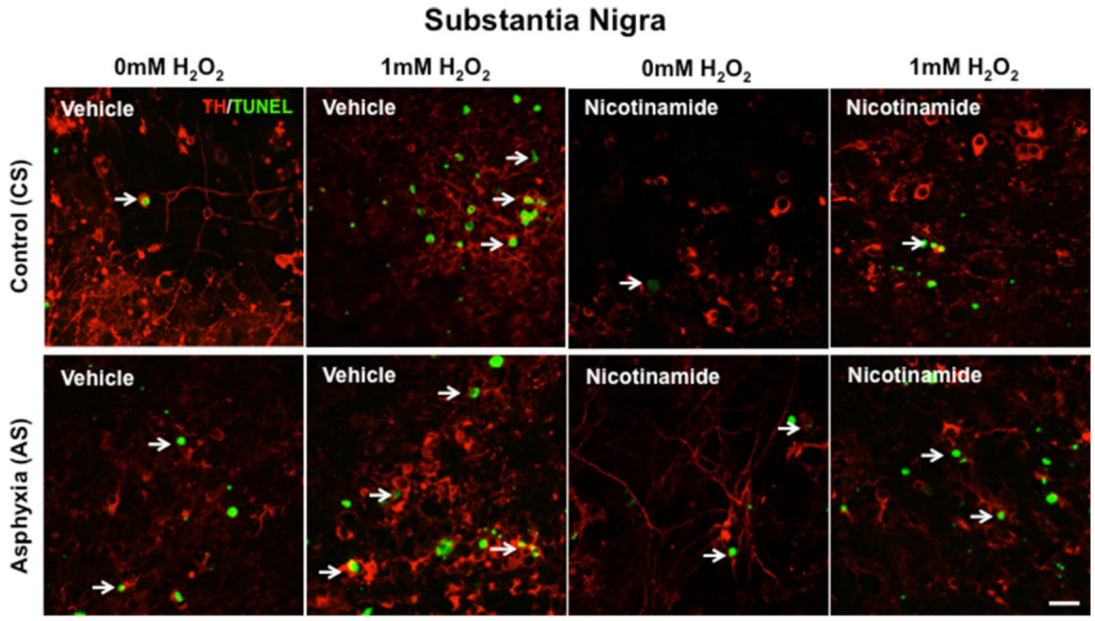

Figure 7. Dopaminergic cells (TH^+) and dopaminergic apoptosis ($TUNEL^+/TH^+$) in substantia nigra of triple organotypic cultures from control and asphyxia-exposed rats after 0 or 1 mM H_2O_2 treatment: effect of nicotinamide.

Representative microphotographs obtained by confocal microscopy showing $TUNEL^+$ (green) and TH^+ (red)- cells in substantia nigra from triple organotypic cultures of control and asphyxia-exposed rats. Arrows show TUNEL/TH double labelling, indicating astroglial apoptosis. *Scale bar:* 20µm.

Neostriatum (Table 5)

In contrast with the findings in SN, no differences were observed in the number of MAP-2^+ cells in AS, compared to that in CS samples, although 1 mM H_2O_2 produced a strong increase in the number of MAP-2/TUNEL$^+$ cells both in CS (>2X) and AS groups (>4X). The number of GFAP$^+$ cells was not decreased by PA, even when GFAP/TUNEL$^+$ cells increased. 1 mM of H_2O_2 increased the number of GFAP/TUNEL$^+$ cells in neostriatum of CS (≈2-fold), but the number of GFAP/TUNEL positive cells was instead decreased (34%) in cultures from AS animals. Nicotinamide prevented both neuronal and astroglial apoptosis as shown by a decreased number of MAP-2/TUNEL$^+$ and GFAP/TUNEL$^+$ cells in CS and AS samples.

Table 5: Effect of perinatal asphyxia on vulnerability (apoptosis-like cell death) to a recurrent metabolic insult evaluated in organotypic cultures: protection by nicotinamide (NAM) in neostriatum. Number of positive cells for neuronal (MAP-2, TH) and astroglia (GFAP) per mm^3 (6-8 samples per brain region, evaluated at 60X with an Olympus Confocal Microscope fv.10i). Multivariate analysis of variance (MANOVA) was performed. Two-way F-ANOVA indicated a significant effect of PA ($F_{(6,26)}$ = 35.465, [a]P<0,001), 1 mM H_2O_2 ($F_{(3,46)}$ = 33.468, [b]P<0,001), and NAM ($F_{(8,31)}$ = 44.924, [c]P<0,001), ns= non-significant.

Experimental groups	**Neostriatum**					
Caesarean delivered (CS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled cell/mm^3**		
	MAP2/mm^3	**GFAP/mm^3**	**MAP2 +TH /mm^3**	**MAP2 /mm^3**	**GFAP /mm^3**	**MAP2 +TH /mm^3**
CS+0mM H_2O_2; n=6	46074±1143.43	80484±1714.61	nd	2805±215.44	6683±283.43	nd
CS+N+0mM H_2O_2; n=6	43637±899.10	65960±4794.81	nd	1121±195.47	6061±536.81	nd
CS+1mM H_2O_2; n=6	43092±1171.82	86100±1530.57	nd	**8225±267.38 (>2.9 X)[b]**	**13097±727.16 (>1.9 X)[b]**	nd
CS+N +1mM H_2O_2; n=6	44358±1203.06	67106±1252.40	nd	**4696±274.40 (by 43%)[c]**	**5277±241.91 (by 60%)[c]**	nd
Asphyxia-exposed (AS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled cell/mm^3**		
AS+0mM H_2O_2; n=6	45695±1065.92	70846±920.15	nd	2445±274.42	**9201±348.14 (>1.99 X)[a]**	nd
AS+N+0mM H_2O_2; n=6	45967±845.79	66855±4750.92	nd	1658±162.15	6235±503.83	nd
AS+1mM H_2O_2; n=6	39995±1207.66	59608±808.65	nd	**13822±548.77 (>4.6 X)[b]**	**6066±278.62 (by 43%)[b]**	nd
AS+N+1mM H_2O_2; n=6	39234±715.81	43301±3400.09	nd	**11322±546.67 (by 20%)[c]**	**4962±429.59 (>1.15 X)[c]**	nd

Neocortex (Table 6)

In neocortex, PA showed no significant effect on $MAP2^+$ and $GFAP^+$ cells. Apoptosis (TUNEL) only increased when co-labelled with neuronal phenotype (>1.6X). H_2O_2 treatment increased neuronal apoptosis (≈3 fold) in CS and AS groups, but surprisingly, astroglial apoptosis increased only in CS samples. Nicotinamide treatment prevented neuronal and astroglial apoptosis in AS and CS samples, but it protected against the effect of 1 mM of H_2O_2 on cells with neuronal (MAP-2/$TUNEL^+$) phenotypes.

Table 6: Effect of perinatal asphyxia on vulnerability to a recurrent metabolic insult evaluated in the of organotypic cultures (apoptosis-like cell death): protection by nicotinamide (NAM) in neocortex (Cx). Number of positive cells for neuronal (MAP-2, TH) and astroglia (GFAP) per mm^3 (6-8 samples per brain region, evaluated at 60X with an Olympus Confocal Microscope fv.10i). Number of positive cells for each marker per mm^3 (6 samples per brain region). Multivariate analysis of variance (MANOVA) was performed. Two-way F-ANOVA indicated a significant effect of PA ($F_{(7,28)}$ = 43.679, [a]P<0,001), 1 mM H_2O_2 ($F_{(8,23)}$ = 21.17, [b]P<0,001), and NAM ($F_{(9,37)}$ = 64.16, [c]P<0,001), ns= non-significant.

Experimental group	**Neocortex**					
Caesarean delivered (CS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled cell/mm^3**		
	MAP2/mm^3	**GFAP/mm^3**	**MAP2 +TH /mm^3**	**MAP2/mm^3**	**GFAP /mm^3**	**MAP2 +TH /mm^3**
CS+0mM H_2O_2; n=6	42693±771.20	71935±916.40	nd	1517±152.86	5976±200.10	nd
CS+N+ 0mM H_2O_2; n=6	31476±2447.96	75047±1503.56	nd	**957±176.65 (by 58%)[c]**	**4868±261.59 (by 22%)[c]**	nd
CS+1mM H_2O_2; n=6	**31836±555.27 (by 25%)[b]**	75475±794.91	nd	**4757±301.68 (>3 X)[b]**	5315±336.14	nd
CS+N+1mM H_2O_2; n=6	33938±1298.56	75693±1942.69	nd	**2386±235.79 (by 50%)[c]**	**7142±326.62 (by 30%)[c]**	nd
Asphyxia-exposed (AS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled cell/mm^3**		
AS+0mM H_2O_2; n=6	41081±981.12	65568±1528.47	nd	**3268±317.47 (>1.6 X)[a]**	6825±268.32	nd
AS+N+0mM H_2O_2; n=6	41106±1081.41	62407±1217.76	nd	**2004±243.26 (by 63%)[c]**	**4826±207.50 (by 41%)[c]**	nd
AS+N+1mM H_2O_2; n=6	37500±1192.39	65151±789.38	nd	**6857±346.72 (>2.7 X)[b]**	6407±244.45	nd
AS+N+1mM H_2O_2; n=6	**33059±1062.25 (by 12%)[b]**	60661±815.55	nd	**3578±364.49 (by 48%)[c]**	6748±278.41	nd

Effect of perinatal asphyxia and nicotinamide on cell metabolism, oxidative stress and cytotoxicity produced by a recurrent metabolic in triple organotypic culture.

To determine changes in energy metabolism, triple cultures were sampled for measuring ADP/ATP, using a bioluminescent ADP/ATP ratio Assay (Sigma-Aldrich, MAK135), evaluating the effect of H_2O_2 on cultures from asphyxia-exposed and control animals. The ADP/ATP ratio was used as an indicator of energy expenditure and cell viability. Increased levels of ATP and decreased levels of ADP indicated proliferating and healthy cells. Conversely, decreased levels of ATP and increased levels of ADP indicated apoptotic or necrotic phenomena.

It was found that PA modified ADP/ATP ratios *in vitro* (Table 8), implying a long-term energetic deficiency, which was confirmed by analysing the effect of 1 mM of H_2O_2, resulting in an increased ADP/ATP ratio in cultures from both AS and CS animals. The metabolic insult increased the ADP/ATP ratio in all experimental groups, but the effect was more prominent in AS samples >5-fold in AS, compared to than that from CS). Similarly, the H_2O_2 insult increased >7-fold the ADP/ATP ratio in CS, but approximately >2-fold in cultures from AS, indicating that AS samples cope better with postnatal stressor than CS.

The effect of PA on the ADP/ATP ratio was significantly decreased by nicotinamide, also when evaluating the effect of 1 mM H_2O_2. The protection by nicotinamide may be explained by PARP-1 inhibition, reported to be an effective strategy to prevent cell death caused by PA and oxidative stress conditions, but also by NAD^+ replacement (Klawitter et al 2006; Trammell et al. 2016; Kawamura et al. 2016).

Effect of a recurrent metabolic insult after perinatal asphyxia and Nicotinamide administration on Oxidative stress.

To explore changes in redox metabolism, reduced (GSH) and oxidized (GSSG) glutathione levels were monitored. GSH is critical for protecting the brain from oxidative stress, acting as a free radical scavenger, also as inhibitor of lipid peroxidation. The total GSH/GSSG ratio is an indicator of physiological homeostasis (Wu et al. 2004). Reduced glutathione is a scavenger of reactive oxygen species (ROS) (Folbergrová et al. 1979), and its ratio with oxidised glutathione (GSSG) can be used as a marker for oxidative stress (Jones, 2002). In this thesis, the cultures were exposed to 1 mM H_2O_2, finding a decrease in the GSH/GSSG ratio by approximately 40% in all experimental groups (Table 7). It was also found that the GSH/GSSG ratio of AS samples was decreased (by approximately 40%), compared to the controls under 0 mM of H_2O_2 condition, suggesting a sustained stress condition elicited by the perinatal hypoxic insult, which was attenuated by nicotinamide treatment.

Effect of a recurrent metabolic insult after perinatal asphyxia and Nicotinamide administration on antioxidant capacity using a colorimetric probe.

To establish the capacity of the tissue to deal with postnatal stressors following PA, total antioxidant capacity was measured using the ferricyanide/Prussian blue method for reducing capacity. This method is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}), which is accomplished in the presence of antioxidants. Substances with a reduction potential react with potassium ferricyanide, forming potassium ferrocyanide, which further reacts with $FeCl_3$, producing an intense Prussian blue complex, with a maximum absorbance at 700 nm. The amount of formed complex is directly proportional to the reducing power of the test sample.

When normalized by the total protein content, it was revealed that cultures from asphyxia-exposed animals showed a decreased reducing capacity (by 37% compared to the controls), which was prevented by nicotinamide (Table 8). The H_2O_2 insult decreased total reducing capacity only in CS samples.

Table 7: Effect of a recurrent metabolic insult after perinatal asphyxia and Nicotinamide administration on antioxidant capacity and LHD release. Asphyxia-exposed animals showed a decreased reducing capacity compared to the controls, which was prevented by nicotinamide. The H_2O_2 insult decreased total reducing capacity only in CS samples. Data expressed as the mean ± SEM. Two-way F-ANOVA indicated a significant effect of PA ($F_{(7,11)} = 19.32$, $^{a}P<0,001$), 1 mM H_2O_2 ($F_{(7,23)} = 31.27$, $^{b}P<0,001$), and NAM ($F_{(7,62)} = 25.62$, $^{c}P<0,001$), ns= non-significant. LDH release assay applied to supernatants from triple organotypic cultures. Values are expressed as percentage of a positive control of LDH. (means ± SEM, n=4). Multivariate analysis of variance (MANOVA) was performed. Two-way F-ANOVA indicated a significant effect of PA ($F_{(3,17)} = 15.19$, $^{a}P<0,001$), 1 mM H_2O_2 ($F_{(3,12)} = 21.11$, $^{b}P<0,001$), and NAM ($F_{(4,22)} = 31.17$, $^{c}P<0,001$), ns= non-significant.

Experimental groups	ADP/ATP ratio	GSH/GSSG ratio	*Potassium Ferricyanide reducing power assay.*	% of LDH release in Triple organotypic cultures / LDH control
Caesarean-delivered (CS)				
CS+0mM H_20_2; n=6	0.072 ± 0.022	85.018 ± 4.97	0.382 ± 0.019	11.452 ± 0.379
CS+NAM; n=6	0.106 ± 0.03	86.39 ± 5.32	0.379 ± 0.011	9.5 ± 0.795
CS+1mM H_20_2; n=6	**0.529 ± 0.02 (>7-fold)^{b}**	**47.82 ± 3.19 (by ≈40%)^{b}**	**0.3003±0.01 (by≈20%)^{b}**	**23.052 ± 0.315 (>2 fold)^{b}**
CS+1nM H_20_2+NAM; n=6	0.588 ± 0.031	46.65 ± 4.01	0.319 ± 0.01	21.867 ± 0.513
Asphyxia-exposed (AS)				
AS+0mM H_20_2; n=6	**0.432 ± 0.058 (>6 fold)^{a}**	**54.27 ± 4.07 (by ≈40%)^{a}**	**0.254 ± 0.011 (by ≈30%)^{a}**	**18.004 ± 0.287 (>1.5 fold)^{a}**
AS+NAM; n=6	0.158 ± 0.014	65.31 ± 3.97	0.324 ± 0.02	16.094 ± 0.423 (by 10%)^{ns}
AS+1mM H_20_2; n=6	**0.793 ± 0.039 (2-fold)^{b}**	**32.45 ± 3.87 (≈40%)^{b}**	0.233 ± 0.006	**26.228 ± 0.307 (>1.5 fold)^{b}**
AS+1mM H_20_2+NAM; n=6	**0.539 ± 0.034 (by 33%)^{c}**	**50.02 ± 4.41 (>1.54)^{c}**	0.311 ± 0.01	21.363 ± 0.628 (by 22%)^{ns}

Effect of a recurrent metabolic insult after perinatal asphyxia and Nicotinamide administration on cell necrosis.

To determine necrotic cell damage in cultured tissue following PA and postnatal stressors, LDH released into culture media was measured by an enzymatic colorimetric method (Chang et al. 2013). This method measures extracellular LDH in culture media using a two-step enzymatic reaction resulting in a red formazan product that can be measured spectrophotometrically. LDH release was expressed as a percentage of a positive control standard (provided by manufacturer). The results revealed that triple organotypic cultures from asphyxia-exposed animals showed a 2-fold increase in cell necrosis (Table 8). Consequently with the assessment of cellular death by viability/cytotoxicity tests and TUNEL, the increase of LDH release following 1 mM H_2O_2 was higher in CS than in AS samples.

Effect of perinatal asphyxia and nicotinamide on the specific vulnerability of substantia nigra to a recurrent metabolic insult evaluated in mono-organotypic cultures

Analysis of neuronal apoptosis

We reported that SN is a vulnerable region to the long-term consequences of PA (Neira-Peña et al. 2015). *In vivo* and *in vitro* studies demonstrated the protective effect of neonatal nicotinamide treatment, mainly in mesencephalon of PA-exposed animals, while telencephalon showed less cell damage in all experimental groups.

Neuronal phenotype was shown to be vulnerable in SN of triple organotypic cultures. Hence, an immunophenotyping analysis was performed in single-mono cultures. The analysis of immunofluorescence is shown in Table 9, demonstrating that PA decreased the number of MAP-2^+ and MAP-2/TH^+ cells, accordingly to that observed in SN of triple organotypic cultures.

The number of $TUNEL^+$ cells was, however, larger in mono-cultures of substantia nigra compared to that shown by the corresponding triple cultures. In agreement, the decrease in the number of MAP-2^+ and TH^+ cells was larger in mono- than in triple cultures (Table 4 and 10 and Figure 9).

The effect of 1 mM H_2O_2 was stronger in triple than in mono cultures. The increase of $TUNEL^+$/MAP-2^+ cells was, however, similar in triple and mono cultures from AS animals. The effect of 1 mM H_2O_2 on ADP/ATP ratio was stronger in samples from CS than that from AS animals, under both triple and the mono-culture conditions. A similar result was observed on the GSH/GSSG ratio and on total antioxidant capacity (Table 11, figure 10).

Table 8. Effect of perinatal asphyxia on vulnerability to recurrent metabolic insults evaluated in substantia nigra organotypic mono-cultures: protection by nicotinamide.

Number of positive cells for each marker per mm^3 (6-8 samples per brain region, evaluated at 60X with an Olympus Confocal Microscope fv.10i). Two-way F-ANOVA indicated a significant effect of PA ($F_{(3,41)}$ = 19.844, $^{a}P<0,001$), 1 mM H_2O_2 ($F_{(3,29)}$ = 23.499, $^{b}P<0,001$), and NAM ($F_{(3,62)}$ = 17.722, $^{c}P<0,001$), ns= non-significant.

Experimental groups	**A. Substantia nigra mono cultures**					
Caesarean delivered (CS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled cell/mm^3**		
	DAPI+ /mm^3	**MAP2+/mm^3**	**MAP2+ TH /mm^3**	**DAPI+ /mm^3**	**MAP2+ /mm^3**	**MAP2+TH+ /mm^3**
CS+0mM H_2O_2; n=5	87216±1938.44	36988±1170.18	10113±472.8	12045±530.4	2556±231.35	965±189.34
CS+NAM+ 0mM H_2O_2; n=5	81761±1534.53	34090±1023.06	**8920±332.58 (by 12%)^{c}**	**9886±330.79 (by 18%)^{c}**	2443 ±264.26	**681±172.92 (by 30%)^{c}**
CS+1mM H_2O_2; n=5	**73295±1229.72 (by 16%)^{b}**	**23352±1265.07 (by 37%)^{b}**	**5284±361.94 (by 48%)^{b}**	**33977±1422. (> 2.8 X)^{b}**	**10284±334.6 (> 4 X)^{b}**	**3920±302.6 (> 4 X)^{b}**
CS+NAM+1mM H_2O_2; n=5	75795 ±1087.78	27954 ±1107.28	**6534±348.55 (> 1.2 X)^{c}**	**26647±984.0 (by 22%)^{c}**	**6250±344.87 (by 40%)^{c}**	**2840±225.7 (by 28%)^{c}**
Asphyxia-exposed (AS)	**Neuronal and astrocyte cell density (cells/mm^3)**			**Neuronal and astrocyte double labelled (cell/mm^3)**		
AS+0mM H_2O_2; n=5	**73579±1202.83 (by 26%)^{a}**	**27840±854.76 (by 25%)^{a}**	**5397±358.17 (by 47%)^{a}**	13068±518.1	**3977±364.05 (>1.5 X)^{a}**	**2045±255.4 (>2.1 X)^{a}**
AS+NAM+ 0mM H_2O_2; n=5	74715±1185.75	**33750±1036.59 (>1.2 X)^{c}**	6079±352.43	12102±475.5	4090±323.52	**1250±182.4 (by 40%)^{c}**
AS+1mM H_2O_2; n=5	68522±1103.29	**14261±788.48 (by 49%)^{b}**	**2500±195.09 (by 54%)^{b}**	**42897±1090.2 (> 3.2 X)^{b}**	**10056±422.5 (> 2.5 X)^{b}**	2443±170.4
AS+NAM+ 1mM H_2O_2; n=5	71420±1154.98	**16250±918.39 (>1.13 X)^{c}**	**3011±264.26 (>1.2 X)^{c}**	**37215±1099.5 (by 14%)^{c}**	**8579±439.92 ç(by 15%)^{c}**	2159±231.7

Table 9: ADP/ATP ratio, GSH/GSSG ratio and Reducing Power measurements in entire sample homogenates from substantia nigra organotypic mono-cultures. Data expressed as the mean ± SEM. Two-way F-ANOVA indicated a significant effect of PA ($F_{(7,18)}$ = 20.41, [a]P<0,001), 1 mM H_2O_2 ($F_{(6,67)}$ = 28.74, [b]P<0,001), and NAM ($F_{(7,32)}$ = 28.101, [c]P<0,001), ns= non-significant.

Experimental groups	ADP/ATP ratio	GSH/GSSG ratio	Potassium Ferricyanide reducing power assay
Caesarean-delivered (CS)			
CS+0mM H_2O_2; n=6	0.068± 0.019	82.115 ± 10.42	0.377 ± 0.030
CS+NAM; n=6	0.125± 0.13	79.094 ± 9.37	0.365 ± 0.047
CS+1mM H_2O_2; n=6	**0.602 ± 0.37 (>9-fold)[b]**	**33.59** ± 5.61 **(by ≈60%)[b]**	0.284 ± 0.029
CS+1nM H_2O_2+NAM; n=6	0.645 **± 0.053**	44.29 ± 7.18	0.295 ± 0.033
Asphyxia-exposed (AS)			
AS+0mM H_2O_2; n=6	**0.505 ± 0.028 (>7.4-fold)[a]**	**59.04** ± 8.77 **(by ≈30%)[a]**	0.265 ± 0.042
AS+NAM; n=6	0.313 **± 0.017**	60.29 ± 8.64	0.309 ± 0.021
AS+1mM H_2O_2; n=6	**0.864 ± 0.079 (>1.7-fold)[b]**	28.43 ± 5.56	0.204 ± 0.017
AS+1mM H_2O_2+NAM; n=6	0.618 **± 0.044 (by 29%)[c]**	41.22 ± 7.12	0.267 ± 0.015

Effect of co-culturing on cellular death

The number of MAP-2/TUNEL+ cells was higher in SN Mono Cultures under both 0 and 1 mM H_2O_2 conditions (Figure 8), compared to that in SN of Triple Cultures. Nicotinamide protected against neuronal apoptosis after PA in single and triple cultures of SN, but 1 mM H_2O_2 treatment did not produce any effect on cell viability.

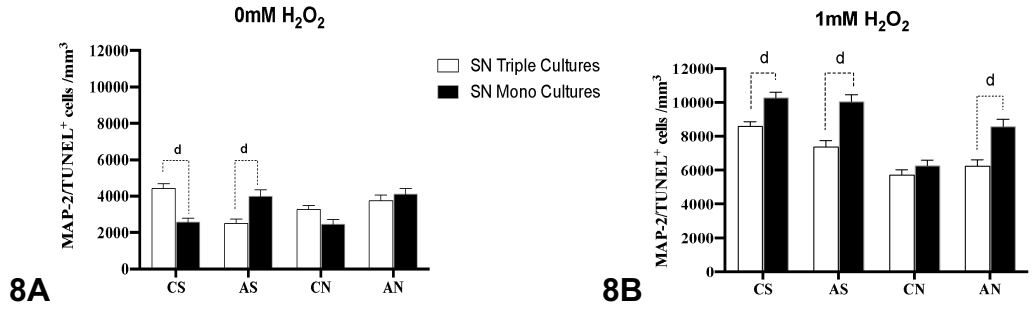

Figure 8. Neuronal apoptosis (MAP-2/TUNEL$^+$ cells) in substantia nigra of triple organotypic cultures and single mono-cultures from control and asphyxia-exposed rats after 0 (a) and 1 mM H_2O_2 treatment (b): effect of nicotinamide. The effect of co-culturing on neuronal apoptosis was evaluated by a multiple comparisons test (MANOVA). Two-way F-ANOVA indicated a significant effect of culture conditions ($F_{(3,12)} = 15.21$, $^dP<0,001$).

Apoptosis of dopaminergic neurons

The number of dopaminergic neurons (MAP-2/TH$^+$) undergoing apoptosis (TUNEL$^+$) in SN under 0 mM H_2O_2 condition was similar to that observed in triple organotypic cultures following PA (Figure 9A). Nevertheless, the effect of 1 mM H_2O_2 on the number of dopaminergic neurons undergoing apoptosis was significantly decreased in AS, compared to that in CS (Figure 9B).

The culturing protocol increased apoptosis of dopaminergic neurons only in AS tissue, showing higher number of MAP-2/TH/TUNEL$^+$ cells/mm^3 in SN monocultures. The 1 mM H_2O_2 condition increased dopamine apoptosis in monocultures of AS, AN and CN samples Interestingly, NAM treatment prevented the effect of PA only following 0 mM H_2O_2, but not the 1 mM H_2O_2 condition.

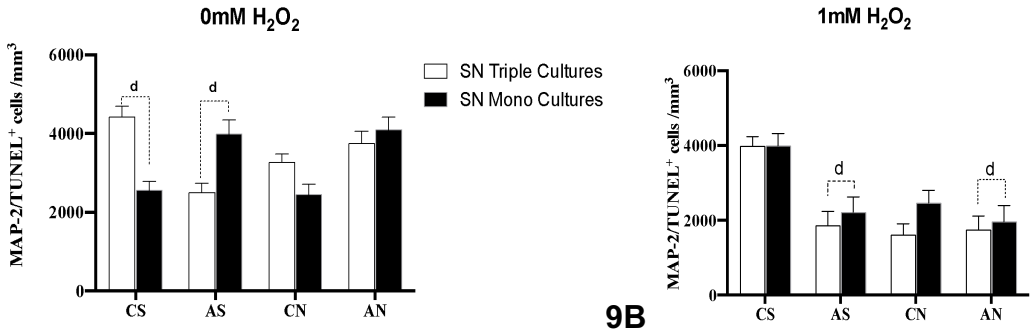

Figure 9. Neuronal apoptosis of dopaminergic neurons (MAP-2/TUNEL/TH$^+$) in substantia nigra of triple organotypic cultures and single mono-cultures from control and asphyxia-exposed rats following 0 (A) and 1 mM H_2O_2 treatment (B): effect of nicotinamide. The effect of co-culturing on apoptosis of dopaminergic neurons was evaluated by multiple comparisons test (MANOVA). Two-way F-ANOVA indicated a significant effect of culture conditions ($F_{(3,23)}$ = 52.488, ^{d}P<0,001).

Assessment of LDH release in SN monocultures

As indicator of necrotic cell death LDH release was analysed in supernatants from single and triple cultures, as described above.

LDH release was increased by PA under both 0 and 1 mM H_2O_2 conditions, but in PA monocultures LDH was already increased under the 0 mM H_2O_2 condition. No differences were observed among any of the groups following 1 mM H_2O_2. (Figure 10).

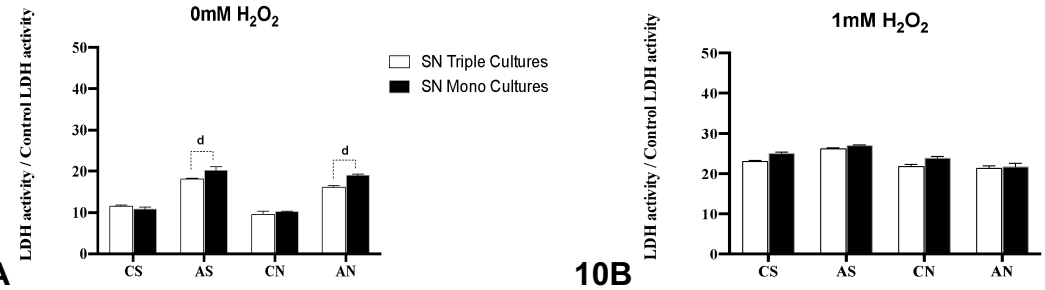

Figure 10. LDH release in substantia nigra from triple and monoorganotypic cultures from control and asphyxia-exposed rats under 0 (A) and 1 mM H_2O_2 conditions (B): effect of nicotinamide. The effect of co-culturing on LDH release was evaluated by multiple comparisons (MANOVA). Two-way F-ANOVA indicated a significant effect of culture conditions ($F_{(3,21)}$ = 15.18, ^{d}P<0,001).

Assessment of cellular vulnerability to oxidative stress in SN monocultures

Response to oxidative stress induced by PA and H_2O_2 treatment. Energy levels were also monitored in SN mono-cultures (Table 9), and comparisons made with data from triple organotypic-cultures (Figure 11 A-D).

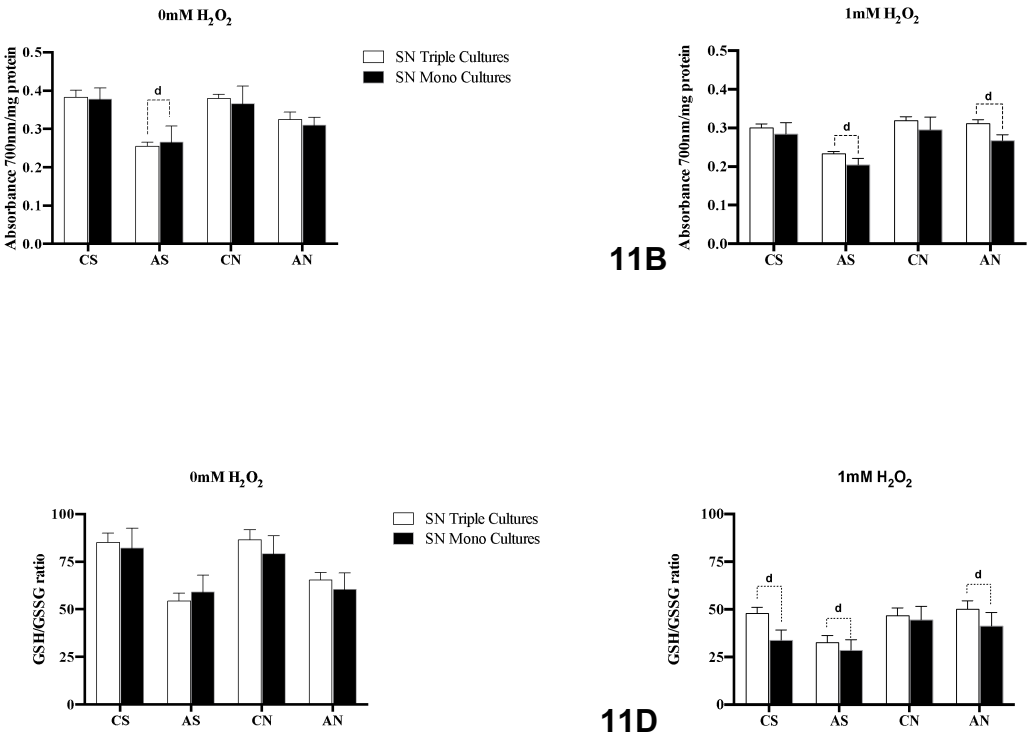

Figure 11. Total antioxidant capacity (11A-B) and GSH/GSSG ratio (11C-D) from triple and mono organotypic cultures from CS and AS rats under 0 mM H_2O_2 (A and C) and 1 mM H_2O_2 conditions (B and D): effect of nicotinamide. The effect of co-culturing on total reducing capacity and GSH/GSSG ratio was evaluated by a multiple comparison test (MANOVA). Two-way F-ANOVA indicated a significant effect of culture conditions ($F_{(3,27)} = 16.42$, $^{d}P<0,001$).

Vulnerability to energetic stress

ADP/ATP ratio was used as an indicator of cell death and tissue viability, monitored in triple and mono cultures. PA induced a long-term energy depletion, revealed as a high ADP/ATP ratio observed under the 0 mM H_2O_2 condition (Figure 12A). 1 mM H_2O_2 increased the ratio in both CS and AS tissue. Mono-cultures of PA SN showed a worsened energy condition evaluated under 0 and 1 mM H_2O_2 (Fig. 12B). For CS tissue, the influence of culture procedure on ADP/ATP ratios was only observed under the 1 mM H_2O_2 condition.

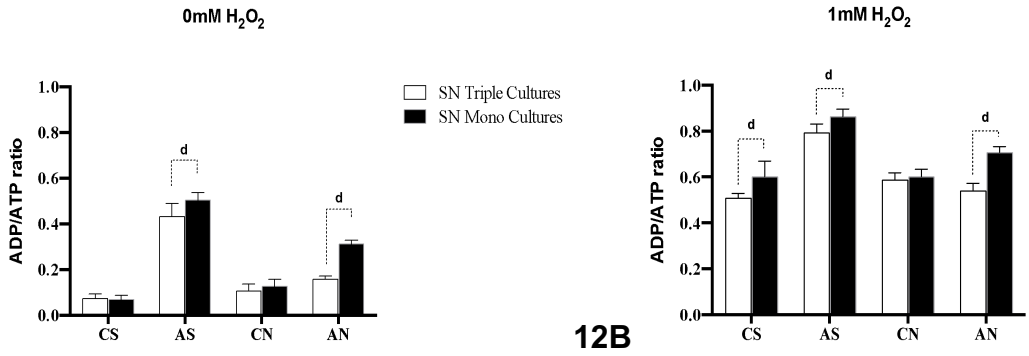

Figure 12. ADP/ATP ratio (12A-B) from triple and mono organotypic cultures from CS and AS rat neonates, under 0 mM H_2O_2 (a-c) and 1 mM H_2O_2 conditions (b-d): effect of nicotinamide. The effect of co-culturing on ADP/ATP ratios was evaluated by multiple comparisons (MANOVA). Two-way F-ANOVA indicated a significant effect of culture conditions ($F_{(3,17)}$ = 21.64, $^{d}P<0,001$).

VIII. DISCUSSION

PA is a severe complication during labour, leading to neurological and behavioural disorders. Many studies have focused on short and long-term effects on CNS following PA, showing a regional vulnerability towards cell death (Pérez-Lobos et al. 2017).

Although long-term consequences affecting dopaminergic neurocircuitries following PA have been reported, it is not clear whether PA affects the coping mechanisms of CNS against postnatal stressors occurring along life. The hypothesis of enhanced vulnerability to oxidative stress by neonates surviving PA was here evaluated by a protocol combining *in vivo* and *in vitro* experiments. Asphyxia exposed (AS) and caesarean-delivered control (CS) rat pups were used for preparing triple organotypic cultures, including tissue samples from mesencephalon (SN) and telencephalon (Str and Cx). At DIV 18, cultures were subjected to a second insult, consisting of H_2O_2 added to the culture medium for 18h. After a 48h recovery period, the cultures were either assessed for (i) cell viability; (ii) neurochemical phenotype; (iii) energy metabolism (ADP/ATP); (iv) oxidative stress (GSH/GSSG); (v) antioxidant capacity, and (vi) LDH release levels, as a necrosis marker (Koh, Choi, 1987; Lobner, 2000; Chan et al. 2013).

It was found that PA produced a long-term energetic deficit associated to regionally specific cell loss, mainly affecting mesencephalic dopamine systems. Indeed, homogenates of triple organotypic cultures from asphyxia-exposed animals (AS) showed a >6-fold increase in ADP/ATP ratio, assayed at DIV 21-22, as compared to culture homogenates from the corresponding controls (CS). The increase in the ADP/ATP ratio reflected a permanent energetic deficit, as a single 1 mM H_2O_2 insult also increased ADP/ATP ratio (>7-fold) in CS culture homogenates. The 1 mM H_2O_2 insult further increased the ADP/ATP ratio in AS culture homogenates. The effect on the ADP/ATP ratio was in parallel with a decrease in GSH/GSSG ratio, observed in AS culture homogenates, mimicked by the H_2O_2 insult in CS and AS cultures. The potassium ferricyanide reducing power was significantly decreased in AS cultures following the 0 mM H_2O_2 condition, suggesting a permanent impairment in antioxidant defences (Lespay-Rebolledo et al., 2018). The effect of potassium ferricyanide reducing power was

also observed in CS cultures, but following 1 mM H_2O_2 only. The decrease in ferricyanide reducing power suggests the involvement of the mitochondrial transition pore, which has been shown to be induced by ROS (Skulachev 1996), and increased by reoxygenation following hypoxia (Duchen et al. 1998; Griffiths and Halestrap 1993; Krasnikov et al. 1997), leading to a permanent mitochondrial deficit and cytochrome oxidation (Krasnikov et al. 1997; Amoroso et al. 2000).
There was a significant increase of LDH release in cultures from asphyxia-exposed, as compared to that from control neonates, suggesting cytolysis. 1 mM of H_2O_2 increased LDH levels in cultures from both control and asphyxia-exposed animals, but the effect of H_2O_2 was more remarkable in control than in asphyxia-exposed cultures, suggesting a pre-conditioning on AS samples, making them more resistant to the insult.

The present results demonstrate that PA leads to a sustained energetic deficit, even when re-oxygenation is stabilized, probably responsible for the long-term deficits after PA reported by several groups (Boksa et al. 1995; Chen et al. 1997; Engidawork et al. 1997; Hoeger et al. 2000; Carloni et al. 2008), involving also the glutathione pathway, since the GSH/GSSG ratio was decreased by 40% in AS compared to CS culture homogenates. A decrease in total glutathione has been reported 24h after hypoxia-ischemia in a model combining a unilateral carotid ischemia and hypoxia at P7 in rats (Wallin et al. 2000), associated to ATP deficit, as shown after ischemia in the adult animal (Rehncrona et al. 1980). In fact, we reported that the glutathione scavenger system is long-term impaired after PA *in vivo* (Lespay-Rebolledo et al. 2018). The association to oxidative stress is supported by the observation that 1 mM H_2O_2 re-produced the effect in the controls, i.e. (i) increasing the ADP/ATP ratio; (ii) decreasing the GSH/GSSG ratio and (iii) decreasing the potassium ferricyanide reducing power in AS culture homogenates, suggesting a permanent mitochondrial deficit (Krasnikov et al. 1997; Amoroso et al. 2000).

Cell vulnerability to PA and to a recurrent metabolic insult: EthD-1-positive cell death in vitro

Cell death was examined directly on the field of a fluorescence inverted microscopy (NIKON T100), counting the number of EthD-1 positive cells in SN, Str and Cx of organotypic cultures from AS and CS rat neonates, treated with saline or nicotinamide 1h after birth, evaluating the vulnerability to a recurrent metabolic insult induced by adding different concentrations of H_2O_2 into the culture medium for 18h, an established method for investigating neurotoxicological issues in organotypic cultures, a model that retains the complex neuronal-astroglial interaction (Bai and Lipski 2010).

It was found that AS cultures were more resistant than CS cultures to the metabolic insult induced by H_2O_2. There was a shift to the right on the concentration-dependence of the H_2O_2 effect on AS cultures. The CS cultures were more vulnerable to H_2O_2, along all tested concentrations. That result was observed in each analysed region. Similar paradoxical results have been observed in models of hypoxia/ischemia (McQuillen et al. 2003), suggesting that hypoxia/ischemia at birth may provide protection to recurrent oxidative stress shown by surviving cells, implying perhaps a preconditioning mechanism (Chang et al. 2008). The significantly larger number of dying cells in brain tissue exposed to PA implicates an enhanced coping capacity of the surviving cells *in vitro*, implying selection (Jian et al. 2004) and/or morphological and functional re-arrangements, as previously suggested (De Torres et al 1997; Van de Berg 2002). The number of dead cells was, however, significantly larger in AS compared to CS cultures under the 0 mM H_2O_2 condition (>3-fold), showing also, as discussed above, an increased ADP/ATP ratio and decreased GSH/GSSG and potassium ferricyanide reducing power compared to CS cultures, confirming a long-term deficit induced by PA (Ikeda et al. 2001). In fact, the tissue reducing power assayed by potassium ferricyanide was significantly decreased in homogenates from AS cultures, not even responding to a powerful oxidative stress as that produced by 1 mM of H_2O_2. The effect of 1 mM H_2O_2 on LDH levels was similar under the control and the post asphyxia-conditions.

Apoptosis (TUNEL) phenotype of dying cells evaluated in formalin fixed cultures
The phenotype of dying cells was investigated in formalin fixed cultures exposed to 0 or 1 mM of H_2O_2, labelling for TUNEL (apoptosis), counterstaining for DAPI

(nuclear staining). The TUNEL/DAPI co-labelling indicates an apoptosis mechanism for dying cells, mainly at the DNA fragmentation stage, considered to be an early (Labat-Moleur et al. 1998) event of apoptosis. Following 0 mM of H_2O_2, TUNEL-positive apoptosis represented ≈10% of DAPI-positive cells in SN of CS and ≈17% in AS cultures. 1 mM of H_2O_2 increased the number of DAPI/TUNEL positive cells, mainly in SN from both CS and AS cultures. In AS SN treated with 1 mM H_2O_2, the number of TUNEL/DAPI cells/mm^3 represented ≈50% of the number of DAPI positive cells/mm^3.

The number of TUNEL/DAPI cells/mm^3 suggests, as a whole, that the increase in the number of EthD-1 positive cells observed in AS versus CS cultures can partially be explained by an apoptotic mechanism, since 1 mM of H_2O_2 induced a similar increase in the number of TUNEL/DAPI positive cells/mm^3 in cultures from both AS and CS animals, mainly affecting SN, in agreement with previous reports (Ezquer et al. 2006). A similar effect of H_2O_2 has been observed in primary neuronal (Whittemore et al. 1994, 1995) and in hippocampus organotypic (Bai and Lipski 2010; Feeney et al. 2008) cultures, proposed to rely on apoptosis-induced oxidative stress (Bai and Lipski 2010; Orrenius et al. 2011).

The issue of cell phenotype was further investigated with immunocytochemistry, labelling for MAP-2 (neuronal phenotype), GFAP (astroglial phenotype), and TH (dopamine phenotype), assessed by confocal microscopy and stereological quantification, as previously reported (Morales et al. 2005; Klawitter et al. 2007). Following 0 mM of H_2O_2, MAP-2 positive cells represented a 30-40% of the number of DAPI-positive cells/mm^3 in all regions and experimental groups, although the number of MAP-2 positive cells/mm^3 was decreased in AS SN, compared to the corresponding control. In SN, MAP-2/TH-positive neurons represented ≈27% of the number of MAP-2-positive cells in CS cultures, while that proportion was decreased to ≈18% in AS SN. No MAP-2/TH positive labelling was observed in Str or Cx, in agreement with the specific localization of dopamine cell bodies in mesencephalon (Ungerstedt 1971), also observed in organotypic cultures (Plenz and Kitai 1996; Gomez-Urquijo et al. 1999). 1 mM of H_2O_2 decreased the number of MAP-2 and MAP-2/TH-positive cells/mm^3, both in CS

and AS cultures, but the effect on MAP-2/TH-positive cells/mm^3 was stronger in AS, compared to CS cultures, further supporting the observation that dopamine neurons are particularly vulnerable to PA (Burke et al. 1992).

The Immunocytochemical phenotype of TUNEL-positive dying cells was monitored by co-labelling with MAP-2, GFAP and TH. In CS cultures, ≈11% of MAP-2 and MAP-2/TH positive cells/mm^3 were also TUNEL positive, while in AS cultures that percentage was increased to 18% for MAP-2 and 26% for MAP-2/TH positive cells/mm^3, respectively. The number of GFAP/TUNEL positive cells/mm^3 was also significantly increased when comparing AS versus CS cultures, indicating a long-term effect of PA on glial cells. 1 mM of H_2O_2 produced a strong increase in the number of MAP-2, GFAP and TH cells/mm^3 co-labelled with TUNEL, indicating apoptosis generated by the H_2O_2 insult, but not on TH/TUNEL co-labelling in cultures from AS animals, suggesting that there is an oxidative stress resistant population of dopamine neurons remaining after the PA insult.

Nevertheless, the effect of 1 mM of H_2O_2 increased neuronal apoptosis in all regions, both in AS and CS cultures, supporting the idea that neurons are susceptible to oxidative stress, as previously reported (Desagher et al. 1996, 1997; Saito et al. 2005). 1 mM of H_2O_2 also increased astroglial apoptosis in SN of both AS and CS cultures, but the effect of 1 mM of H_2O_2 in Str was only observed in cultures from CS animals. No increase of apoptosis was observed in Cx of any of the experimental groups, confirming that the consequences of oxidative stress, on glial and neuronal phenotypes, are regionally dependent (Gavrieli et al. 1992; Mischel et al. 1997; Dell'Anna et al. 1997; Nedergaard et al. 2003; Klawitter et al. 2005; Matyash and Kettenmann 2010).

Under the present experimental conditions, apoptotic-necrotic like (Yue et al. 1997; Northington et al. 2005) and/or autophagy (Smith et al. 2011; Weis et al. 2014) mechanisms were not monitored, which may explain some of the differential results among different regions. It can also be argued that TUNEL identifies cells undergoing early stages of apoptosis, not labelling cells during the late stages (Crowley et al. 2016). Furthermore, the estimation of necrotic cell

death by the LDH release assay did not allow to identify the neurochemical phenotype of the affected cells.

The present results also point out to different pathophysiological mechanisms of PA and pro-oxidative conditions (H_2O_2), implying different redox (Ferriero 2001) and energy (Palmer et al. 1990) imbalance. Indeed, it has been reported that hypoxia and oxidative conditions, as consequences of perinatal hypoxia/ischemia, can modify mitochondrial function (Jendrach et al. 2008), biogenesis (Yin et al. 2008) and cell energy metabolism (Gilland et al. 1998), suggesting that the response of the CNS to PA and the coping capacity against postnatal stressors may rely on multiple metabolic pathways, responding differently according to regional vulnerability (Mischel et al. 1997), cell phenotype (Wang and Michaelis 2010) and stage of development (Vannucci et al. 2004).

It has been reported that PA leads to increased PARP-1 activity, triggering a pro-inflammatory signalling cascade associated to cell death (Neira-Peña et al. 2015). As a proof of concept, it was shown that a single dose of the PARP-1 inhibitor nicotinamide (0.8 mmol/kg, i.p.),1h post-delivery, prevented the effect of PA on PARP-1 activity and on cell death in mesencephalon of asphyxia-exposed animals (Allende-Castro et al. 2013; Neira-Peña et al. 2015; Vio et al. 2018), supporting the idea that pharmacological inhibition of PARP-1 can restore metabolic functions, improving energy viability (Yang 2002; Kauppinen and Swanson 2007; Morales et l. 2010; Bai et al. 2011; Kauppinen et al. 2011; Allende-Castro et al. 2013; Neira-Peña et al. 2015; Vio et al. 2018).

In the present study, nicotinamide was administrated *in vivo* 1h after delivery, before preparing the organotypic cultures, finding that nicotinamide decreased the effect of PA and that of H_2O_2 on cell viability assessed *in vitro,* monitored by the number of EthD-1 positive cells/mm^3, mainly in SN and Cx. The prominent effect of nicotinamide on SN cell death evaluated in organotypic cultures agrees with previous reports (Østergaard and Zimmer 1995; Klawitter et al. 2007; Allende-Castro et al. 2013; Neira-Peña et al. 2015).

Nicotinamide also protected against apoptosis following 1 mM H_2O_2 treatment in SN, Str and Cx of both AS and CS cultures, suggesting a general effect on the ability of the tissue for coping with oxidative stress, without consideration whether there was or not a previous perinatal insult. That agrees with the idea of PARP-1 inhibition, as a strategy to improve NAD^+ metabolism and to reduce cellular stress (Mukherjee et al. 1997; Chong et al. 2004; Luo and Kraus 2011). Accordingly, nicotinamide also prevented neuronal apoptosis induced by 1 mM of H_2O_2 in SN, Str and Cx of AS and CS cultures, although the effect of nicotinamide on dopamine-positive cell death was only observed in AS cultures. In fact, the use of PARP-1 inhibitors was first reported to diminish H_2O_2 toxicity in human peripheral lymphocytes (Schraufstatter et al. 1986). Nicotinamide did not prevent changes induced by hypoxia and/or H_2O_2 on LDH levels.

It can be speculated that PA primes the defence mechanisms of dopaminergic neurons, delaying the pro-apoptotic cascades elicited by pro-oxidative insults, such as that induced by high H_2O_2 concentrations (Chen et al. 2009). Although no studies have been carried out to prove the effect of PA on preconditioning of dopamine neurons, several groups have already shown that postnatal hypoxic conditions can induce neuroprotection (Gidday 2006) against recurrent metabolic challenges (Vannucci et al. 1998; Ara et al. 2011).

The present results demonstrate that PA induces long-term changes in metabolic pathways related to oxidative stress, priming cell defence mechanisms, with both neuronal and glial phenotypes. The effects observed were region dependent, being the SN more prone than other evaluated regions to cell death. Thus, we analysed the effect of perinatal asphyxia and of 1 mM H_2O_2 on energy and oxidative stress, as well as on LDH levels in mono-organotypic cultures of the substantia nigra, finding, however, that the effects were very similar under both culturing conditions (single versus triple condition).

Delayed cell death induced by PA has been the subject of a large number of reports, starting with the pioneering paper by Dell'Anna et al. (1997), that referred to an enhanced vulnerability of neurocircuitries of the basal ganglia. The idea that PA implies a long-term vulnerability to recurrent metabolic insults has already

been discussed (Herrera-Marschitz et al. 2014; Marriott et al. 2016), but not yet demonstrated. Several studies, however, have shown long-term consequences following PA, including delayed cell death (Dell'Anna et al. 1997; Van de Berg et al. 2002; Morales et al. 2005), and neuroinflammation (Foster-Barber et al. 2001; Neira-Peña et al. 2015), suggesting that vulnerability implies impaired defence mechanisms against metabolic challenges probable to occur along life (Marriot et al. 2016).

Assessment of cell death was a main endpoint to be evaluated in this thesis. It was found that the effect of PA, associated or not to a recurrent metabolic insult, was different in mesencephalon and telencephalon of cultures from asphyxia-exposed or control animals. Indeed, PA was associated with a decreased number of cells showing neuronal phenotype (MAP-2), but only in substantia nigra and neocortex (by approximately 20%). That effect was significantly enhanced when co-labelling MAP-2 and TH, showing that dopamine neurons were decreased in substantia nigra from asphyxia-exposed, compared to that from controls (by approximately 50%). The regional dependence of the long-term effects elicited by PA was evident when analysing the vulnerability to a recurrent metabolic insult.

In substantia nigra, as shown about, it was found that the number of dopamine neurons was decreased when comparing cultures treated with 0 mM of H_2O_2, from asphyxia-exposed versus control animals in mono and triple organotypic co-cultures. Co-labelling phenotype markers with TUNEL, the assay for apoptosis, indicated that apoptosis did not explain the cell death observed in substantia nigra. Neuronal apoptosis was increased by 1 mM H_2O_2, in cultures from both AS and CS animals, but this result was not replicated for dopaminergic neurons, because the number of TUNEL/TH positive cells was not affected by 1 mM H_2O_2, further supporting the idea that PA can enhance, in substantia nigra, the resistance of remaining alive dopaminergic neurons against postnatal stressors.

In neostriatum, PA did not have any effect on the number of neurons, however differences were observed between AS and CS after 1 mM of H_2O_2 treatment. In agreement, the number of neurons undergoing apoptosis was increased by 1 mM

H_2O_2, in cultures from both AS and CS animals. That was also observed for astroglial apoptosis, but only in neostriatum from CS. Proving once again that PA enhances regionally-dependent vulnerability in CNS. Similar, but not the same results were obtained in Cx.

As previously described, different results were shown after PA and/or 1 mM H_2O_2 treatment in samples from AS and CS animals, revealing variations in: (i) amount of neurons and astroglia cells, and (ii) neuronal and astroglial apoptosis. These effects were region dependent and could be explained by different oxidative and apoptotic responses occurring in mesencephalon and telencephalon of rat brain following PA *in vivo* (Lespay-Rebolledo et al. 2018). Surprisingly, after 1 mM H_2O_2 samples from AS animals showed a diminished number of apoptotic positive astroglia, possibly indicating enhanced metabolic resistance induced by PA, which has been proposed to promote glial proliferation and astrocyte reactivity (Rojas-Mancilla, Thesis, 2015), although this issue requires further investigation. Likewise, it has already been reported that astrocytes can protect neurons from H_2O_2 toxicity and redox misbalances (Desagher et al. 1996).

As already discussed, neonatal nicotinamide was systemically administered, as a proof of concept, studying the relation between an initial metabolic insult at birth, and the coping capacity of the surviving cells in CNS. It was found that nicotinamide treatment prevented some of the effects induced by PA and by the recurrent metabolic insult, mainly on cell viability, revealing enhanced protection in all regions, although that was more prominently observed in substantia nigra, which was also previously reported *in vivo* (Neira-Peña et al. 2014). In substantia nigra nicotinamide protected against the consequences of PA on the number of dopamine neurons, also preventing apoptotic cell death.
Nicotinamide prevented the effect of 1 mM H_2O_2 on MAP-2 and on MAP-2/TH co-labelling. Nicotinamide prevented the effect of 1 mM H_2O_2 on MAP-2/TH cell number, but not on the effect of PA on MAP2/TH/TUNE, suggesting that the effect of PA on dopamine neurons was apoptotic-independent. Interestingly, however, when MAP-2/nNOS co-labelling was also simultaneously monitored, it was found that the MAP-2/nNOS co-labelling increased following PA, effect that was reversed by nicotinamide (no published observation).

In neostriatum of formalin fixed cultures, nicotinamide prevented the increase o MAP-2/TUNEL and GFAP/TUNEL co-labelling observed in control cultures treated with 1 mM of H_2O_2. No significant effect was observed in cultures from asphyxia-exposed animals. In neocortex, nicotinamide prevented the effect on MAP-2 observed following PA and the increase of MAP-2/TUNEL co-labelling induced by 1 mM H_2O_2 in control cultures. That effect was even more prominent in cultures from asphyxia-exposed animals, decreasing the number of MAP-2/TUNEL co-labelling by approximately 50%. Indeed, the prevention of the effect of 1 mM H_2O_2 by nicotinamide was prominent on MAP-2/TUNEL positive cells/mm^3 in all regions, indicating that that protection may rely on neuronal rather than on astroglial phenotypes.

Thus, morphological analysis led us to the conclusion that nicotinamide administrated *in vivo* has long-term protective effects, even protecting from a high H_2O_2 concentration. It has been proposed that nicotinamide prevents cell death by inhibiting PARP-1 over activation, reversing a sustained energy depletion, as indicated by the ADP/ATP ratio. In agreement, nicotinamide has been proposed to improve mitochondrial function in models of stroke (Klaidman et al. 2003). Nicotinamide also increased the GSH/GSSG ratio in all experimental groups, along with increased antioxidant power measured in cells homogenates, suggesting that the protection induced by nicotinamide relies on enhanced antioxidant mechanisms, involving PARP-1 related pathways, and relaying on NAD^+ replacement.

Energy depletion and oxidative stress are concomitant phenomena occurring in CNS after PA, and in tissue undergoing metabolic imbalance. It has been observed that the GSH/GSSG ratio and the total reducing capacity is altered by PA *in vivo* (Lespay-Rebolledo et al. 2018), which can be enhanced by a postnatal metabolic insult, in this study induced by a high H_2O_2 concentration, suggesting a common mechanism linked to the buffer capacity and defences of the vulnerable tissue.

In summary, nicotinamide protected against the effects induced by PA, and/or by a second metabolic insult, decreasing: (i) neuronal and astroglial cell death; (ii) dopamine neuronal death; (iii) decreasing TUNEL-positive cell death.

The vulnerability of the dopamine phenotypes deserves additional studies. To enlighten this phenomenon, substantia nigra mono-cultures were used to assess the role of telencephalic tissue and striato-nigral pathways on substantia nigra (Herrera-Marschitz et al 2010). Nevertheless, a decrease in the number of MAP-2^+ and TH^+ cells was also observed in substantia nigra from mono-cultures, in all groups, and changes in metabolic and cytotoxic parameters were not dramatically different when comparing mono- versus triple-cultures. Minor changes were observed between metabolic parameters in mono and triple organotypic cultures, and the amount of TH^+/$TUNEL^+$ cells was higher in mono cultures, suggesting that the dopaminergic input may have influenced the response of substantia nigra to postnatal stressors. This could be explained by the presence of D_1 receptors on spiny-medium sized projecting neurons (Herrera-Marschitz and Ungerstedt, 1984; Mordecai et al. 1987, You et al. 1996; Zhang et al. 2008), playing perhaps a role in brain hypoxia, by an interaction between the synaptic dopaminergic input from TH^+ neurons in substantia nigra and the metabolism of postsynaptic neurons in telencephalon, responding differently to PA and postnatal recurrent insults.

IX. CONCLUSIONS

In summary, the main conclusions of this thesis are:

(i) PA primes the vulnerability of brain tissue exposed to postnatal stressors, being the substantia nigra more vulnerable than other simultaneously monitored regions.

(ii) The effect of PA and 1 mM H_2O_2 treatment on cell survival was more prominent on cells with neuronal than astroglial phenotypes in all experimental groups.

(iii) TH^+ neurons from AS neonates showed resistance to postnatal metabolic insults compared to that observed in cultures from CS neonates, suggesting a stress-induced preconditioning, specific for the dopamine phenotype.

(iv) PA induced specific changes in metabolic parameters, differently to those induced by recurrent metabolic insults, suggesting a sustained and specific impairment of cell homeostasis in substantia nigra.

(v) Nicotinamide treatment *in vivo* protected from the priming effect of PA, enhancing the neuronal capacity to cope with metabolic insults *in vitro*.

(vi) Nicotinamide protection was observed on both apoptotic and necrotic cell death, evaluated by both TUNEL and LDH assays. The effect of 1 mM H_2O_2 was particularly prominent on TUNEL-positive cell death evaluated in organotypic cultures from asphyxia-exposed animals.

(vii) Nicotinamide provided relevant protection against the long-term effects elicited by PA.

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