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THE ROLE OF GAP JUNCTIONS IN RATS SUBMITTED TO NEONATAL ANOXIA

SANTO ANDRÉ

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DEDICATION

I dedicate this project to my family and
friends from the anoxia team.

ACKNOWLEDGMENTS

I thank my parents, Sergio and Kátia, my sister Mayara, for all their unconditional love, support and encouragement to study during all these years.

I thank my supervisor, Silvia and my co-supervisor, Alexandre for all the knowledge, support, encouragement and advice throughout my career at the Neurogenetics Laboratory.

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I thank the Federal University of ABC, mainly, the professors and colleagues who participated and contributed directly and indirectly to my academic formation.

“Science never solves a problem without creating at least ten more.”

(George Bernard Shaw)

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LIST OF ABBREVIATIONS

pre-OLs	oligodendrocyte progenitor cells
Cxs	connexins
GJs	gap junctions
CBX	carbenoxolone
PBS	sodium phosphate buffer solution
FJC	Fluoro-Jade C®
TUNEL	Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick End Labeling
ANOVA	analysis of variance

ABSTRACT

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Neonatal anoxia, which is the lack of oxygen at birth, is considered an important public health problem because it affects 0.1 to 0.3% of neonates born at term and approximately 60% of premature infants with low birth weight. About 20-50% of newborns suffering from neonatal anoxia do not survive, and those who survive, 25% have some permanent neurological sequelae such as cerebral palsy, cognitive, hearing and visual deficits. The oxygen deprivation in the immature brain can lead to neuronal death by apoptosis, necrosis, autophagy and excitotoxicity; it can also lead to the death of glial cells such as astrocytes and oligodendrocyte progenitor cells, especially in vulnerable regions such as the hippocampus and cerebral cortex. One hypothesis for the contribution of neurodegeneration after oxygen deprivation involves the participation of connexins, which are transmembrane proteins with the ability to form intercellular channels in the gap junctions and hemichannels located in the cell membrane. Studies with different models of injury and connexin blockers confirmed the crucial involvement of gap junctions in injury from hypoxia-ischemia in the mature brain as during its development. Among the connexins, the Cx43 seems to be expressed most abundantly in tissues and in the central nervous system is present in astrocytes and in oligodendrocytes, whose communication through gap junctions plays a key role in the process of myelination during development. Studies have shown that Cx47 phosphorylation and stability of oligodendrocytes are present in astrocyte-dependent Cx43 expression. Therefore, changes in glial gap junctions resulting from oxygen deprivation during development may influence neuronal and glial cell death. In this project, we analyzed by immunohistochemistry the distribution and location of Cx43 marker 24h after neonatal anoxia. The quantification of pixels has been performed and the following results were found: 24 hours after anoxia, we observed an increase of Cx43 immunoreactivity in the oriens layer. We analyzed the effects of the use of gap junctions blocker, carbenoxolone, in hippocampal cell death of rats subjected to neonatal anoxia and it was effective to prevent cell death. We analyze the somatic development and sensorimotor reflexes, anxious behavior, locomotor activity and spatial memory of rats submitted to neonatal anoxia and intrahippocampal injection of carbenoxolone or vehicle (PBS), but we could not observe differences. It seems that by blocking the gap junctions we can prevent acute cell death, but this blockade was not enough to minimize functional sequelae in adult life. We hope that these results may contribute to a better understanding of the complex mechanism of cell death and functional impairment after neonatal anoxia.

Keywords: connexins, neurodegeneration, synapses, perinatal asphyxia, apoptosis, development of the nervous system.

1. INTRODUCTION

1.1 Neonatal anoxia

The neonatal anoxia, which is the lack of oxygen at birth, is considered an important public health problem because it affects from 0.1 to 0.3% of children born at term (Kurinczuk, 2010) and about 60% of premature children low birth weight (Vannucci, 2000; Gluckman, 2011; Volpe, 2009). Prematurity is closely associated with the occurrence of neonatal anoxia (Vannucci, 2000; Gluckman, 2011; Volpe, 1998), since the normal development of the alveoli and pulmonary vascularization of these children is interrupted by premature birth. Also, the lungs of premature infants are more prone to respiratory complications due to deficiency in surfactants (Suguihara, 2005).

About 20 to 50% of neonates who suffer neonatal anoxia do not survive and, of those who do survive, 25% have some permanent neurological sequelae (Wilson-Costello, 2005), such as cerebral palsy, cognitive, auditory and visual deficits (Vannucci, 2000; Back, 2014). In a recent study of perinatal asphyxia in Brazil (Almeida, Kawakami et al 2017), the mortality rate between 2005 and 2010 was 0.65 newborns per 1,000 live births.

The brain is very sensitive to decreases in oxygen levels, as it is responsible for a large part of energy consumption. When oxygen levels are compromised, a cascade of biochemical events triggers the injury or death of the most susceptible cells of the central nervous system, leading to neuronal cell death by apoptosis, necrosis, autophagy, and excitotoxicity; it can also lead to the death of glial cells, such as astrocytes and oligodendrocyte progenitor cells (pre-OLs), especially in more vulnerable regions, such as the hippocampus and cerebral cortex (Back, 2014; Northington , 2011; Peterson, 2012).

The pathophysiological mechanisms triggered by oxygen deprivation in the developing brain are still not well established. The metabolic changes involve a drastic reduction in the cell's energy reserves and Na^{+} gradients, resulting in glutamate reuptake failures and a toxic increase in extracellular glutamate, leading to cell death from excitotoxicity (Choi and Rothman 1990), in both neuronal and glial cells.

Oxygen deprivation leads to the onset of mitochondrial dysfunction, excitotoxicity and apoptotic pathways; reperfusion is followed by the latent phase, which is characterized by the relative recovery of metabolic processes; finally, 6 hours after oxygen deprivation, the secondary phase begins, in which there is massive cell death and production of pro-inflammatory cytokines (Inder and Volpe 2000, Volpe 2012), continual uptake of intracellular calcium as well as the release of reactive oxygen species. A third phase has been described as another chronic phase of injury that occurs within days and continues for months, up to years. This phase includes astrogliosis, chronic inflammation, and tissue repair and remodeling, which further contribute to the loss of brain cells and cerebral atrophy (Bo Li et al., 2017).

The great dependence on energy supply in developing neurons is related to the maintenance of connections and survival in the perinatal period (Li, 1998). Oxygen deprivation and interruptions in the development of these connections in target regions such as the hippocampus can cause cell death in the immature brain. The neurodegeneration by deprivation of target regions is one of the propositions for the late occurrence of neurodegeneration in hypoxia-ischemia models (Geddes, 2011).

1.2 Gap junctions channels

One of the hypotheses for the contribution of neurodegeneration after oxygen deprivation involves the participation of connexins (Davidson, 2013). Connexins (Cxs), pannexins and innexins are described as three families of proteins that form the conductance channels, being regulators of communication between neighboring cells by the gap and/or hemichannel junctions (D' Hondt, 2013).

The connexins are transmembrane proteins encoded by a large gene family, with at least 21 members in the human genome (11 of them expressed in the central nervous system), while pannexin family contains only three members. Among the structural and functional similarities of connexins and pannexins, the ability to form channels of gap and hemichannel junctions is included (Davidson, 2013; D' Hondt, 2013).

The combination of six connexins form a hemichannel or connexon and the coupling of two hemichannels in affixed cellular membranes constitute the gap junctions (Davidson, 2013; D' Hondt, 2013).

Changes in the expression of the different connexins during development, their distribution according to the cell type in different regions of the brain used to study the role of the gap junctions in cell death (Paschon, 2012) or after hypoxia-ischemia were explored in previous works, using different animal models of oxygen deprivation. In the vast majority of these studies, gap junction blockers were used, such as octanol (Rawanduzy , 1997; Cotrina, 1998; Contreras, 2002; Nodin, 2005), halothane (Rami, 2001) and carbonexolone (CBX), (Nodin, 2005; de Pina- Benanou, 2005; Thompson, 2006), confirming the crucial involvement of the gap junctions in hypoxia-ischemia injuries in both the adult brain and during their development.

Among connexins, Cx43 appears to be expressed more abundantly in tissues; moreover, in the central nervous system, astrocytes are extensively coupled by gap junctions made up of proteins co-located with Cx43 and 30 (Nagy, 2000). The coupling between astrocytes can be influenced by a variety of neurotransmitters and neuromodulatory substances, many of which are released by neurons (Nagy, 2000).

The communication of astrocytes with oligodendrocytes through heterologous gap junctions plays a fundamental role in the process of myelination during development. Studies have shown that the phosphorylation and stability of Cx47 present in oligodendrocytes is dependent on the expression of astrocytic Cx43 (May, 2013) and the use of Cx43 blocking mimetic peptides has improved the survival rate of oligodendrocytes in the intragiral region and in the periventricular white matter of sheep submitted to prenatal hypoxia-ischemia (Davidson, 2012).

Thus, the hypotheses of this project are:

Hypothesis 1 (H1): changes in the coupling between astrocytes and, consequently, in the distribution and location of Cx43 can be expected to occur in response to the cascade triggered by oxygen deprivation and influence the pattern of neurodegeneration and glial changes;

Hypothesis 2 (H2): Blocking gap junctions by carbenoxolone may be effective against hippocampal neurodegeneration due to oxygen deprivation;

Hypothesis 3 (H3): Blocking gap junctions by carbenoxolone can mitigate the functional sequelae in juvenile and adult rats submitted to neonatal anoxia.

1.3 Text organization

This final undergraduate project is organized as follows: topic 2 presents the general objectives, while topic 3 presents the specific objectives of the project. Topic 4 presents the scientific methodology used to investigate the hypotheses. Topic 5 presents the results obtained that will be discussed in topic 6. Finally, topic 7 presents the final considerations of the project and future perspectives.

2. GENERAL OBJECTIVES

Evaluate the distribution and quantification of Cx43 and the possible effects of the use of the gap junctions blocker, carbenoxolone, in the hippocampal neurodegeneration and in the functional sequelae of rats submitted to neonatal anoxia.

3. SPECIFIC OBJECTIVES

This project aimed to:

i) determine possible changes in the distribution and location of connexin 43 (Cx43) arising from the neonatal anoxia by immunohistochemistry, using anti-Cx43.

ii) verify the role of gap junctions in cell death following neonatal anoxia using carbenoxolone (CBX) intrahippocampal injection and Fluoro-Jade C® (FJC) and Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) staining.

iii) analyze the effects of blockade of gap junctions formed by connexins with carbenoxolone (CBX) or vehicle (PBS) after neonatal anoxia in the somatic and sensorimotor development.

iv) analyze the effects of blockade of gap junctions formed by connexins with carbenoxolone (CBX) or vehicle (PBS) after neonatal anoxia in locomotor activity and anxious behavior in juvenile rats.

v) analyze the effects of blockade of gap junctions formed by connexins with carbenoxolone (CBX) or vehicle (PBS) after neonatal anoxia in spatial reference memory in adult rats.

4. MATERIALS AND METHODS

4.1 Animals

For the experiments, pups of 8 albino rats couples were used (Rattus norvegicus, Wistar), maintained in the vivarium of the Federal University of ABC - campus São Bernardo do Campo, with constant temperature (22 ° C ± 1 ° C), light / dark cycle of 12: 12h, the beginning of the course at 7:00h, and water and food *ad libitum*. In total, about 40 neonates, aging about 30 hours of birth (P1-P2), weighing between 6 and 8 g as described by Takada et al., 2011 (Takada, Sampaio et al. 2011) were used. The procedures are in accordance with the Ethical Principles of Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee on Animal Use of the UFABC, protocol 05/2014.

4.2 Neonatal anoxia

For anoxia, the system described by Takada et al (2011) was used with some modifications. It was constituted by a semi-hermetic polycarbonate chamber with the gas inlet and outlet, coupled to a regulator and the 100% nitrogen gas cylinder. As a modification of the original model, the chamber temperature was maintained at 35 and 37°C by the heater plate and not by the water bath system.

Initially, the chamber was completely saturated with 100% nitrogen gas at a flow of 11,5 L/ minute (Takada et al., 2011). The animals remained in the chamber, exposed to nitrogen gas for 25 minutes. After animals' recovery (color recovery, respiration, and active drive), which occurs in the first 5 minutes after removal of the chamber, the pups were returned to the mother or underwent intrahippocampal blocker or vehicle injection. The control group was exposed to the same experimental conditions, but without having air exchange within the chamber, i.e. remained in room air.

4.3 Intrahippocampal injection

For intrahippocampal injection of the blocker carbenoxolone (CBX), immediately after the insult (anoxia or control), neonates were anesthetized by

cryoanesthesia and fixed by a modified stereotaxic for neonate rats, with rubber bars for head fixation. Once stabilized, two small holes were performed bilaterally using a fine needle attached to a microsyringe (Hamilton Co., Nevada, USA) and CBX (1mM, experimental group) or vehicle only (sham group) was injected in the amount of 0.3 µL in the following range of established coordinates for Fitting et al. (right hemisphere -0.3 mm anterior to bregma, 0.7 mm medial to bregma, -2.0 mm dorsal from dura; left hemisphere -0.3 mm anterior to bregma, -0.7 mm lateral to bregma, -2.0 mm dorsal from dura) (Fitting, Booze et al., 2007) so that the hippocampus could be reached bilaterally. After injection, the neonates remained on the hot plate at 33°C until full recovery and then returned to the mother.

4.4 Processing of biological material

To study the distribution and location of Cx43 in the hippocampus of neonates, they were perfused transcardially 24 hours after neonatal anoxia (n=5/group). For transcardial perfusion, the animals were deeply anesthetized with the anesthetic cocktail of ketamine (75 mg/kg) and xylazine (10 mg / kg). The pups were transcardially perfused with saline (0.9%, pH 7.4) and then fixative solution composed of paraformaldehyde (1% in phosphate buffer, pH 7.4, 4°C). The brains were dissected and cryoprotected in sodium phosphate buffer solution (PBS, 0.1 M, pH 7.4) plus 30% sucrose until the time of cryosection at cryostat (Leica CM 1850) in frontal sections 12 µm thick, collected in 20 series and mounted on gelatinized slides.

4.5 Immunohistochemistry for Cx43

After washing with 0.1 M PBS, the slides containing the sections were incubated in a blocking solution, composed of PBS 0.5% Triton and 3% NDS (normal donkey serum). They were then incubated for 24 hours at room temperature in a primary antibody solution anti-Cx43 (Zymed/Invitrogen, cat.#71-0700, 1:300).

After this period, they were washed in 0.1 M PBS and incubated in a solution containing DAPI and fluorescent secondary antibody conjugated to Alexa Fluor® 488 for 2 hours at room temperature. They were washed again in 0.1 M PB and, after drying,

covered with glycerol carbonate solution. It should be emphasized that the specificity of anti-Cx proposed in this project was validated in experiments on animals knockout (KO).

4.6 Fluoro-Jade C® (FJC) and Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay

The FJC staining solution was prepared using 0.0005% of the stock solution to 99 ml of 0.1% acetic acid. Brain sections were washed in PBS 0.1M and placed in Triton 0.3M + DAPI solution for 2 hours. Then, after washing, brain sections remained to dry at a temperature of 50 °C for 1 hour. After this period, sections were buffered in alcohol 99.9%, and then alcohol 70% and submitted to two washes in distilled water, for 10 minutes each immersion. The sections were transferred to the 0.06% potassium permanganate solution for 10 minutes and agitated gently and, after washing in distilled water, transferred to FJC working solution (Millipore Corporate Headquarters, Billerica, MA, EUA) for 20 minutes. After that, slides were washed three times in distilled water, 2 minutes each washing, and placed to dry in an oven at 50 °C. After completely dry, they were immersed in xylene for at least one minute and overlaid with coverslips using DPX.

Histological analysis by Tunel technique is characterized by the incorporation of deoxyuridine trisphosphate fluorescein-12 (12-d-UTP) at the DNA 30-OH ends, whose signal is amplified by the reaction involving the enzyme terminal deoxynucleotidyl transferase (rTdT), and the fragmented DNA marked by the 12-dUTP fluorescein becomes visible under the fluorescence microscope. We used the in situ cell death detection kit, TMR Red (Roche, USA), for coronal sections mounted on 12-μm-thick gelatinized slides (n = 5 per group). The slides were washed in 0.05MPB and then incubated for 2 min in 1% sodium citrate solution in 0.05MPB at 4°C. After additional washes in 0.1MPB, 50 μL of the Tunel reaction mixture was pipetted onto each slide. The slides were kept for 60 min at 37°C in the dark. After this procedure, they were washed again with 0.1MPB, incubated in DAPI solution (1:65000) for 5 min, washed in 0.1M PB, allowed to dry and then covered with glycerol.

4.7 Somatic development and sensorimotor reflexes ontogeny

24h after exposure to anoxia and intrahippocampal injections, male pups underwent somatic development evaluation and assessment of sensorimotor reflexes ontogeny. The pups were uniquely identified within each litter with a small tattoo on one or more of the paws and allowed until weaning on P21.

In order to evaluate the somatic development, the parameters previously established by Fox (Fox 1965) were evaluated, such as: pinna detachment, auditory conduit opening, eyes opening, the eruption of the lower and superior incisors. The body weight and length and the lateral and anterior-posterior axis of the head were assessed daily too. The maturation age of a particular feature was defined as the day when it occurred for the first time.

The reflexes were tested according to Smart and Dobbing (Smart and Dobbing 1971) and were carried out daily: **1. surface righting reflex** - pup is placed in the supine position on a flat surface and the test was considered positive when pup turned to prone support on four paws, within a maximum period of 10 s; **2. palmar grasp reflex and disappearance** – pup grasps a thin metal rod with forepaws when stroked; **3. vibrissae placing** - the pup is suspended by the tail in such a way that its vibrissa lightly touches the edge of a table. It is considered a positive response when the animal, in the maximum time of 10 s, laid its forefeet on the table trying to walk; **4. cliff avoidance** - the pup is placed with its front paws on the edge of a flat and high surface to sense the cliff. It is considered a positive response if the animal in the maximum time of 10s, moved to 45°-side featuring cliff aversion; **5. negative geotaxis** - the pup is placed in the center of a ramp of 45° tilt and its head will be downward. The reflex response is considered positive when it turns upright within a maximum period of 10 s and is able to rotate the body by placing its head upward; **6. startle reflex** - the pup is subjected to a sharp crack, produced by percussion of two metal structures at a distance of 10 cm from the animal. The response is considered positive when there is a simultaneous decrease with rapid and involuntary detention of the animal body, characteristic of fear or scare. **7. freefall righting reflex** - the pup is held upside down by four legs with the back facing down at a distance of 30 cm over a bed of synthetic foam (30 x 12 cm) and then it is released and its free-fall is observed.

The response is considered positive when, during the fall, the animal completely spun its body leaning on all four paws on foam. The first of a series of three consecutive days in which the response was present was considered to be the day of the consolidation of the reflexes.

4.8 Open Field Test

The Open Field test was performed on a 60 cm diameter and 50 cm high platform, divided by two circular areas, one peripheral and one central, in addition to the quadrants within the circles (12 quadrants). Each animal from vehicle (n=8) and CBX (n=8) groups, at P30, was placed in the center of the open field and was observed for 10 minutes. At the end of the test, the arena was cleaned with 70% alcohol solution.

4.9 Barnes Maze Test

Sixteen adult rats (P60) were used to perform the Barnes Maze spatial reference memory test and were divided into two groups: vehicle anoxia (n = 8) and CBX anoxia (n = 8).

The protocol to be followed was described by Sunyer and colleagues in 2007 (http://www.nature.com/protocolexchange/protocols/349#/procedure), with minor modifications. The animal was placed in the center of the platform (diameter 120 cm), initially in a dark cylinder, where it remained for 1 minute. The cylinder was removed, causing in the animal the aversive response against the brightness of the room, which should be well lit. The time limit to explore is 180 seconds and when the animal finds the escape box it should remain inside for 60 seconds. If it did not find the escape box in this period, it was gently led to the same, where it remained for 1 minute. The training sessions were held for 4 days (sessions), each session consisting of 4 trials with 15-minute intervals between them. The escape box remained in the same place (in relation to the runways of the environment) every day. On the 5th and 12th days, when the "probe trails" were performed, the escape box was removed and the animals had 90s to find their old location, thus allowing verification of short and long-term memory retention, respectively.

The recording and analysis of the data were carried out with the camera coupled to a computer containing Noldus software, Ethovision XT 7.

To analyze the data, we compared the path length, latency and number of errors that each animal in each group performed, according to the methodology described in the literature. To quantify the rates of error and accuracy of the animals, we classified the path covered in some categories:

- Direct (D): the animal proceeded straight to the escape box;

- Direct Error (ED): the animal went through up to 2 holes until reaching the escape box;

- Quadrant (Q): the animal proceeded straight to the quadrant where the escape box was located (quadrant with 3 holes to the right and 3 to the left in relation to the escape box);

- In Series (S): the animal followed around the perimeter, beginning with some wrong hole until finding the right hole;

- In series and random (SA): the animal apparently presented a random strategy but will continue in series around the perimeter until finding the right hole;

- Random (A): the animal followed randomly, with no apparent strategies.

Each classification received a score (D: 6 points, Q: 5 points, ED: 4 points, S: 3 points, SA: 2 points, A: 1 point).

4.10 Analysis of results

Sections were examined with a fluorescence microscope (DM 5500, Leica Microsystems, Wetzlar Germany) using the frequency excitation 450-490 nm. The capture of the photomicrographs was held using the camera (DFC 365 FX, Leica Microsystems, Germany) coupled with the computer deconvolution system Leica Application Suite Advanced Fluorescence software (LAS AF, Leica Microsystems). The pixel density was measured with the same software using standard boxes randomly distributed by structures of interest (hippocampus) (4 sections per animal, 6 boxes per

section). FJC+ and TUNEL+ cells were counted manually in the whole extension of bilateral hippocampal slices.

We used the ANOVA with post hoc Tukey for statistical analysis of pixel density and Barnes Maze data; one-way ANOVA for developmental parameters; T-Student test for Tunel, FJC, and Open Field data.

5. RESULTS

5.1 Cx43 immunohistochemistry 24 hours after neonatal anoxia

To study the distribution and location of Cx43 in the hippocampus of neonates, they were perfused transcardially 24 hours after neonatal anoxia, the brains were dissected, cryosectioned at cryostat and mounted on gelatinized slides. Immunohistochemistry using anti-Cx43 was performed. After the capture of the photomicrographs, the pixel density was measured and the values (n=5 per group) were entered into a two-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey's HSD test). The results obtained are shown in figure 1.

The quantification of pixels of Cx43-immunoreactive cells, 24 hours after neonatal anoxia (control, n = 5, anoxia, n = 5) showed an increase in the oriens layer CA3 in animals subjected to anoxia (45.20 ± 6.45) compared to control (26.60 ± 3.15; p = 0.035). No differences were observed in other layers and other subfields (p>0.05)

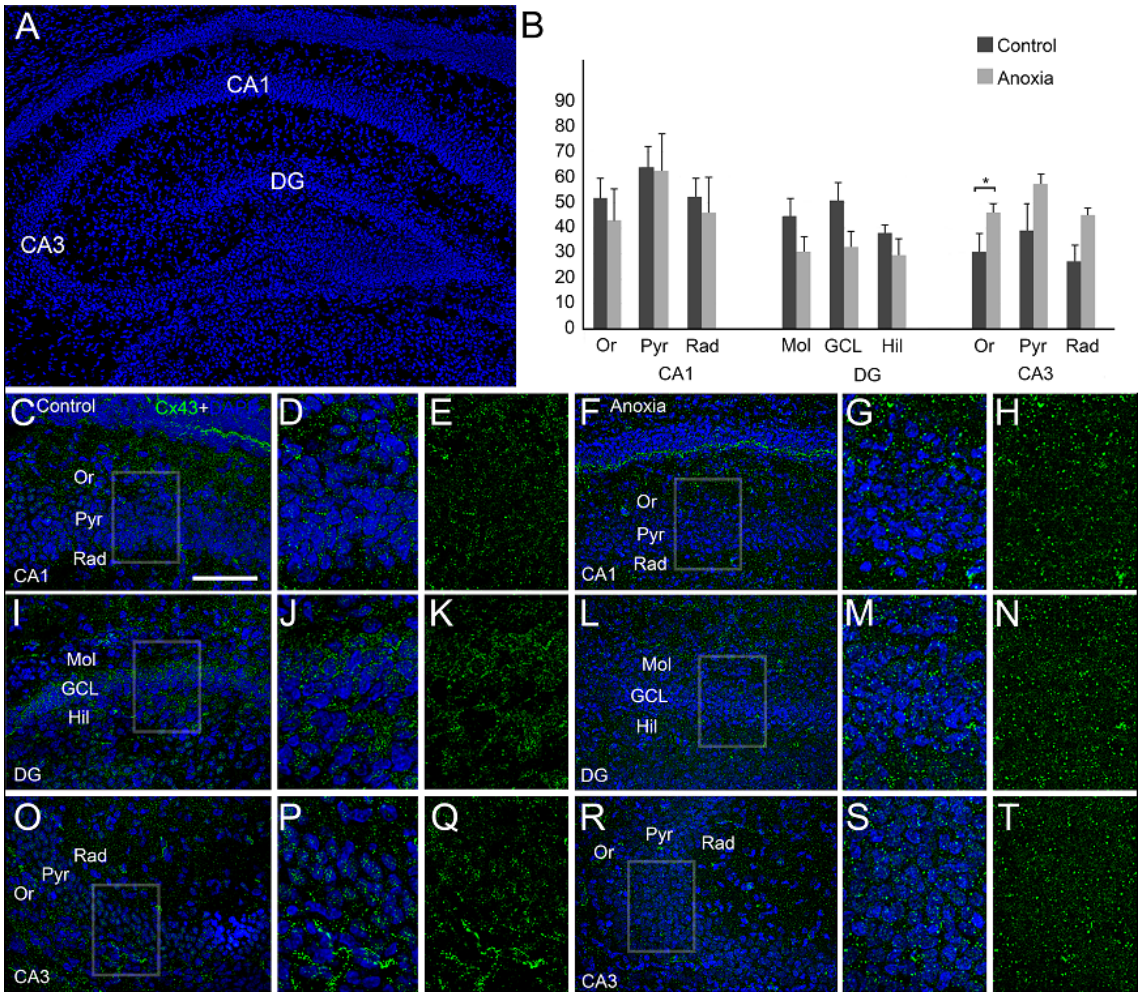

Figure 1: Cx43 distribution pattern in rat hippocampus 24 hours after neonatal anoxia. (A) DAPI stained coronal slice of hippocampus region showing the subfields CA1, CA3 and DG considered in the pixels quantification procedure. (B) Values (n=5 per group) were entered into a two-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey's HSD test). Neonatal anoxia caused an increase in anoxia group in CA3 in oriens layer. *p<0.05. Cx43 distribution pattern in Control (CA1, C-E; CA3, I-K; DG, O-Q) and Anoxia (CA1, F-H; CA3, L-N; DG, R-T). CA1: Cornus Ammonis 1; CA3: Cornus Ammonis 3; DG: Dentate Gyrus.

5.2 Bilateral intrahippocampal CBX injection decreased FJC+ cells after neonatal anoxia

To study hippocampal neurodegeneration 24h after neonatal anoxia and intrahippocampal injection of CBX or vehicle (PBS), they were performed FJC and TUNEL staining. After the capture of the photomicrographs, FJC+ and TUNEL+ cells were counted manually, and T-Student test was performed. The results obtained are shown in figure 2.

Bilateral intrahippocampal injection of CBX resulted in decreased neurodegeneration caused by neonatal anoxia, as evidenced by a reduction in the number of FJC + cells (vehicle group, n = 5, CA1: 9.3 ± 3.67, CA3: 16 ± 2.84, DG: 9.6 ± 6.4; CBX group, n = 5, CA1: 4 ± 0.42, CA3: 11.125 ± 1.23, DG: 2.375 ± 0.063). The number of TUNEL + cells 24 h after CBX injection was not altered (vehicle group, n = 4, CA1: 1.75 ± 0.24, DG: 1.50 ± 0.59; CA3: 2.25 ± 0.43; CBX group, n = 4, CA1: 3.00 ± 0.20, DG: 3.75 ± 0.43, CA3: 2.50 ± 0.43; p-value>0.05).

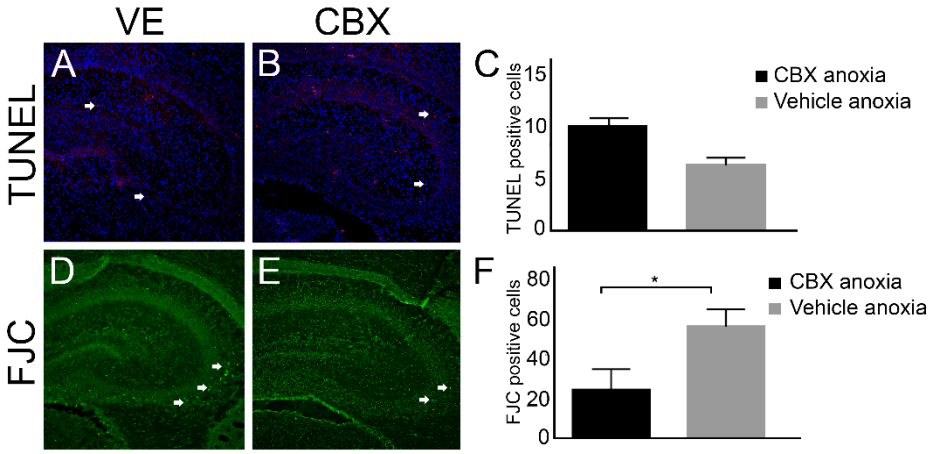

Figure 2: FJC+ and TUNEL+ cells quantification of anoxia animals treated with PBS (vehicle) or carbenoxolone (CBX). (A) hippocampal coronal slice of vehicle pup showing TUNEL+ cells (white arrows) in red; (B) hippocampal coronal slice of CBX pup showing TUNEL+ cells (white arrows) in red; (C) No differences were observed in the quantity of TUNEL+ cells between vehicle and CBX groups; (D) hippocampal coronal slice of vehicle pup showing FJC+ cells (white arrows); (E) hippocampal coronal slice of CBX pup showing FJC+ cells (white arrows); (F) T-Student test evidenced decrease in FJC+ cells in the hippocampus of CBX group.

5.3 Somatic development

The animals submitted to neonatal anoxia and intrahippocampal injection of CBX had a reduction of body weight at the ages of P18 ($p < 0.001$), P19 and P20 ($p < 0.05$) in relation to the anoxia group injected with vehicle solution (Figure 3A). In the anteroposterior axis of the skull, the CBX group had a larger diameter in relation to the vehicle group in P4, and it was the same in all other ages ($p < 0.001$; Figure 3C). There were no changes in body length (Figure 3B) and neither in the lateral axis of the skull (Figure 3D).

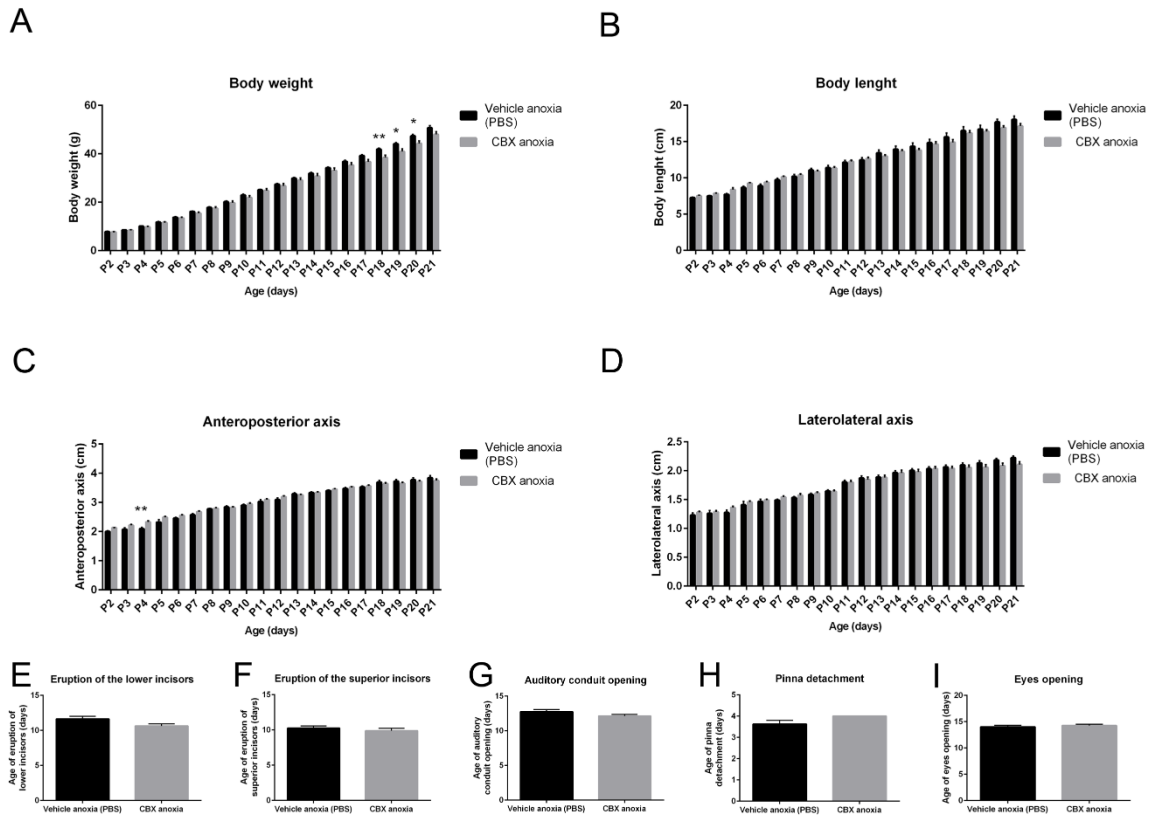

Figure 3: Analysis of somatic development in rats subjected to neonatal anoxia and intrahippocampal injection carbenoxolone (CBX) or vehicle (PBS 0.1M). There was a reduction in body weight in P18, P19 and P20 animals of the CBX group and an increase in the anteroposterior axis of the skull at P4, and there were no changes in the length of the body or the lateral-lateral axis of the skull.

5.4 Ontogeny of sensorimotor reflexes

The analysis of the sensorimotor reflexes of the animals submitted to neonatal anoxia and subsequent intrahippocampal injection of CBX or vehicle showed a delay in the acquisition of the cliff avoidance reflex of the CBX group when compared to the vehicle group ($p = 0.05$, Figure 4C). There was no change in the other tested reflexes.

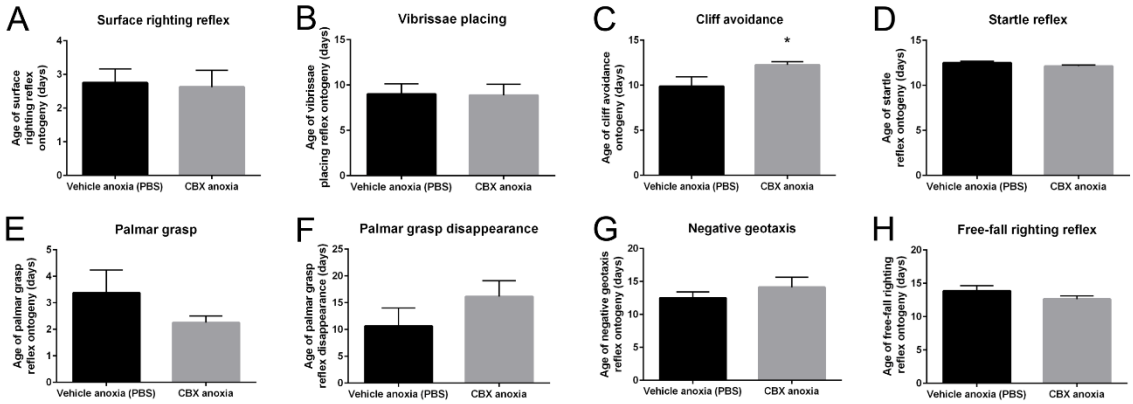

Figure 4: Analysis of sensorimotor reflexes in rats subjected to neonatal anoxia and intrahippocampal injection carbenoxolone (CBX) or vehicle (PBS 0.1M). There was a delay in the acquisition of the cliff aversion reflex in the CBX group in relation to the vehicle group (p=0,05, 4C). In the other reflexes, no differences were observed.

5.5 Locomotor activity and anxiety

The open-field behavior evaluation did not show differences between the CBX and vehicle groups (Figure 5), although there was an apparent tendency of the CBX group to present a longer latency in the center (p = 0.0882), indicating a possible decrease in anxiety-like behavior (Figure 5B).

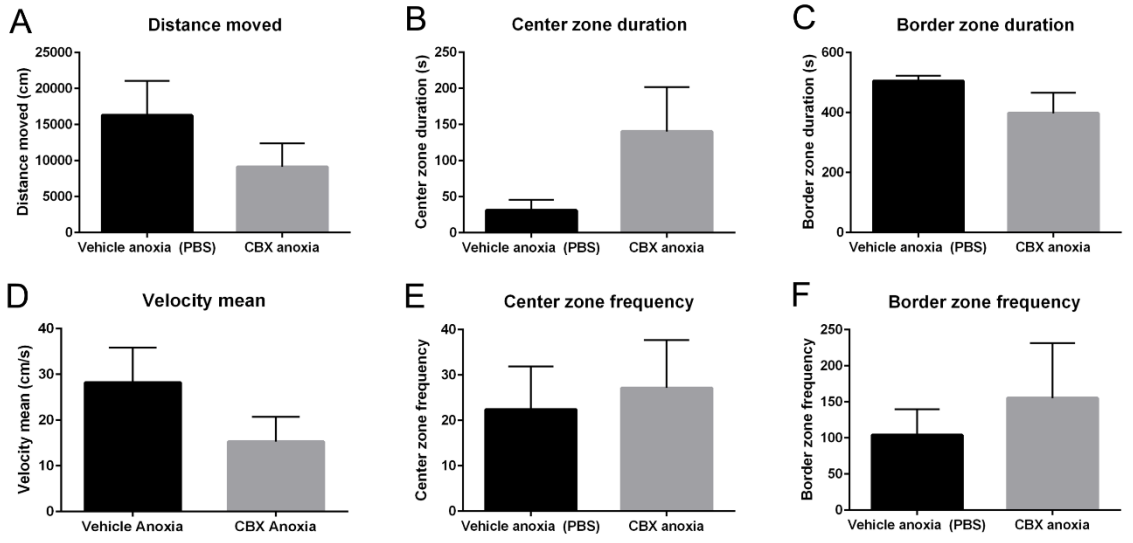

Figure 5: Analysis of the locomotor activity and the anxious behavior in rats subjected to neonatal anoxia and intrahippocampal injection carbenoxolone (CBX) or vehicle (PBS 0.1M). There was no difference between the CBX and vehicle (PBS) groups in any of the analyzed parameters.

5.6 Spatial reference memory

The analysis of the spatial memory of the animals submitted to neonatal anoxia and subsequent intrahippocampal injection of CBX or vehicle showed a reduction in path length on the second and third test day, on the first and third trial respectively in the CBX group compared to the vehicle group (PBS) (p-value <0.05, Figure 6B). In the other analyzed parameters, there were no observed differences.

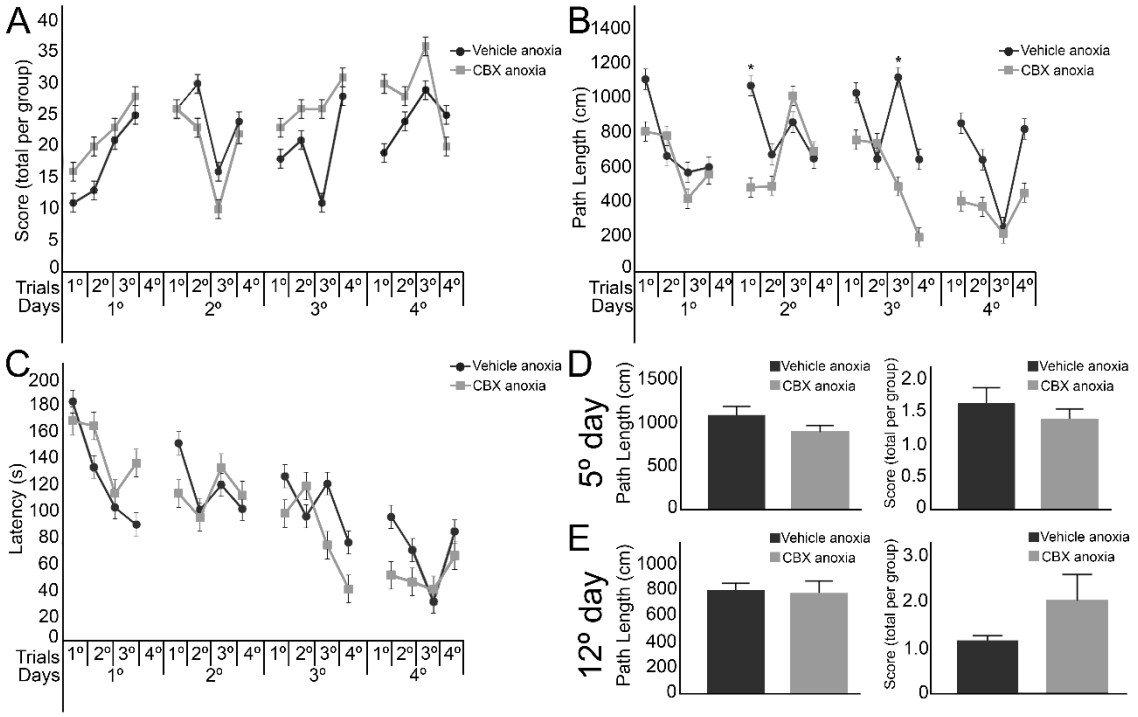

Figure 6: Analysis of spatial reference memory of rats submitted to neonatal anoxia and intrahippocampal injection of carbenoxolone (CBX) or vehicle (0.1M PBS). There was a reduction in path length on the second and third test days in the CBX group compared to the vehicle group (PBS). In the other analyzed parameters, there were no observed differences. * p-value <0.05.

6. DISCUSSION

In our project, we aimed to evaluate the distribution and quantification of Cx43 and the possible effects of the use of the gap junctions blocker, carbenoxolone, in the hippocampal neurodegeneration and in the functional sequelae of rats submitted to neonatal anoxia. In the introduction, we presented the hypotheses of our project:

Hypothesis 1 (H1): changes in the coupling between astrocytes and, consequently, in the distribution and location of Cx43 can be expected to occur in response to the cascade triggered by oxygen deprivation and influence the pattern of neurodegeneration and glial changes;

Hypothesis 2 (H2): Blocking gap junctions by carbenoxolone may be effective against hippocampal neurodegeneration due to oxygen deprivation;

Hypothesis 3 (H3): Blocking gap junctions by carbenoxolone can mitigate the functional sequelae in juvenile and adult rats submitted to neonatal anoxia.

The pixels quantitative analyzes of immunoreactive cells to Cx43 showed interesting changes caused by neonatal anoxia: 24 hours of anoxia, an increase of Cx43 in oriens layer of CA3. The increase of Cx43 triggered by neonatal anoxia, plus the peak cell death in the same period (after 24 hours), as shown by Takada et al (Takada, Dos Santos Haemmerle et al. 2015) suggests the correlation between Cx43 and hippocampal cell death. Also, the location of the largest amount of Cx43 24 hours post-anoxia coincides with the result observed with Fluoro-Jade B ®, a neurodegeneration marker, shown in the same article.

We observed a reduction of Fluoro Jade C + cells in anoxiated pups submitted to intrahippocampal injection of carbenoxolone, a gap junction blocker and did not observe differences in the number of TUNEL+ cells after gap junction blockade. de Pina-Benabou and colleagues (de Pina-Benabou, Szostak et al. 2005) performed in vitro and in vivo studies about the effects of gap junctions blockade in slices submitted to oxygen-glucose deprivation and in the administration of intraperitoneal injection of CBX in neonate rats submitted to intrauterine asphyxia. They observed that CBX led to reduced cell death in CA1 assessed by propidium iodide 24h later with the in vitro experiment. This result was also observed in vivo. However, there was decreased activation of Caspase-3 after CBX

injection in neonate rats, suggesting that TUNEL labeling, which is admittedly an apoptosis marker, might be decreased. Takada and colleagues (Takada, dos Santos Haemmerle et al. 2015) did not observe differences in estimated activated caspase-3-positive cells after neonatal anoxia, so we should consider the possibility that our anoxia model does not cause caspase-3-dependent cell death.

Our results showed a reduction in body weight in P18, P19 and P20 animals of CBX group and an increase in the anterior-posterior axis of the skull at P4 and a delay in the acquisition of cliff aversion in the CBX group relate to the vehicle group. We did not observe differences in the length of the body or the lateral-lateral axis of the skull or other reflexes analyzed. These transient changes in the skull axis and delay in cliff aversion acquisition may reflect the influences of gap junctions on brain development (for review see (Bruzzone and Dermietzel 2006)). In the evaluation of anxious behavior and locomotor activity, there were no differences between the groups. However, in the spatial reference memory, we observed a reduction in the path length in the CBX group compared to the vehicle group on the second and third test days. In the other analyzed parameters, no differences were observed. We had a high value of standard error mean, so probably the significance of this result could be reached using a larger number of animals.

7. FINAL CONSIDERATIONS

Neonatal anoxia is a public health problem with severe and permanent sequelae. In our project, we analyzed the role of gap junctions formed by connexins in cell death, somatic development, sensorimotor reflexes, anxious behavior, locomotor activity and spatial memory in rats submitted to neonatal anoxia.

We observed an increase of Cx43 in oriens layer of CA3 24h after neonatal anoxia and a reduction of Fluoro Jade C + cells in anoxiated pups submitted to intrahippocampal injection of carbenoxolone, a gap junction blocker. We did not observe significant differences in locomotor activity, anxious behavior and spatial reference memory between the vehicle group (PBS) and CBX group.

In face of these results, we can conclude that gap junctions are involved in the acute phase hippocampal cell death after neonatal anoxia, but its blockade may not reverse the functional deleterious effects resulting from neonatal oxygen deprivation in juvenile and adult rats.

Further studies are needed to elucidate whether oxygen deprivation causes changes in Cx43 gene expression and protein levels after 24 hours. We hope that these results may contribute to a better understanding of the complex mechanism of cell death and functional impairment after neonatal anoxia.

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