Title: Prefrontal cortex dysfunction in hypoxic-ischemic encephalopathy contributes to executive function impairments in rats – potential contribution for attention-deficit/hyperactivity disorder

Short title: Hypoxia-ischemia contributes to ADHD in rats

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Abstract

Objectives: The attention-deficit/hyperactivity disorder (ADHD) compromises the quality of life of individuals including adaptation to the social environment. ADHD etiology includes perinatal conditions such as hypoxic-ischemic events; preclinical studies have demonstrated attentional deficits and impulsive-hyperactive outcomes after neonatal hypoxic and/or ischemic intervention but data are missing to understand this relationship. Thus, the aim of this study was to evaluate executive function (EF) and impulsivity, and tissue integrity and dopaminergic function in the prefrontal cortex (PFC) of rats submitted to hypoxia-ischemia (HI). **Methods**: At postnatal day (PND) 7, male Wistar rats were divided into control (n=10) and HI groups (n=11) and the HI procedure was conducted. At PND60, the animals were tested in the *attentional set-shifting* (ASS) task to EF and in the *tolerance to delay of reward* for assessment of impulsivity. After, morphological analysis and the dopaminergic system were evaluated in the PFC. **Results**: Animals subjected to HI had impairments in EF evidenced by a behavioral inflexibility that was correlated to PFC atrophy. Moreover, HI animals presented reduced D2 receptors in the ipsilateral side of ischemia in the PFC. **Conclusions**: Animals submitted to HI presented impaired EF associated with tissue atrophy and dopaminergic disturbance in the PFC.

Keywords: attentional set-shifting; behavioral flexibility; tolerance to delay of reward; ADHD; D2 receptor.

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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disorder affecting 8 to 12% of children (Faraone et al. 2003) and 4% of adults (Kessler et al. 2006). The symptoms include inattention, hyperactivity and impulsivity; often associated with a primary deficit in executive functions (EF) such as impulse control, vigilant attention and set shifting (Barkley 1997; Castellanos and Tannock 2002; Castellanos et al. 2006; Lezak 1982). The “Intradimensional/Extradimensional (ID/ED) shifting task” is commonly used to assess EF in humans, in which the main goal is shift responses between different exemplars into the same stimulus dimension (ID shift) or between a novel, previously irrelevant dimension (ED shift) (Owen et al. 1991). In this task, ADHD individuals have demonstrated poorer performance in comparison to healthy subjects, representing their EF impairment (Coghill et al. 2014; Gau and Chang 2010a; Gau and Chang 2010b; Kempton et al. 1999).

The concept of "impulsivity" covers a range of different maladaptive behaviors that can be broadly divided into two categories: impulsive action and impulsive choice (Winstanley et al. 2006). The impulsive choice can be measured by the intolerance to delayed reward (Winstanley et al. 2006). In this paradigm, both a smaller but immediate reward and a larger but delayed reward are offered, and impulsive choice is defined by the reduced ability to select the large delayed gratification (van Gaalen et al. 2006; Winstanley et al. 2006). In many ADHD individuals, the impulsive choice is exhibited (For review Luman et al. 2005; Pauli-Pott and Becker 2011).

With regards to the disorder neurobiology, several studies have indicated the prefrontal cortex (PFC) as the core region implicated in the ADHD symptoms (Arnsten 2007; Cubillo et al. 2012). Moreover, the theory of dopamine (DA) dysfunction in fronto-subcortical pathways has been widely accepted. Alterations in different

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components required for an efficient dopaminergic neurotransmission such as the dopamine D2 receptor (Volkow et al. 2009) and the dopamine transporter (DAT) have been shown (Cheon et al. 2003; Dougherty et al. 1999; Krause et al. 2000). Supporting the DA theory, methylphenidate is widely used to relieve ADHD-related symptoms by blocking DAT, thereby increasing DA concentration in the synaptic cleft (Fredriksen et al. 2014; Greenhill et al. 2002; Sonders et al. 1997).

Several environmental factors have been associated with an increased risk of developing ADHD. They occur mainly during prenatal and/or perinatal period, such as drug exposure by the mother, poor fetal growth, and neonatal hypoxia-ischemia (HI) (Millichap 2008; Schmitt and Romanos 2012). Especially, several clinical trials have related perinatal hypoxic-ischemic events and the development of ADHD-like characteristics (Zhu et al. 2016; Dopwell et al. 2014; Getahun et al. 2013; Lou 1996; Marlow et al. 2005). In preclinical studies, hyperactivity was observed after neonatal repeated hypoxia (Oorschot et al. 2007; Oorschot et al. 2013) and attentional deficits and impulsive-hyperactive outcomes were found after the hypoxia-ischemia procedure (Miguel et al. 2015, Smith et al. 2014; Ikeda et al. 2001). We have previously demonstrated that attention deficits and impulsivity in adult rats that underwent neonatal HI was associated to brain atrophy in different regions (Miguel et al. 2015). However, some behavioral assessments and the neurobiological mechanisms involved are still poorly understood. Therefore, the aim of this study was to explore behavioral aspects related to ADHD in adult rats submitted to neonatal HI using the *attentional set-shifting task*, developed for rats based on the ID/ED shifting task, and the *tolerance to delay of reward* to evaluate impulsive choice. Furthermore, morphological analysis and the dopaminergic system were evaluated in the PFC area.

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Materials and Methods

Animals

Twenty one male Wistar rats were used in this study. On the 7th postnatal day (PND), pups were randomly distributed into control (CT, n=10) and HI (n=11) groups. Animals were maintained in a constant room temperature (22-24°C) with 12h light–dark cycle. Standard rat chow and water *ad libitum* were available to the dams during all period and to the weaned pups until PND60; after this period, food restriction was applied to the behavioral tasks (see details below). All procedures were approved by the Institutional Ethics Committee on Animal Use (No. 25709) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023).

HI Procedure

Neonatal HI model was based on Levine method (Levine 1960) adapted for rat pups by Rice (Rice et al. 1981). At PND7, animals were anesthetized with halothane (2–4%), an incision was made on the neck and the right common carotid artery was occluded with a silk thread. Past two hours interval with their dams, the pups were placed in chambers partially immersed in a 37°C water bath, in which they received hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90min. Sham surgery was applied in control group, i.e., animals received only anesthesia and neck incision (Miguel et al. 2015; Pereira et al. 2007).

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Behavioral Tasks

In adult phase (60 PND) animals were tested in the *attentional set-shifting* and *tolerance to delay of reward* tasks. Food restriction was applied (15-20g/rat/day) one week prior and throughout both behavioral tasks; daily weighing was conducted for certifying that animals maintained a body weight at 85% to 90% of their free-feeding weight (Birrell and Brown 2000; Chase et al. 2012).

Attentional set-shifting (ASS)

Animals were trained to dig into two bowls filled with a variable medium and odor in order to receive a food reward. Firstly, the animals must respond to changes in odor, regardless of the medium in which it is exposed. After, there was a change in stimulus dimension, being the medium the relevant stimulus, independent of the odor (Birrell and Brown 2000).

The a*pparatus* consisted of a black acrylic box (70 x 40 x 40cm) divided in two sections by a removable panel: the starting and choice area (two thirds of the box, where the bowls were presented). In the choice area, another removable panel separated the bowls (terra cotta bowls with 7cm in diameter and 4cm in depth) (Birrell and Brown 2000).

Habituation/pre-training: In the first day, animals were allowed to explore the apparatus for 15 minutes (habituation). From the second day onwards (pre-training), two bowls filled with wood shaving were presented with portions of reward (1/4 Froot Loops, Kellogg's®) placed on the top of wood shaving. Throughout the trials, the reward was progressively buried and once the animals had successfully found the reward (10 digging in alternating sides) they started the simple discrimination training (McCoy et al. 2007). Rats required between 4 and 6 habituation/pre-training daily sessions before starting the SD training.

Simple Discrimination (SD) training: In this stage, two simple discriminations were presented: two different odors with the same digging medium and two different mediums with the same odor. For each one of the discriminations, animals should reach 6 consecutive correct trials before moving on to the next discrimination, excluding the first 4 trials that were considered discovery trials (when animals could explore both bowls). Examples of discriminations adopted in this training were not used in the test phase (Birrell and Brown 2000; McCoy et al. 2007). A single session was performed at this stage.

Test phase: In the following day of SD training, animals performed the test phase. Here, animals were evaluated in a series of 7 discriminations performed in sequence, in a single daily session. The criterion to move to the next discrimination was the same employed in the SD training.

In SD testing the bowls differed by odors, being the reward always baited in a specific odor (e.g, oregano). In compound discrimination (CD) a second dimension (digging medium) was introduced along with the odor previously presented, but the reward remained baited in the oregano bowl, independent of the digging medium. In reversal 1 (Rev1), odors and mediums remained the same, but the odor that was previously associated with the reward, now was not. New exemplars of odors and mediums were used in intradimensional (ID) and extradimensional (ED) shift. The difference is that in ID shift, odor remained the relevant dimension whereas in ED shift, the dimension changed, being medium the relevant dimension. Both ID and ED discriminations were followed by reversals: reversal 2 (Rev2) and reversal 3 (Rev3), respectively. The test paradigm is represented in Figure 1. Additionally, odors and mediums exemplars used in each discrimination phase are described in Table 1. The number of trials required to achieve the criterion of six consecutive correct trials and the

number of errors made in each discrimination were evaluated. For the complete attainment of ASS task, animals required 6 to 8 daily sessions.

Tolerance to delay of reward

Two days after ASS, animals were started in the *tolerance to delay of reward* protocol - adapted from Thiébot et al. (1985). This task measures impulsive choice by offering two different magnitudes of food reward: small amount delivered immediately or large amount delayed (Bizot et al. 2007; Thiébot et al. 1985).

The *apparatus* consisted of a black T-maze with a starting runway and two arms equipped with removable doors at the entrance (proximal door 1 - d1) and distal portion of the arm (distal door 2 - d2). Each arm measures 50 cm long, 40 cm high and 10 cm internal width.

Habituation: In the first day, all the doors (d1 and d2) were open and animals were allowed to explore the maze for 5 minutes and eat the food reward (same amount) at the end of both arms.

Pre-training: From the second day onwards, the right arm of the maze contained a large amount of reward (8 pieces of ¼ Froot Loops, Kellogg's®) whereas the left arm had a small amount (2 pieces of ¼ Froot Loops). All the doors remained open in the pre-training phase and the animals could eat the reward from both arms. After the food was eaten in both arms, rats were removed from the maze and returned to their home cage for a 2-3 min intertrial interval. This stage was conducted for 3 days with 2 sessions of 5 trials/each.

Training: In the training phase, both doors remained closed; at the moment that the animal chose one arm, d1 of the respective arm was closed and d2 was opened, revealing the reward. After the food was eaten, the animal was returned to its home cage for a 2-3 min intertrial interval. Rats had to learn the paradigm choosing the arm leading

to the large reward in 80% of the times (in one day, 8 out of 10 trials). It was stipulated that the animals would spend a minimum of 3 days in this phase, with 2 sessions of 5 trials/each, before proceeding to the Test.

Test: At this stage (10 days, one session of 5 trials/day), when animals chose the arm leading to the large reward, a delay was introduced before accessing the food. Animals were confined between d1 and d2 during 15 seconds (in the first 5 days) or 30 seconds (in the last 5 days); if they chose the arm leading to the small reward, no delay was applied. The number of choices to the small-and-immediate reward or to the large-but-delayed reward was counted. The inability to wait for the large gratification is described as a measure of impulsive-related behavior (Bizot et al. 2007). At the end of the test session, animals received rat chow *ad libitum* for at least 2 days before euthanasia.

Tissue collection

Animals were decapitated, a coronal cut was made (+3.7mm from Bregma, according to Paxinos and Watson 2004) and the PFC (anterior of this cut) was dissected out, instantaneously placed in liquid nitrogen and stored at -80ºC until western blot analysis.

Brain atrophy and behavioral performance correlation

The brain area, at the level of the cut, was photographed in coronal position for estimating the tissue atrophy. This gross morphological measure was performed in the same brains for the Western Blot analysis. Two images of the same coronal position were analyzed per rat and the area of both hemispheres was calculated using Image J software (NIH, USA). The final result of each rat's hemisphere area was the mean of

the areas from the two images. A percentage of brain atrophy for each animal was calculated by relating the left hemisphere (contralateral to the lesion) with the right hemisphere (ipsilateral to the lesion) areas. The percentage of brain atrophy was correlated to the total number of trials and errors in the ASS task, using the Pearson correlation analysis.

Western Blot analysis

Western Blot was performed to verify the levels of tyrosine-hydroxylase (TH) - the rate-limiting enzyme for DA synthesis, dopamine transporter (DAT), responsible for terminating the dopamine action by rapid reuptake into the presynaptic neuron, and dopamine D2 receptor. PFC samples were homogenized in cytosolic extraction buffer with protease (Complete, Roche) and phosphatase inhibitors (Phostop, Roche). After that, the samples were centrifuged at 3.000 rpm (4ºC) for 10 min for cytosolic protein extraction and thereafter at 13.000 rpm (4ºC) for 30 min for purification of the cytosolic fraction. Supernatant of this centrifugation was used to quantify the total protein in the sample, using a BCA protein assay with bovine serum albumin as standard (Thermo Scientific). Aliquots containing 20 µg (DAT) or 50 µg of protein (TH and D2 receptor) were incubated with LDS (Invitrogen) and DTT (Sigma-Aldrich) and protein denaturation occurred boiling the samples at 99ºC for 3 min. They were then loaded on 4–12% polyacrylamide gradient gels (Invitrogen) and a standard molecular weight marker (Magic Marker®, Invitrogen) guided the right position of protein weights. Samples were submitted to electrophoresis, transferred to a nitrocellulose membrane (GE Healthcare) and blocked in Tris-buffered saline with 1% Tween-20 (Sigma) and 5% nonfat dry milk. The membranes were incubated overnight at 4ºC with the following primary antibodies: anti-tyrosine hydroxylase (Millipore, AB152, 1:2000 dilution), anti-dopamine transporter (Sigma-Aldrich, AB1591P, 1:500) and anti-

dopamine D2 receptor (Millipore, AB5084P, 1:500). Secondary antibodies anti-mouse (Cell Signaling, 7076s, 1:2000) or anti-rabbit (Cell Signaling, 7074s, 1:2000) were incubated for 2h in room temperature. After that, chemiluminescence signal was detected using ECL (ECL western blotting analysis system, GE healthcare, RNP2106) and the bands intensity was quantified by densitometry using the ImageJ® software (National Institute of Health, USA). Results were expressed as the ratio between the protein of interest and β-actin (Sigma-Aldrich, A4700, 1:1000) on the same membrane.

Statistical Analysis

The behavioral performance in ASS task, western blot analysis and brain atrophy were evaluated by unpaired *t* test. Repeated-measures analysis of variance (ANOVA) was applied to analyze the performance in the "Tolerance to delay of reward" throughout the days in each time, with Group as the between-subjects factor and Day as the repeated measure. In this task, the means in each waiting time were also analyzed using two-way ANOVA, with Group and Delay time as factors. When required, analyses were followed by the post hoc Tukey's test for multiple comparisons. The correlation between attention performance and brain atrophy was assessed by Pearson correlation. All variables were expressed as mean ± standard error of the mean (S.E.M.), and results were considered significant when $p < 0.05$. All analyses were performed using the Statistica software package (StatSoft, Tulsa, OK), version 10.

Results

Attentional set-shifting

HI animals needed more trials to reach criterion of six consecutive correct trials in reversal 2 ($t(19)= -2.52$; $p < 0.05$) and reversal 3 stages ($t(19)= -3.96$; $p < 0.001$), when compared to the CT animals. There were no differences between groups on the others stages (Figure 2A). Assessing the total number of trials carried out over the entire

task, HI animals required more trials than CT animals ($t(19)= -5.71$; $p < 0.0001$) (Figure 2B).

Unpaired *t* test demonstrated a higher number of errors in HI animals in reversal 2 ($t(19)= -2.67$; $p < 0.05$) and reversal 3 stages ($t(19)= -4.59$; $p < 0.001$), in agreement with higher number of trials to reach criterion. The number of errors in extradimensional shift was also higher in HI animals, when compared to CT group ($t(19)= -2.36$; $p < 0.05$) (Figure 2C). When evaluating the total number of errors throughout the task, the HI group showed twice as many errors as the CT group ($t(19)= -5.53$; $p < 0.0001$), as shown in Figure 2D.

Tolerance to delay of reward

During training, the number of choices to the large reward during two daily sessions over three days, without any delay, was registered. Repeated-measures ANOVA showed a significant main effect of Day ($F(2,38) = 10.34$, $p < 0.001$) and Tukey's post hoc comparisons revealed that the number of choices to the large reward increased on the third day of training, when compared to the first day, in both CT and HI groups (Figure 3A).

During the test phase, one session per day was held over ten days (five days with 15s waiting time and 5 with 30s). Analyzing the delay of 15 seconds, repeated-measures ANOVA showed a main effect of Day ($F(4,76) = 16.65$, $p < 0.0001$) and Tukey's post hoc comparisons demonstrated that the number of choices to the large reward decreased starting from the third day of test, compared to the first day, in both groups (Figure 3B). When the delay of 30 seconds was applied, repeated-measures ANOVA revealed no significant differences throughout the days, in none of the groups (Figure 3C); that is, the groups already showed lower preference for the large reward from the first session.

Mean values of choices to the large reward in each delay time (0, 15 or 30 seconds) were also shown (Figure 3D). Two-way ANOVA demonstrated a main effect of Delay time ($F(2,57) = 334.91$, $p < 0.0001$) and Tukey's post hoc comparisons pointed out that there was a decrease in the number of choices to the large reward when any delay to receive it was inserted. This decrease was progressive in relation to delay time and also similar between groups (Figure 3D).

Brain atrophy and behavioral performance correlation

As expected, the percentage of brain atrophy in PFC region was higher in HI group than CT group ($t(19)= 7.89$; $p < 0.0001$), as shown in Table 2. After this analysis, a correlation between brain atrophy and behavioral performance in ASS task was made for each animal. A significant positive correlation between the total number of trials to complete the ASS task ($p < 0.001$ and $r = 0.66$; Figure 4A), as well as the total number of errors in the task ($p < 0.01$ and $r = 0.65$) and the percentage of brain atrophy in the PFC region were found (Figure 4B).

Western Blot analysis

Protein levels of DAT, dopamine D2 receptors and TH in the PFC are shown in Figure 5. Unpaired *t* test showed a decrease in D2 levels in the HI group when compared to CT group in the ipsilateral PFC ($t(6)= 2.32$; $p < 0.05$). No difference was found in D2 levels in the contralateral region ($t(6)= 1.11$; $p = 0.3$) (Figure 5A). TH and DAT levels were not affected by HI procedure, on both hemispheres (Figure 5B and C).

Discussion

The purpose of the present study was to investigate behavioral characteristics related to ADHD in adult rats that underwent HI in the neonatal period and correlate these deficits with structural and functional abnormalities in the PFC region. Animals exposed to HI showed impaired EF evaluated in the ASS task. The PFC proved to be a vulnerable region to hypoxic-ischemic injury and the atrophy size positively correlated with the performance on the ASS task. In addition, dopamine D2 receptors are diminished in this region, which would decrease the effectiveness of the postsynaptic dopamine action. EF is a term used to describe a wide range of cognitive processes which comprises the attentional set-shifting representing behavioral flexibility (Cao et al. 2012). In humans, a good option to analyze the attentional set-shifting is the "Intradimensional/Extradimensional (ID/ED) shifting task" (Downes et al. 1989), which earned a version to be applied in rats by Birrel and Brown (2000), named "attentional set-shifting" (ASS) task. It has been used to assess behavioral flexibility related in several contexts such as frontal cortex lesion (Birrel and Brown 2000), centrally acting drugs (Tunbridge et al. 2004), genetic models of ADHD (Cao et al. 2012), intrauterine growth restriction (Alves et al. 2015) and bilateral common carotid artery occlusion (Kim et al. 2016). Until now, there was no study assessing EF by this recognized task following hypoxic-ischemic events in rodents. We have previously demonstrated long-term attention deficits and inhibitory control failures in the 5-choice serial reaction time task (5-CSRTT) in rats that underwent neonatal HI, and these results indicate that this neonatal injury could contribute to the development of behavioral characteristics observed in ADHD (Miguel et al. 2015). Given that impairments in EFs are the core deficit in ADHD individuals, to evaluate these aspects in subjects who have had

neonatal HI is very important in order to elucidate environmental factors that could contribute to the development of ADHD.

The present results indicated that animals submitted to neonatal HI and assessed in the ASS task showed a clear impairment in reversals stages of the task and also had made a higher number of errors in the extradimensional shift stage, when the relevant stimulus changed from odor to the digging medium (previously irrelevant). Since the ID shift stage was not affected by the HI procedure, impairments observed in the reversals and ED shift stages cannot be interpreted as a general learning deficit. These findings indicate that neonatal HI selectively compromises discriminations that required attention shift between different dimensions (ED - medium to odor) or even a shift within the same dimension (REV - odor 1 to odor 2). In accordance with our results, van der Kooij and coworkers (2010) found impaired cognitive flexibility, without significant learning deficits, in HI rats evaluated in the *modified hole board* at PND 45. The observed errors in reversal stages in our study seem to result from a behavioral inflexibility consequent to a greater perseveration to the rule employed in the previous stage of the task. Perseverative responses have been already observed in HI animals in the 5-CSRTT (Miguel et al. 2015) and in the choice reaction time task (CRTT) (Ikeda et al. 2001; Mishima et al. 2004) - tasks used for evaluation of attention processes. Thus, the acquired results in the ASS task reinforce the idea that neonatal HI alters behavioral flexibility of adult animals and this characteristic is well-described in ADHD patients (Coghill et al. 2014; Gau and Chang 2010a; Gau and Chang 2010b; Kempton et al. 1999).

Several studies have proved that the PFC plays an essential role in carrying out tests that require shifting an attention set, in either clinical trials or experimental studies. In humans, these conclusions resulted from studies that assess brain activity by

electrophysiology (Mestrović et al. 2012), positron emission tomography (PET) (Sawada et al. 2012) and functional magnetic resonance imaging (fMRI) (Lie et al. 2006). In animal models, some studies have also dissociated different frontal regions underlying different phases of this task. Flexibility in reversal stages depends on the orbitofrontal cortex (OFC) integrity, while the flexibility in ED shift depends on lateral PFC integrity in monkeys (Dias et al. 1996) or medial PFC (mPFC) in rats (Birrell and Brown 2000) and mice (Bissonette et al. 2008). Considering that HI causes an extensive brain cortical atrophy (Alexander et al. 2014; Miguel et al. 2015; Schuch et al. 2016), it is difficult to dissociate the different cortical regions; we thus analyzed the total PFC atrophy. There was a significant percentage of PFC atrophy in the ipsilateral side of the ischemia in HI animals. As both flexibility in reversals and ED stages in the ASS task were impaired in HI animals and a total atrophy of the PFC was observed, we can suggest that both OFC and mPFC subregions must be compromised by the HI procedure. PFC atrophy positively correlated with the total number of errors and trials needed throughout the ASS task (figure 10). PFC atrophy has been observed in HI animals (Taniguchi and Andreasson, 2008; Zhang et al. 2014), with intense neuronal death that has an early onset in this region demonstrated 24h (Chang et al. 2013) or 48h post-HI (Gencpınar et al. 2011). The main mechanisms underlying neuronal death following hypoxic-ischemic conditions include glutamate excitotoxicity, oxidative stress and inflammation (McLean and Ferriero, 2004). Interestingly, a decrease of frontal cortex volume associated with cell death is also observed in the spontaneously hypertensive-rat (SHR) strain, the most recognized animal model for ADHD (Mignini et al. 2004). Similarly to HI rats, inflammation and oxidative stress were identified in the brain of these animals (Kaiser et al. 2014; Lee et al. 2004; Polizio and Peña 2005; Sun et al. 2006), as well as hyperfunctional glutamatergic system in their PFC (Miller et

al. 2014). These data in experimental models can be translated into the clinic, since higher ratio of glutamate (Courvoisie et al. 2004; Hammerness et al. 2012; MacMaster et al. 2003; Moore et al. 2006), oxidative imbalance (Ceylan et al. 2010; Kul et al. 2015; Selek et al. 2008) and inflammation (Donev and Thome 2010; Hariri et al. 2012; Mitchell and Goldstein 2013) were also described in ADHD patients. Considering that the main mechanisms related to HI damage show similarities with the dysregulation that occurs in ADHD patients, the idea that neonatal HI can be seen as a good animal model for ADHD is further reinforced.

Considering ADHD pathophysiology, the theory of dopaminergic dysfunction in fronto-subcortical pathways has been widely accepted (for review see Del Campo et al. 2011). For this reason, we evaluated three major components for an efficient dopaminergic transmission in the PFC: TH, DAT and D2 receptors. TH and DAT are considered presynaptic markers, which permit to infer about the presence of DA in the synaptic cleft, and D2 is an essential postsynaptic indicator of DA transmission (Picetti et al. 1997). Our results demonstrated a reduced level of D2 receptors in the ipsilateral side of ischemia in HI animals, without any changes in TH and DAT levels. Considering that both presynaptic markers are unchanged in HI group, we can infer that the synthesis and reuptake of DA are similar between groups and therefore the down-regulation of the D2 receptors was not related to synaptic DA levels. The current evaluated dopaminergic mesocortical pathway projects neurons from the midbrain region of the brainstem to the frontal cortex (Bissonette and Roesch 2015). It is known that the brainstem (pathway origin) is a less vulnerable site to HI due to its blood supply by the vertebro-basilar arteries (Quattrocchi et al. 2016; Vannucci et al. 1988). Contrarily to this condition, cortical areas as PFC (pathway destination) are profoundly affected by this ischemic event (Zhang et al. 2014). Then it is reasonable to propose that

DAT and TH are unaltered because presynaptic mesencephalic neurons are preserved. Conversely we can assume that decreased D2 receptor levels are consequent to the neuronal damage in the PFC area. As well as our results, other studies applying the Levine-Rice HI model in rats have also shown a decrease in D2 receptors density and unaffected presynaptic markers of dopaminergic systems in the ipsilateral side of ischemia in the striatum - a region that also receives dopaminergic inputs from midbrain (Filloux et al. 1996; Johnston 1983; Johnson et al. 1994; Kostic et al. 1991; Przedborski et al. 1991). Interestingly, Floresco and co-authors (2006) demonstrated that blockade of D2 receptors in the PFC impairs the performance of rats in a set-shifting task. This blockade increased perseverative errors, indicating that D2 receptors play a critical role in behavioral flexibility. In clinical trials, systemic administration of a D2 antagonist also impairs the performance in attentional set-shifting (Mehta et al. 2004) and in ADHD patients, the impairments in set-shifting were alleviated after the use of methylphenidate, a drug that increase DA transmission in the PFC (Yang et al. 2004). Our data are in consonance with these experimental and clinical data, as we demonstrated an increase of perseverative errors in the HI group on the ASS task in association with decreased DA signaling in the PFC, since the D2 receptors are reduced in this region.

In the tolerance to delay of reward task, there was no difference in the impulsivity level between control and HI rats. The results showed only a gradual decrease in selecting the arm that predicts the large reward when the waiting time was inserted before the food delivery, in both groups. In previous work, we showed that adult animals that underwent neonatal HI had impulsive action in the 5-CSRTT, identified by increased premature responses (Miguel et al. 2015), but this aspect of impulsivity differs from the analyses made in the current study. As described by

Winstanley and colleagues (2006), the term "impulsivity" can be broadly divided in impulsive action and impulsive choice. In impulsive action, there is a failure in motoric inhibition, involving slower cognitive mechanisms; while in impulsive choice there are decision-making processes involved. Considering that, we can infer that neonatal HI seems to trigger an impulsive action of these animals in adulthood, but does not interfere in the impulsive choice. The most accepted animal model for ADHD, the SHR strain, also presented no general differences in impulsivity choice when compared to their controls Wistar–Kyoto (WKY) rats in a delay-discounting operant task (Adriani et al. 2003; Garcia and Kirkpatrick 2013). Furthermore, we must consider that there are several studies in the literature that did not find differences in impulsive choice between ADHD patients and controls (Bidwell et al. 2007; Marco et al. 2009; Paloyelis et al. 2009; Scheres et al. 2008; Scheres et al. 2006; Solanto et al. 2007).

The *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.; American Psychiatric Association, 2013) classifies the ADHD into three presentations: predominantly inattentive, predominantly hyperactive-impulsive, and combined form. In our previous work (Miguel et al. 2015) we had demonstrated that animals submitted to neonatal HI had attention deficit and impulsive action. Additionally, the current result had indicated attentional inflexibility – as a parameter of EF. Taken these findings together we can infer that the neonatal HI model seems to trigger an ADHD-combined presentation. Different from this data, a model of neonatal repeated hypoxia - which simulates extreme prematurity – resulted in predominantly hyperactive ADHD presentation, without attention deficit, that was correlated with cerebral myelin injury (Oorschot et al. 2007; Oorschot et al. 2013). We must consider that there are significant differences between this last model and the HI model applied in our study: 1) the frequency of hypoxic insult (acute vs. repeated exposition); 2) the Levine-Rice model

used in our study comprises ischemia and hypoxia resulting in extensive cortical atrophy consequent to neuronal death (Miguel et al. 2015; Chang et al. 2013); and 3) the study using repeated hypoxia was carried out in an earlier period equivalent to extreme prematurity (PND1-3) differently from our intervention (PND7). It is important to consider that some studies demonstrated that applying the same Levine-Rice model at PND3 does not cause a morphological damage as prominent as our model at PND7 (Sanches et al. 2015; Alexander et al. 2014). Thus, it is evident that the behavioral discrepancies found in these two protocols are based on different pattern of morphological damage to neural tissue of animals.

In summary, our results demonstrated that animals submitted to neonatal HI had impaired EF in adulthood and this behavior was a result of PFC atrophy and dopaminergic disturbance. These data bring further insights about the mechanisms involved in the association between the HI intervention and the development of ADHD-like characteristics in rats. Certainly more studies are needed to consolidate the relationship between neonatal HI and ADHD and involved mechanisms.

Acknowledgements

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Statement of Interest

None to declare.

Table 1: Order of discriminations and exemplars used in the attentional set shifting task.

DISCRIMINATION	ODOR	MEDIUM
Simple Discrimination (SD)	**oregano**/clove	wood shavings
Compound Discrimination (CD)	**oregano**/clove	button/shredded Styrofoam
Reversal 1 (Rev1)	**clove**/oregano	button/shredded Styrofoam
Intradimensional shift (ID)	**strawberry**/nutmeg	straw/beads
Reversal 2 (Rev2)	**nutmeg**/ strawberry	straw/beads
Extradimensional shift (ED)	vanilla/cinnamon	**chopped cardboard**/ shredded paper
Reversal 3 (Rev3)	vanilla/cinnamon	**shredded paper**/ chopped cardboard

The correct exemplars are presented in bold.

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Table 2: Percentage of brain atrophy in prefrontal cortex region.

Groups	% of brain atrophy in the PFC
CT	1.11 ± 0.65
HI	17.54 ± 6.54 *

Results are expressed as mean ± S.E.M, *t* test, $p<0.05$. *Difference between groups. CT: control; HI: hypoxia-ischemia.

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Figures

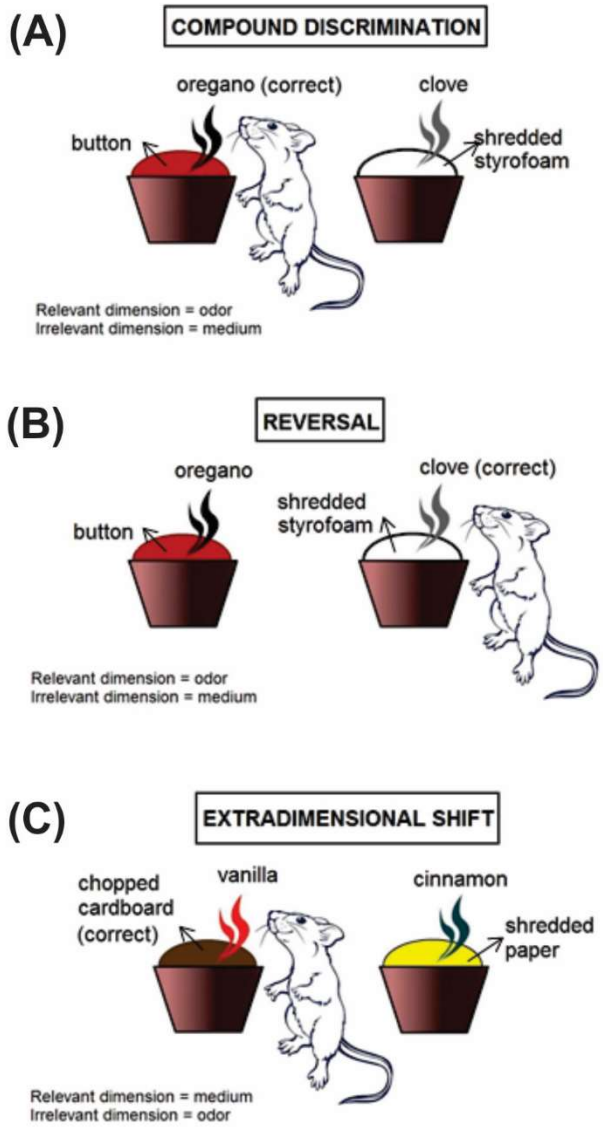

Figure 1: Schematic representation of the attentional set-shifting paradigm. In the Compound Discrimination (A), the relevant dimension is the odor and, in this example, the odor of oregano predicts the reward. In the Reversal Stage (B), the relevant stimulus

remains the same (odor) but now the right exemplar is clove, previously incorrect. In Extradimensional Shift (C), the relevant dimension changes, being medium the relevant dimension.

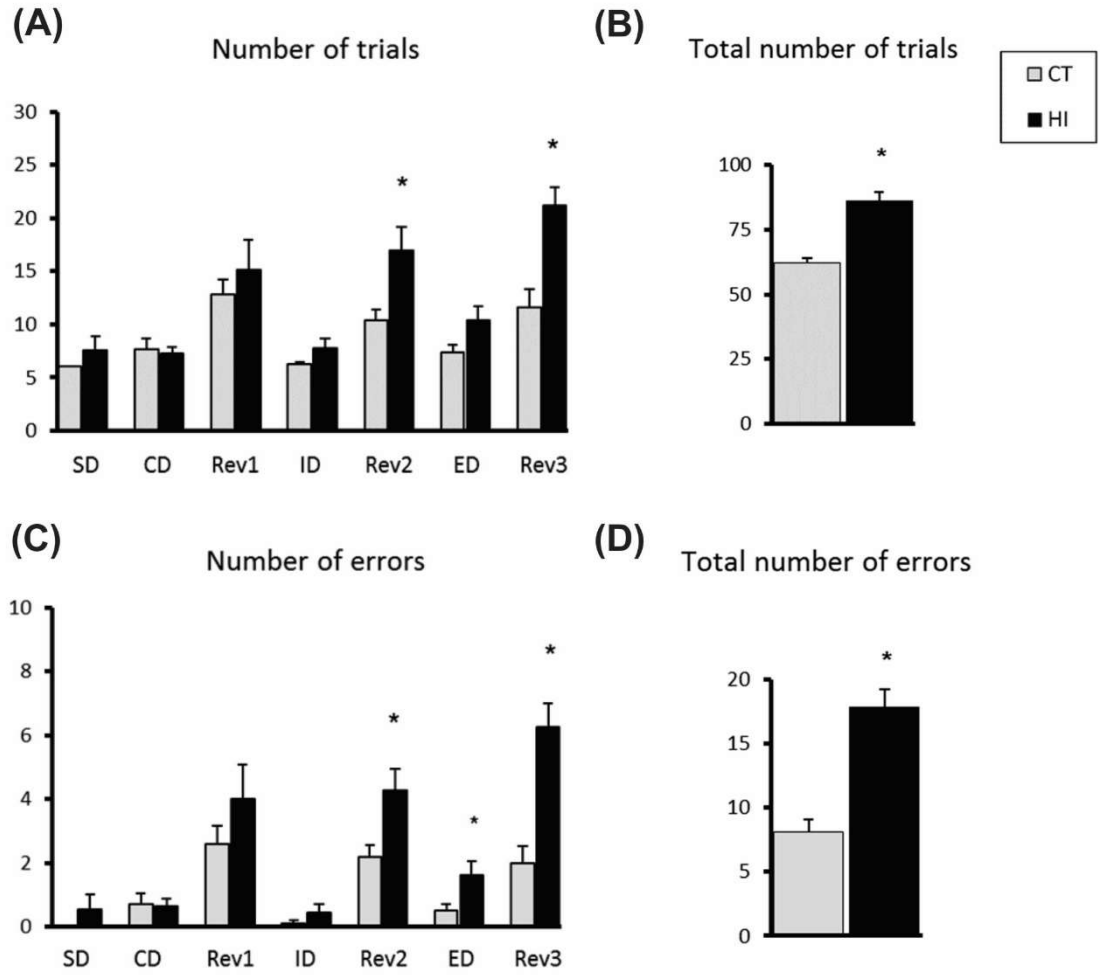

Figure 2: Performance in the attentional set-shifting task. Data are the number of trials (A) and errors (C) in each stage to reach the proposed criterion and the total number of these variables at the end of the task (B and D). Results are expressed as mean ± S.E.M. *Difference between groups, *t* test, $p<0.05$. SD: simple discrimination; CD: compound discrimination; Rev1: reversal 1; ID: intradimensional shift; Rev2: reversal 2; ED: extradimensional shift; Rev3: reversal 3; CT: controls; HI: hypoxia-ischemia.

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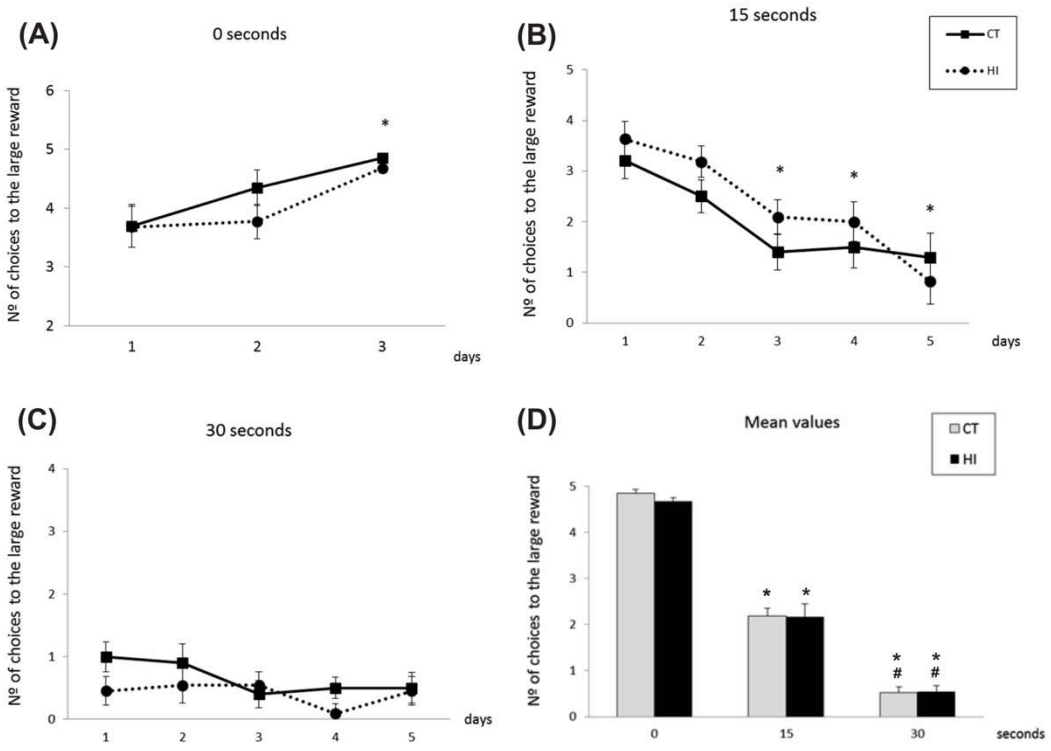

Figure 3: Number of choices to the large reward in the Tolerance to delay of reward task. Rats are submitted to different waiting times to reach the large reward: 0 (A), 15 (B) and 30 seconds (C). *Different from the first day. Repeated-measures ANOVA, $p<0.05$. (D) Mean values of choices to the large reward during the three different waiting times. *Different from 0 seconds. #Different from 15 seconds. Two-way ANOVA, $p<0.05$. Results are expressed as mean ± S.E.M. CT: controls; HI: hypoxia-ischemia.

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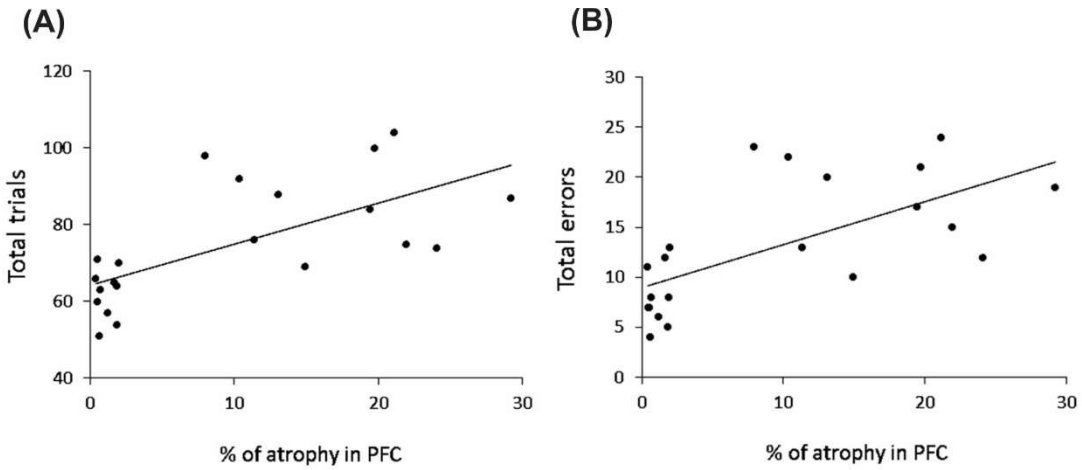

Figure 4: Pearson correlation analysis of percentage of brain atrophy and the performance in the attentional set-shifting task. Correlations were evaluated between prefrontal cortex atrophy and total number of trials (A) and errors (B) in the ASS. A positive correlation was observed in the number of trials (p<0.001 and r=0.67) and errors (p=0.001 and r=0.65).

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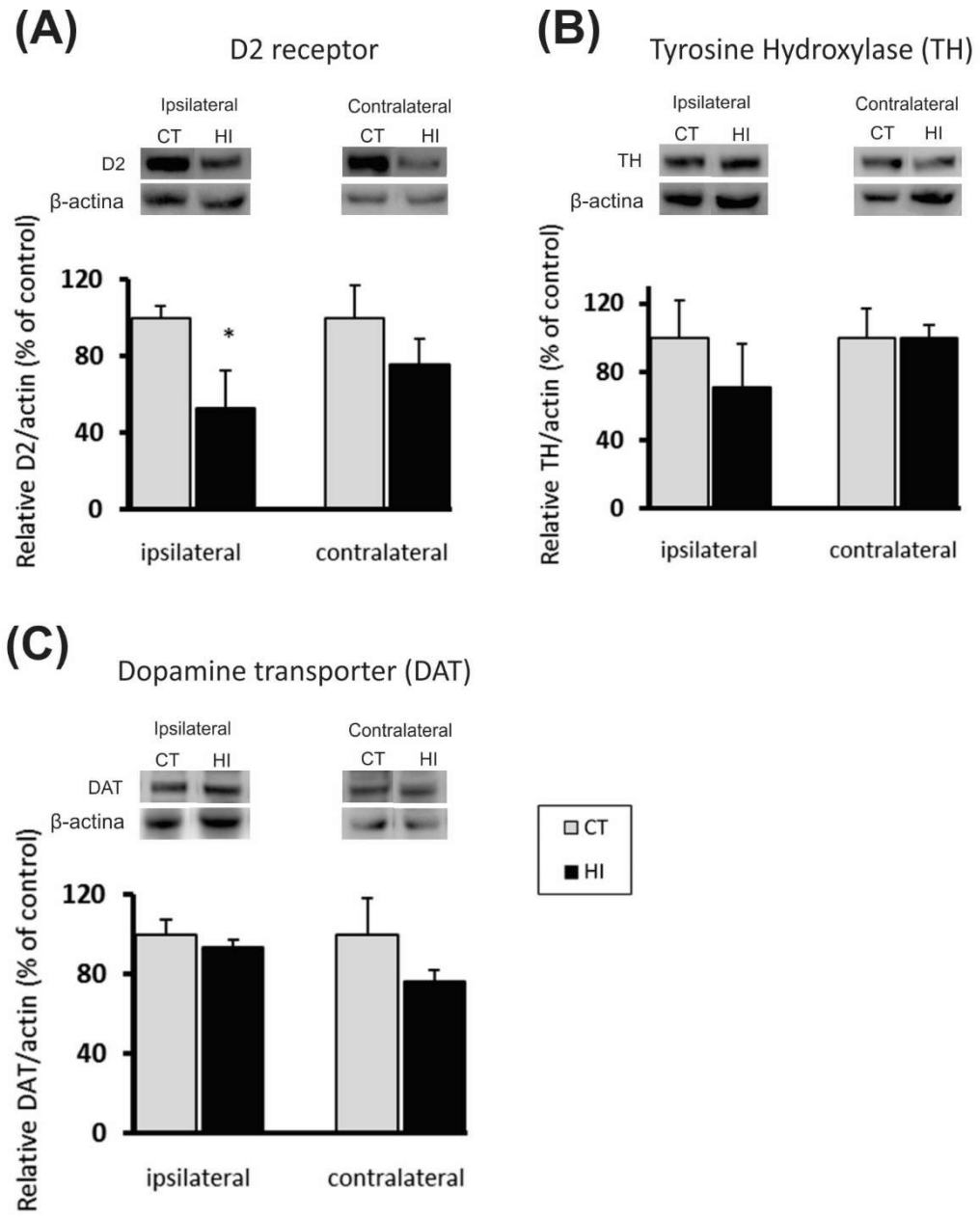

Figure 5: Protein expression of dopamine D2 receptor (A), tyrosine hydroxilase (B) and dopamine transporter (C) in the prefrontal cortex. Results are expressed as mean ± S.E.M. *Difference between groups in the ipsilateral cortex, *t* test, $p<0.05$. CT: control; HI: hypoxia-ischemia.

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