Methylphenidate administration reverts attentional inflexibility in adolescent rats submitted to a model of neonatal hypoxia-ischemia: predictive validity for ADHD study

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Abstract

Perinatal complications such as birth asphyxia were associated with a higher risk for Attention-Deficit/Hyperactivity Disorder (ADHD) in humans. Data from a rat model of neonatal hypoxia-ischemia (HI) have revealed inattention, impulsive behavior and dopamine (DA) disturbances in the prefrontal cortex (PFC), confirming the face validity and construct validity for ADHD study. However, the predictive validity (similar therapeutic efficacy of the pharmacological treatment available in the clinic) should be considered. Therefore, we aimed to investigate the effects of methylphenidate (MPH) - the treatment of choice for ADHD - on exploratory and attentional flexibility behaviors and DA-related proteins in the PFC of animals submitted to neonatal HI. Male Wistar rats were divided into four groups: control saline (CTS, n=12), control MPH (CTMPH, n=12), HI saline (HIS, n=13) and HIMPH (n=12). The HI procedure was conducted at postnatal day (PND) 7 and behavioral measures between PND 30-40, followed by protein analysis in the PFC. The MPH administration (2.5 mg/kg, i.p.) occurred 30 minutes prior each behavioral session and euthanasia for western blot analysis. We observed that the MPH increased the locomotor activity in the open field especially in HI rats. In the attentional-set shifting task, the MPH reversed the HI- induced attentional inflexibility, but impaired the task acquisition in control rats. Neonatal HI resulted in lower DA D2 receptors expression but also decreased DA transporter (responsible for DA reuptake) and increased pTH (phosphorylated-tyrosine hydroxylase) levels in the PFC, probably to compensate the dysfunctional DA transmission. This compensation was higher in the HIMPH group and it could explain the improvement in the attentional flexibility as well as the increased locomotor activity in this group. Taken this data together, we can assume the predictive validity of the HI model for the ADHD study concerning the impact of MPH treatment on attentional parameters.

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Keywords: attentional set-shifting; open field; locomotor activity; attention-deficit/hyperactivity disorder; D1; D2; dopamine transporter; DAT; tyrosine hydroxylase; TH

Abbreviations:

5-CSRTT: 5-choice serial reaction time task

ADHD: attention-deficit/hyperactivity disorder

ASST: attentional set-shifting task

CTMPH: control treated with methylphenidate

CTS: control treated with saline

DA: dopamine

DAT: dopamine transporter

DAT1: dopamine transporter gene

EF: executive functions

HI: hypoxia-ischemia

HIMPH: hypoxia-ischemia treated with methylphenidate

HIS: hypoxia-ischemia treated with saline

I.P.: intraperitoneally

IUGR: intrauterine growth restriction

MPH: methylphenidate

PFC: prefrontal cortex

PND: postnatal day

pTH: phosphorylated-tyrosine hydroxylase

SHR: spontaneously hypertensive rat

TH: tyrosine hydroxylase

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Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder characterized by inattention and/or hyperactive-impulsive behaviors (American Psychiatric Association, 2013). It is the most commonly diagnosed neurobehavioral disorder of childhood (Centers for Disease Control and Prevention, 2013) and the ADHD worldwide prevalence rate has been maintained in the last three decades around 5% (Polanczyk et al., 2007; 2014). The ADHD etiology is complex, comprising genetic and environmental contribution (Biederman and Faraone, 2005). Among the environmental factors, perinatal complications such as birth asphyxia have been associated with a higher risk for ADHD (Ketzer et al., 2012; Mikkelsen et al., 2017).

The psychostimulant methylphenidate (MPH), commercially known as Ritalin®, is the first-line pharmacological treatment for ADHD patients (National Institute for Health and Care Excellence, 2018) and its effectiveness in reducing ADHD symptoms is strongly reported in the literature (Jadad et al., 1999; Oh et al., 2018; Sudnawa et al., 2018). Although it appears paradoxical, psychostimulants such as MPH are shown to decrease hyperactivity in ADHD patients, but this effect seems to be due to a focusing action of these drugs (Porrino et al., 1983; Swanson et al., 2002). MPH blocks the dopamine transporter (DAT), responsible for the dopamine (DA) reuptake from the synaptic cleft, then increasing the extracellular DA levels (Schmeichel and Berridge, 2013; Volkow et al., 1995a). This effective psychostimulant therapy reverses the dopaminergic dysfunction underlying the ADHD pathophysiology (Del Campo et al., 2011; Levy, 1991). When used at low and clinically relevant doses, MPH preferentially increases DA release in the prefrontal cortex (PFC) (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012). This brain area is the main

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region involved in executive functions (EF) - a set of skills necessary for the cognitive control of behavior, such as cognitive flexibility, planning, and inhibition (Barkley, 1997; Diamond, 2013). In ADHD patients, impairments in EF are commonly described (Holmes et al., 2010; Marzocchi et al., 2008; Reader et al., 1994) as well as abnormalities in the PFC (Cubillo et al., 2012; Proal et al., 2011; Rubia, 2018).

Attempting to better understand the neurobiological basis of the disorder, as well as to guide clinical drug development, several animal models to study ADHD have been proposed (Bari and Robbins, 2011; Russell, 2011). A reliable animal model should mimic the behavioral characteristics of the human disorder (face validity), its mechanisms (construct validity), and the pharmacological treatment response available in the clinic (predictive validity) (van der Staay et al., 2009; Willner, 1986). Considering that perinatal hypoxic-ischemic conditions are related to ADHD development in humans (Zhu et al., 2016), a neonatal model of anoxia has been appointed as an animal model for ADHD, but it failed to succeed on the validities modalities or does not have enough data available (Russell, 2011). Recently, our group has been examining ADHD-related outcomes in a rat model of neonatal hypoxia-ischemia (HI), a widely used model proposed by Levine (Levine, 1960) and modified by Rice and colleagues (Rice et al., 1981; Vannucci and Vannucci, 2005). Attentional impairments and inhibitory control failures (impulsivity and compulsivity observed in the 5-choice serial reaction time task/5-CSRTT) associated with a general brain atrophy in the ipsilateral side to the ischemic lesion were observed in adult animals (Miguel et al., 2015). Moreover, we demonstrated attentional inflexibility correlated to PFC atrophy and dopaminergic dysfunction in this structure also in adult animals that underwent neonatal HI (Miguel et al., 2017). Then we have already established the face validity and construct validity of the Levine-Vannucci model of HI as a possible rat model to study ADHD.

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Therefore, intending to investigate the predictive validity of the HI animal model for the ADHD study (using the pharmacological treatment adopted for ADHD) the aim of this work was to evaluate the effects of MPH administration on ADHD-related outcomes: behavioral measures (exploratory and attentional flexibility) and dopaminergic system parameters in the PFC of animals submitted to neonatal HI. Our previous data reporting attentional inflexibility in HI animals was conducted in adult animals (Miguel et al., 2017); considering that ADHD is more prevalent in children and adolescents, we delineated the current study with young animals, starting the analysis on the 30th postnatal day (PND). We hypothesize that the MPH treatment will improve the behavioral deficits and dopaminergic transmission in the PFC of animals submitted to neonatal HI.

Materials and Methods

Animals

Forty-nine male Wistar rats were used in this study and they were obtained from the Central Animal House of the Institute of Basic Health Sciences (Universidade Federal do Rio Grande do Sul). On the 7th PND, pups were randomly distributed into control and HI groups and then subdivided in saline and MPH treatment, resulting in four experimental groups: control treated with saline (CTS, n=12), control treated with MPH (CTMPH, n=12), HI treated with saline (HIS, n=13) and HI treated with MPH (HIMPH, n=12). Animals were maintained with their dams until PND 21 when they were weaned and housed in 2-3 per cage (Plexiglas cages). In all stages, they were maintained in a controlled room temperature (22–24°C) on a 12:12h light/dark cycle, with food and water available *ad libitum* until the PND 30, when a protocol of food restriction was started for the attentional set-shifting task. All procedures were approved by the Institutional Ethics

Committee on Animal Use (No. 29750) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023), the guide of the Federation of Brazilian Societies for Experimental Biology and the Arouca Law (nº 11.794/2008).

Hypoxia-ischemia

The HI procedure was conducted on the 7th PND of the rat, a period that is comparable to 32 to 36 weeks' gestational age of the human infant (Patel et al., 2014). Animals were anesthetized with halothane (2– 4%) and an incision on the ventral surface of the neck was made to permit access to the right common carotid artery, that was permanently occluded with a surgical silk thread. Following a 2-h interval with their dams to recover, the pups were placed in chambers partially immersed in a 37°C water bath, where they were exposed to a hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90 min. After the HI procedure, the animals returned immediately to maternal care. Control animals were submitted to sham surgery, i.e., animals received only anesthesia and neck incision (Miguel et al., 2017; Miguel et al., 2015).

MPH administration

Methylphenidate hydrochloride (MPH) (Ritalina®, Novartis, Brazil) was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (i.p.) at a volume of 1 ml/kg, 30 minutes prior to each behavioral session and 30 minutes prior to euthanasia. Saline injections consisted of only 0.9% NaCl solution administered i.p. in the same volume (1 ml/kg). The MPH dose of 2.5mg/kg, adopted in this study, corresponds to a

medium dose (Dafny and Yang, 2006) and it was effective in improving attentional deficits in the attentional-set shifting task in rats (Cao et al., 2012).

Open Field test

The open field is one of the most widely used tasks to measure locomotor activity and spontaneous exploration of a novel environment in animal studies (Seibenhener and Wooten, 2015). At the 30th PND, rats were exposed individually to a wood square arena (54cm length, 38cm width and 45cm height) facing the corner wall of the apparatus and their free exploration was recorded for 5 minutes (Deniz et al., 2018). The software ANY-Maze video-tracking system 4.70 (Stoelting Co., Wood Dale, IL) was used to analyze the following parameters: total distance travelled, average speed, number of rotations of the animal's body (entire rotations of 360°), latency to the first entry into the central zone, number of entries into the central zone and total number of entries into the central and peripheral zone (Mestriner et al., 2013; Miguel et al., 2015). The number of rearings (a measure of vertical exploration) was evaluated by a blind researcher.

Attentional set-shifting task (ASST)

Immediately after the open field assessment (30th PND), animals started a food restriction protocol (12-15g of food/rat/day) required to motivate the animal to eat the reward in the ASST. The food restriction started four days prior to the habituation on the ASST maze and at this period, daily weighing was conducted to certify that animals maintained a body weight at 85% to 90% of their free-feeding weight (Birrell and Brown, 2000; Miguel et al., 2017).

The attentional flexibility was measured using a maze-based set-shifting task that allows animals, in a sequence of trials, to turn left or right on a "T" maze to obtain a food reward. The apparatus consisted of a wood four-arm maze and each arm measures 55cm long, 9.5cm wide and 40cm high. A bowl (7cm in diameter and 4cm in depth) was situated at the end of each arm, in which the food reward was provided. The four-arm maze was used only during the habituation process; during the turn bias and testing, it was made into a "T" maze by placing a wood block at the entrance of one of the arms (Floresco et al., 2006; Ragozzino et al., 2002).

Habituation

Two days prior to their initial exposure to the maze, each animal received four sweetened food rewards (Froot Loops, Kellogg's®) in their home cages, to avoid food neophobia. On the first day of habituation, two pieces (1/4 Froot Loops) of reward were placed in each arm and one piece in each bowl, and the rat was allowed to explore the apparatus and eat the froot loops for 15 minutes. If a rat did not consume the rewards in 15 minutes, the same habituation session was conducted in the following days. In the next habituation stage, the reward was provided only in the bowls. As soon as the rat consumed the reward on the bowl, it was picked up and placed in a different arm to habituate the animal to being handled in the maze after consuming the reward. The criterion to move to the next stage (the turn bias) was to consume all four pieces of reward at least four times in 15 minutes, in 2 consecutive days.

Turn Bias

For assessing the turn bias, the cross maze was made into a "T" maze such that animals could only turn left or right to obtain the food reward – that was available in both left and right sides. This stage measures the animal natural turn preference for one of the sides. The rat was placed in the start arm and after it chose an arm and consumed the reward, it was picked up and placed again in the start arm until it chose the other arm and consumed the reward. After choosing both arms, the rat was returned to the holding cage and the visual cue (white stripes cardboard; 20cm long, 9.4cm wide and 1cm thickness) was placed randomly in the right or left arms' floor when assessing the turn bias. This procedure was conducted to avoid neophobia to the visual cue during the test in the following day.

In the turn bias evaluation, in each trial the rat was placed in the start arm and allowed to consume the reward that was available in both sides but the turn that the rat made first during the initial choice of each trial was recorded and counted toward its turn bias. After consuming the reward in both sides, the rat was picked up and returned to the holding cage, the maze rebaited and a new trial started. Seven trials were applied and the direction (right or left) that the rat turned four or more times was considered its turn bias, i.e., the natural turn preference. The test started on the following day.

Test 1: Egocentric response (Acquisition Learning)

In the first day of testing, the animal was required to turn in the opposite direction as its turn bias to obtain the food reward, regardless of the presence of the visual cue placed in one of the choice arms. The experimenter room had at least one spatial cue in each wall and to discourage animals from using an allocentric spatial strategy, the maze was placed on a rotating platform that easily allows us to turn the maze and modify the

position (south, east, west) of the start arm. We never started a rat on the north position during the tests and this position was used only for the probe trial. The order of the start position and the location of the visual cue for each trial were determined pseudorandomly and taken from a preset sequence that was identical for each animal.

In each trial, the rat was placed on the start arm and as soon as it consumed the reward or realized its mistake, it was picked up and placed back in the holding cage for approximately 10 seconds (the inter-trial interval). The criterion to complete this test was 10 correct consecutive choices and a probe trial was applied after the animal achieved this criterion. In the probe trial, the rat started from the north position and the visual cue was inserted in the opposite arm to the correct response. The test ended if the rat turned to the appropriate direction but if the rat made a mistake, the egocentric testing was continued until the rat made an additional five correct consecutive choices, and a subsequent probe trial was performed. For each rat, we analyzed the number of trials to reach the criterion, the number of errors, time to complete the test and number of probe trials.

Test 2: shift to visual-cue discrimination (Attentional flexibility)

In the second testing day, the strategy used previously changed and now the correct response was the arm that contained the visual cue, regardless of the side. Thus, this stage required attentional flexibility (Supplementary Video 1). The protocol was very similar to the test 1: the location of the cue and the start position were determined pseudorandomly, the criterion to complete the test was 10 correct consecutive responses and the same variables were analyzed.

Additionally, in Test 2, the errors were further broken down into three error

subtypes: perseverative, regressive and never-reinforced. The perseverative and regressive errors were counted when a rat continued to make the same previous egocentric response on those trials that required the rat to turn to the opposite direction (with the visual cue). The difference is that in regressive errors, the animal started to use an alternative strategy, i.e., it made some correct answers to the opposite direction of the egocentric response. To discriminate these two types of errors, we separated the trials in blocks of 16 consecutive trials and perseverative errors were scored until less than six of them were made within a block (Brady and Floresco, 2015). Beginning with the next block and continuing through the end of the task, subsequent errors of this type were counted as regressive. Never-reinforced errors were scored when a rat entered the incorrect arm on trials where the visual cue was placed in the same arm that the rat had been trained to enter on the previous day. The combination of regressive and never-reinforced errors has been used as an index of the animals' ability to maintain a new strategy (Floresco et al., 2006) while perseverative errors occurring early in testing reflect an animal's inability to abandon the previous strategy (Floresco et al., 2008).

Tissue collection

At the end of the ASST (approximately PND 40), animals received rat chow *ad libitum* in their home cage. In the following day, they were euthanized by decapitation 30 minutes after the MPH or saline injection and the PFC was dissected out, instantaneously placed in liquid nitrogen and stored at -80˚C until western blot analysis.

Western Blot

The Western Blot technique was applied to analyze the expression of different proteins involved in the dopaminergic signaling in the PFC region. Assessments of the DA transporter (DAT, responsible for the DA reuptake), D1 and D2 receptors, tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) and phosphorylated-TH (pTH; Ser40) expression were conducted in both ipsilateral and contralateral hemispheres to the arterial occlusion. PFC samples were homogenized in cytosolic extraction buffer with protease (Complete, Roche) and phosphatase inhibitors (Phostop, Roche). The samples were then centrifuged at 3000rpm (4˚C) for 10min for cytosolic protein extraction (for TH and pTH) and thereafter at 13000rpm (4˚C) for 30 min for purification of the cytosolic fraction (for DAT, D1, and D2). The supernatant of the centrifugation processes 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 40µg of protein were incubated with lithium dodecyl sulfate (LDS, Invitrogen) and dithiothreitol (DTT, Sigma-Aldrich), and protein denaturation occurred by boiling the samples at 99˚C for 3 min. They were then loaded on 4–12% polyacrylamide gradient gels (Invitrogen) and a standard molecular weight marker (Magic MarkerVR, 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% non-fat dry milk. The membranes were incubated overnight at 4˚C with the following primary antibodies: anti-DA transporter (Sigma-Aldrich, AB1591P, 1:500), anti-DA D1 receptor (Millipore, AB9141, 1:500), anti-DA D2 receptor (Millipore, AB5084P, 1:500), anti-TH (Millipore, AB152, 1:2000) and anti-pTH (Invitrogen, 1:1000). Secondary antibodies anti-mouse (Cell Signalling, 7076s, 1:2000) or anti-rabbit (Cell Signalling, 7074s, 1:2000) were incubated for 2h at room temperature. The chemiluminescence signal was then detected using ECL (ECL Western Blotting

Analysis System, GE healthcare, RNP2106) and the intensity of the bands was quantified by densitometry using Image J® software (National Institute of Health, USA). Results were expressed as the ratio between the protein of interest and ß-actin (Sigma-Aldrich, A4700, 1:1000) or α-tubulin (Sigma-Aldrich, 1:2000) on the same membrane.

Statistical Analysis

Two-way ANOVA followed by Tukey's post-hoc, with lesion and treatment as factors, was used to analyze the open field test, ASST performance and protein quantification by western blot. All variables were expressed as mean±standard error of the mean (SEM), and the results were considered significant when $p<0.05$. The analyses were performed using the Statistica software package (StatSoft, Tulsa, OK, USA), version 10.

Results

Table 1 presents the statistical results concerning the main effects and interactions for all dependent variables considered in this study.

Open Field

In the open field test, we observed a significant lesion effect and a trend towards the treatment effect for the variables total distance travelled (lesion $F(1,45)=8.53$, $p=0.005$; treatment $F(1,45)=3.49$, $p=0.06$), average speed (lesion $F(1,45)=8.21$, $p=0.006$; treatment $F(1,45)=3.45$, $p=0.06$) and rotations of the animal´s body (lesion

F(1,45)=10.46, p=0.002; treatment F(1,45)=3.39, p=0.07) in the total 5 minutes of exploration. The Tukey post hoc demonstrated that the HIMPH group travelled longer distances, in a higher speed, and rotated more than the CTS and CTMPH group (Figure 1 A, B and C, respectively). Considering the total number of entries into the central and peripheral zone, we found a lesion (F(1,45)=7.82, p=0.007), treatment (F(1,45)=6.31, p=0.01) and a trend towards lesion x treatment effect (F(1,45)=3.41, p=0.07), indicating that the HIMPH group had a higher number of entries compared to all other groups (Figure 1D). A treatment effect was observed for number of rearings (F(1,45)=5.64, p=0.02) and the post hoc pointed out that the HIMPH animals had an increase in the number of rearings compared to the HIS group (CTS: 49.58±4.51, CTMPH: 52.33±3.55, HIS: 40.23±3.55, HIMPH: 57.83±5.35).

Analyzing only the exploration on the periphery, the same general pattern was observed: a lesion effect for distance travelled (F(1,45)=6.21, p=0.01) and average speed (F(1,45)=6.94, p=0.01) demonstrating that the HIMPH had longer distance travelled and higher speed when compared to both control groups – CTS and CTMPH (Table 2). In the central zone, a lesion effect was again observed for the variable distance travelled (F(1,45)=4.06, p=0.04) with the post hoc showing a trend for the HIMPH group exploring more this area than the CTS group (p=0.08). A significant lesion x treatment interaction was also found for the latency to the first entry in the central zone (F(1,45)=7.08, p=0.01) and the post hoc demonstrated that the HIMPH group had a tendency to decreased latency to entry in the center when compared to the HIS group (p=0.052). Considering the number of entries into the central zone, a treatment effect was observed (F(1,45)=4.78, p=0.03) with the MPH administration increasing the number of entries (see Table 2).

Attentional set-shifting task

No differences were observed between groups in relation to the number of habituation days in the ASST, neither on the first habituation stage (lesion $F(1,45)=0.83$, $p=0.36$; treatment $F(1,45)=0.83$, $p=0.36$) nor the second habituation stage (lesion $F(1,45)=0.004$, $p=0.95$; treatment $F(1,45)=1.18$, $p=0.28$).

In the Test 1 we measured the egocentric response acquisition, i.e., the animal had to choose the opposite direction of its turn bias in the T maze, regardless of the visual cue. In this test, we found a lesion x treatment interaction for the number of trials ($F(1,45)=10.79$, $p=0.001$), number of errors ($F(1,45)=10.05$, $p=0.002$) and time to complete the task ($F(1,45)=7.11$, $p=0.01$). Surprisingly, the Tukey post hoc demonstrated that the CTMPH group had a higher number of trials and errors than CTS and HIMPH groups. The CTMPH animals also took longer to complete the task than the HIMPH group (Figure 2A-C). A treatment effect was observed for the probe trial ($F(1,45)=6.67$, $p=0.01$) but no post hoc interaction was detected.

In the following day (Test 2), the visual-cue discrimination was assessed, requiring behavioral flexibility. Here, we observed a lesion effect ($F(1,45)=5.06$, $p=0.02$) and lesion x treatment interaction ($F(1,45)=6.29$, $p=0.01$) for number of trials and number of errors (lesion $F(1,45)=4.22$, $p=0.04$; lesion x treatment $F(1,45)=5.81$, $p=0.02$). Lesion ($F(1,45)=6.48$, $p=0.01$), treatment ($F(1,45)=7.7$, $p=0.007$) and lesion x treatment interaction ($F(1,45)=5.64$, $p=0.02$) was detected for time to complete the task. Different from the Test 1, the post hoc showed that the HIS group made a higher number of trials in a longer time when compared to all other groups. The total number of errors was also higher in the HIS group in relation to the CTS and HIMPH groups (Figure 2D-F). No effect was observed for the number of probe trials on Test 2. Additionally, when segregated the different types of errors in this phase, we observed a trend toward a

treatment effect (F(1,45)=3.22, p=0.07) for regressive errors, without any effect for never-reinforced and perseverative errors (Figure 3).

Western Blotting

Two-way ANOVA showed a lesion effect for the D2 receptor (F(1,21)=6.26, p=0.02) and DAT expression (F(1,21)=4.9, p=0.03) only on the contralateral side to the ischemia, demonstrating that these proteins are decreased in the HI groups (Figure 4B and C). The D1 receptor was not affected by lesion or treatment factors in both hemispheres (Figure 4A). In the same way, the TH enzyme levels were not affected by any factor in both hemispheres (Figure 5A). Nonetheless, we found a significant lesion effect for the pTH levels in both hemispheres: contralateral (F(1,22)=5.2, p=0.03) and ipsilateral (F(1,24)=10.85, p=0.003). The HI groups showed an increased expression of pTH levels and the post hoc demonstrated that the HIMPH group has higher expression levels compared to the CTS group in the ipsilateral hemisphere (Figure 5B).

Discussion

In the present study, we aimed to investigate the effects of MPH administration on ADHD-like behaviors and dopaminergic signaling in the PFC of young animals that underwent neonatal HI. We observed that the MPH administration increased the locomotor activity in the open field especially in HI animals. Confirming our hypothesis the MPH reversed the attentional inflexibility caused by the neonatal HI but impaired the task acquisition in control rats in the ASST. Hypoxic-ischemic animals had lower DA D2

receptors and transporter (DAT) and higher pTH enzyme levels in the PFC, suggesting a disruption of the DA signaling in this brain region.

MPH administration increased locomotor activity in adolescent HI animals

The present findings did not demonstrate a strong impact of the HI on exploratory activity in young rats, corroborating previous data in young HI animals (Carletti et al., 2012; Kim et al., 2017; Schuch et al., 2016b). However, hyperactivity has been a recurrent finding in adult rats submitted to neonatal HI (Deniz et al., 2018; Markostamou et al., 2016; Rojas et al., 2013; Sanches et al., 2015). The occurrence of divergent results in young and adult animals could be explained by the progressive brain damage that occurs following the neonatal HI, which was also associated with continuous cognitive impairment (Diaz et al., 2016; Mishima et al., 2004). Further studies should address this issue and evaluate locomotion in adult animals and using different MPH doses.

It is important to observe that only HI animals under MPH effect increased their locomotor activity in relation to both control groups. This result indicates that the MPH has a potentiating effect in hypoxic-ischemic rats, since the CTMPH group did not demonstrate this pattern of behavior. We should consider that the MPH is a psychostimulant drug in the same class as cocaine and amphetamine – drugs well-recognized to increase locomotion (McKinzie et al., 2002; Wellman et al., 2002). In a meta-analysis conducted by Askenasy (Askenasy et al., 2007) it was showed MPH-induced hyperactivity in 93% of the studies evaluating the MPH's acute locomotor effects in rats. We can infer that our findings in HIMPH group are in line with the literature data. It could be considered an unexpected effect because psychostimulants are used to treat hyperactivity in ADHD patients. However, it has been reported that the effects of

stimulants depend on the behavior elicited by the environment. For example, dextroamphetamine use in children decreased activity during controlled classroom settings (with rules and planned activities); however, during physical education (where a free exploration is allowed), there was a significant drug-induced increase in motor activity (Porrino et al., 1983). Also, MPH treatment in ADHD children decreased expressively the activity in classroom but this effect was smaller in playground activities (Swanson et al., 2002). Taking together, these results suggest that the "calming effects" designated to psychostimulants are due to an improved focusing of activity rather than a decreased motor activity. This fact is recognized as "the paradoxical calming effect of psychostimulants" (Gainetdinov et al., 1999; Napolitano et al., 2010). Interestingly, the data of the present study are strictly in accordance with this statement since the open field is a new environment where a free exploration is allowed. Therefore, measures of the MPH effects in different contexts and with different stimulus should be cautiously interpreted.

The hyperactivity consequent to MPH treatment was also observed in the central zone of the open field apparatus. MPH administration increased the number of entries in the center in both groups and decreased the latency to the first entry in the central zone only in the HI group. An increased exploration in the central zone could be interpreted as a risk behavior since rodents display a natural aversion to open areas that ethologically mimic a situation of predator risk (Choleris et al., 2001; Prut and Belzung, 2003). Then, the increased locomotor activity of the HIMPH group observed even in the central area of the open field could enhance their exposure to danger.

A particular question is that the stimulant effect of MPH was observed especially in hypoxic-ischemic rats. This could be explained by the vulnerability of the striatum region to the Levine-Vannucci model of HI (Miguel et al., 2015; Schuch et al., 2016a)

since that region has a fundamental role in motor activity (Grillner et al., 2005). Previous reports demonstrated loss of dopaminergic fibers (Park et al., 2013) and alteration in the DA receptors D1 and D2 (Filloux et al., 1996) in this model. In newborn piglets, the HI brain insult resulted in a transient increase in striatal DAT (Zhang et al., 2011). Thus, we can infer that alterations in the DA signaling in the striatum of HI animals would contribute for a higher response to MPH-induced hyperlocomotion, when compared to control animals. Interestingly, similar findings were observed in the recognized model of ADHD: the spontaneously hypertensive rat (SHR) respond more to the MPH administration (increasing locomotion) than their control group (Chelaru et al., 2012; Yetnikoff and Arvanitogiannis, 2013). Striatal dysfunction was also found in SHR animals such as polymorphisms on the DAT gene (DAT1) (Mill et al., 2005), higher DAT1 gene expression and higher striatal DAT density compared to control group (Roessner et al., 2010; Watanabe et al., 1997). Thus, the results observed in HI animals are in harmony with the findings from the most recognized animal model for ADHD, supporting the idea of the HI model as a potential option for the ADHD study.

Neonatal HI did not alter ASST acquisition learning (Test 1) but impaired attentional flexibility (Test 2)

The attentional set-shifting task (ASST) was used to measure executive function parameters such as learning process and attentional flexibility in rats. In Test 1, essentially involving learning skills (Ragozzino et al., 2002), we did not observe any impairment in hypoxic-ischemic animals. Conversely, in Test 2, HI animals demonstrated cognitive inflexibility. This result is in agreement with our previous report showing that HI animals had no learning deficits in another ASST (using digging mediums and odors) despite the

inflexibility identified in further stages of the task (Miguel et al., 2017). Such results were correlated to PFC atrophy found in HI animals. In agreement, other studies have revealed that lesions in the PFC in primates or rats do not affect initial discrimination learning, but profoundly impair the ability to inhibit an old strategy and utilize a new one (Brown and Bowman, 2002; Dias et al., 1996; Floresco et al., 2008; Ragozzino et al., 2002). In order to interpret the findings in the HI group, it should be considered that differential response to reward could influence on attention to task performance. Although there are no studies investigating directly food responses in the HI animal model, our previous report demonstrated that HI animals had perseverative responses in the 5-CSRTT that uses sweet pellet as reward and it could be an indication of higher wanting for the sweet food (Miguel et al., 2015). Then, this higher motivation for palatable food could improve acquisition in the first stage of ASST used in the present study. This behavior was already observed in rats that suffered intrauterine growth restriction (IUGR), which had higher dopamine response to sweet food resulting in better performance in the reversal learning of an ASST (Alves et al., 2015). The authors suggested that attention (salience) and wanting for the sweet food, a term called "incentive salience" (Berridge and Robinson, 1998; Wise, 2006), was greater in IUGR animals, causing them to become more focused on the task and, consequently, to perform better. In light of our objective of correlating the findings observed in HI rats with ADHD characteristics, clinical reports have shown a significant association between adult ADHD and obesity/overweight, eating disorders and bulimia nervosa (Cortese and Tessari, 2017; Nazar et al., 2016; Seitz et al., 2013). This idea could explain the standard performance in the acquisition phase in HI animals and reinforce our hypothesis of an association between HI and ADHD-related outcomes.

Attentional flexibility was measured in the ASST Test 2, when animals had to shift rule from the egocentric response to the visual-cue discrimination. A clear

impairment in this test was observed in hypoxic-ischemic animals. Interestingly, this finding reveals that only highly cognitive demanding are compromised in this early age in HI animals since neither learning abilities nor locomotor activity were affected by the hypoxic-ischemic event. We have previously identified attentional inflexibility in adult animals that underwent neonatal HI (Miguel et al., 2017) and the present study demonstrated that these executive function impairments already exist in young animals (approximately 40 PND). This is interesting data considering that the ADHD prevalence is more common in children and adolescents.

MPH impaired acquisition learning in control animals (Test 1) but reversed HI-induced cognitive inflexibility (Test2)

Our results regarding the Test 1 demonstrated that the MPH administration impaired the task rule acquisition only for control animals. Although this is not an expected result, current literature has described the ability of stimulants to induce cognitive deficits particularly depending on the dose used (Wood et al., 2014). MPH is the first pharmacological option for ADHD treatment and it is recognized to increase DA levels in the PFC (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012). As seen in humans, an increased in PFC DA levels was also observed in rats performing the initial rule acquisition in the ASST, demonstrating that this stage *per se* increased DA levels in the PFC (Stefani and Moghaddam, 2006). Thus, we can propose that the supplementary stimulation on DA pathway in control animals - by ASST and MPH treatment - could explain the learning impairment observed in CTMPH group. Accordingly, an “inverted-U” curve relationship has been proposed for

PFC DA levels and cognition, where too little or too much DA levels have detrimental effects on performance (For review see (Cools and D'Esposito, 2011; Floresco, 2013).

Interestingly, the impairment seen in CTMPH group in Test 1 (rule acquisition) was not observed in Test 2 (attentional flexibility), indicating that higher DA levels for flexibility demands did not prejudice the animals' performance. In the same way, Stefani & Moghaddam (Stefani and Moghaddam, 2006) showed that higher PFC DA levels were associated with better performance during the rule shift in the ASST, indicating that the "inverted-U" shape is not applicable for attentional flexibility. In this highly cognitive demanding process, that requires not only rule acquisition but the inhibition of responding according to the previous rule, higher levels of DA facilitate performance, in a curvilinear manner (Floresco, 2013; Stefani and Moghaddam, 2006).

Hypoxic-ischemic animals demonstrated a profound impairment in the cognitive flexibility measures (Test 2) of the ASST and the MPH reversed these deficits. Such effect of MPH seems to be associated with a decrease of regressive and never-reinforced errors in HIS group. The combination of these types of errors has been considered as an index of the animals' ability to maintain a new rule strategy (Floresco et al., 2008; Floresco et al., 2006). Thus, HI animals showed difficulty to comprehend and sustain the new strategy and an MPH-induced improvement was reveal in these aspects. MPH is a drug well recognized to improve attentional deficits in ADHD patients (Sunohara et al., 1999; Yang et al., 2004) and we could translate the clinical features to our experimental model, demonstrating the predictive validity of the HI model to the ADHD study. Non-pharmacological strategies have been also adopted to treat the ADHD symptoms; for example, aerobic exercise resulted in benefits for cognitive function in children with ADHD (Ludyga et al., 2016; 2017; 2018). It has been well established that attentional flexibility is a process essentially dependent on the DA signaling in the PFC (Brozoski et

al., 1979; Floresco, 2013). Aiming to interpret the current behavioral findings and to support the construct validity of the HI model, we measured different proteins involved in the dopaminergic signaling in the PFC region.

Dopaminergic system was disrupted in the PFC of HI animals

Different proteins responsible for an efficient DA transmission in the PFC were evaluated in both ipsilateral and contralateral hemisphere to the ischemic occlusion: 1) DA transporter (DAT, responsible for the DA reuptake), 2) DA receptor D1, 3) DA receptor D2, 4) tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) and 5) phosphorylated-TH (pTH, the TH isoform phosphorylated on serine 40). Our findings demonstrate that HI animals had lower D2 and DAT levels in the contralateral PFC, when compared to control animals. These findings revealed a disruption of the DA signaling in the PFC of HI rats that are probably associated with the attentional inflexibility observed in HIS group.

The implication of D2 receptors for cognitive flexibility performance are documented in both experimental (Floresco et al., 2006) and clinical trials (Mehta et al., 2004; van Holstein et al., 2011). Previously, we had demonstrated decreased in PFC D2 receptors in further developmental stage (adulthood) of HI animals, that was also associated with attentional inflexibility (Miguel et al., 2017). In the present study, although the D2 receptor was altered by the neonatal HI, we did not observe alterations in D1 expression in both PFC hemispheres. It is known that D1 and D2 receptors are differently distributed in pyramidal neurons of the rat PFC, with little overlap between these receptors (Gaspar et al., 1995; Santana et al., 2008; Vincent et al., 1993). For cognitive flexibility, reports have identified that D1 stabilizes network activity, whereas

D2 attenuates inhibitory influences, allowing PFC to process multiple stimuli. Thus, D2 activity places networks in a more labile state that facilitates flexible patterns of behavior, having then a key role in cognitive flexibility when compared to the D1 subtype (Durstewitz et al., 2000; Durstewitz et al., 2010; Floresco, 2013; Seamans and Yang, 2004). Therefore, we can infer that neonatal HI impaired cognitive flexibility by damaging neurons expressing D2 rather than D1 receptors.

DAT regulates the strength and duration of dopaminergic transmission and it is the main target for many psychostimulants that increase DA signaling (Schmeichel and Berridge, 2013; Volkow et al., 1995b). Interestingly, a DAT knock-out mice have been proposed as an ADHD model, showing spontaneous hyperactivity and impulsivity (Leo and Gainetdinov, 2013). Thus, the current findings, revealing an alteration in DAT expression in the contralateral PFC in HI animals, might be a contributing feature to the behavioral outcomes observed in this group.

Phosphorylated tyrosine hydroxylase (pTH) at Ser40 is positively related to the speed of dopamine synthesis (Dunkley et al., 2004) and HI animals had higher expression of pTH in both PFC hemispheres, indicating an increase in DA synthesis. Activation of dopamine D2 receptors results in selective inhibition of TH phosphorylation at Ser40 in the striatum (Lindgren et al., 2001) and D2 antagonists have opposite effects, increasing TH phosphorylation at Ser40 (Salvatore et al., 2000). We suggest that the upregulation observed in the pTH enzyme is a compensatory mechanism resulting from the downregulation of the D2 receptor in an attempt to improve DA signaling in this brain region. Although this is a molecular strategy that occurs to improve dysfunctional processes, it does not seem to be effective in HIS group, for its attentional flexibility deficits. Taking together, we demonstrated that neonatal HI had the ability to impact on

DA system-related proteins in the PFC, giving additional support to the construct validity of the HI model for the ADHD study.

Methylphenidate increased pTH levels in the PFC of HI rats

MPH administration appears to be also related to compensatory upregulation of pTH enzyme in HI animals, that was higher in the HIMPH group in the ipsilateral PFC, when compared to the CTS group. Higher pTH enzyme levels indicate higher TH activity and probably higher DA levels in the PFC, that we could presume to be associated with the improved attentional flexibility observed in this group. MPH is well recognized to increase DA levels in the PFC (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012) and we assume that an increase in the DA signaling in the PFC of HIMPH animals was responsible for their attentional improvements. Although MPH administration was able to reverse attentional impairments caused by the neonatal HI, the drug did not impact on protein levels of DA receptors and DAT. Our treatment comprehended approximately 7 days of MPH administration and we propose that this short period was insufficient to alter brain protein expression.

The higher DA synthesis in HIMPH group could be also generalized to the striatum, a region that demonstrates higher levels of DAT (Piccini, 2003; Sesack et al., 1998) and consequently is more sensitive to the MPH effect. Therefore, higher DA levels in the HIMPH group might be also associated with the increased locomotor activity observed in the open field test.

In conclusion, our findings showed that the MPH administration reversed the impairments in attentional flexibility in adolescent rats that underwent neonatal HI. This

result supports the predictive validity of the rat model of HI as an animal model to study ADHD. Alterations in proteins involved in the DA signaling in the PFC of hypoxic-ischemic rats also confirmed the construct validity of the Levine-Vannucci model of HI. Given that ADHD is the most diagnosed disorder in children and negatively impacting on their quality of life new experimental options to study the etiology, neurobiology and effective therapies are necessary and urgent.

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Tables

Table 1: Statistical Results.

Dependent variables	Lesion effect	Treatment effect	Lesion x Treatment interaction
Open field – total exploration			
Distance travelled	$F(1,45)=8.53$, $p=0.005$, partial $\eta^2=0.15$	$F(1,45)=3.49$, $p=0.06$, partial $\eta^2=0.07$	$F(1,45)=2.09$, $p=0.15$, partial $\eta^2=0.04$
Average speed	$F(1,45)=8.21$, $p=0.006$, partial $\eta^2=0.15$	$F(1,45)=3.45$, $p=0.06$, partial $\eta^2=0.07$	$F(1,45)=2.12$, $p=0.15$, partial $\eta^2=0.04$
Number of rotations of the animal's body	$F(1,45)=10.46$, $p=0.002$, partial $\eta^2=0.18$	$F(1,45)=3.39$, $p=0.07$, partial $\eta^2=0.07$	$F(1,45)=0.64$, $p=0.42$, partial $\eta^2=0.01$
Number of entries into the central and peripheral zone	$F(1,45)=7.82$, $p=0.007$, partial $\eta^2=0.14$	$F(1,45)=6.31$, $p=0.01$, partial $\eta^2=0.12$	$F(1,45)=3.41$, $p=0.07$, partial $\eta^2=0.07$
Number of rearings	$F(1,45)=0.20$, $p=0.65$, partial $\eta^2=0.004$	$F(1,45)=5.64$, $p=0.02$, partial $\eta^2=0.11$	$F(1,45)=3.00$, $p=0.09$, partial $\eta^2=0.06$
Open field – central exploration			
Distance travelled	$F(1,45)=4.06$, $p=0.04$, partial $\eta^2=0.08$	$F(1,45)=2.20$, $p=0.14$, partial $\eta^2=0.04$	$F(1,45)=0.38$, $p=0.53$, partial $\eta^2=0.009$
Average speed	$F(1,45)=3.16$, $p=0.08$, partial $\eta^2=0.06$	$F(1,45)=0.27$, $p=0.60$, partial $\eta^2=0.006$	$F(1,45)=3.13$, $p=0.08$, partial $\eta^2=0.06$
Latency to the first entry	$F(1,45)=0.77$, $p=0.38$, partial $\eta^2=0.01$	$F(1,45)=1.09$, $p=0.30$, partial $\eta^2=0.02$	$F(1,45)=7.08$, $p=0.01$, partial $\eta^2=0.13$
Number of entries	$F(1,45)=1.35$, $p=0.25$, partial $\eta^2=0.02$	$F(1,45)=4.78$, $p=0.03$, partial $\eta^2=0.09$	$F(1,45)=0.007$, $p=0.93$, partial $\eta^2=0.0001$
Open field – peripheral exploration			
Distance travelled	$F(1,45)=6.21$, $p=0.01$, partial $\eta^2=0.12$	$F(1,45)=2.29$, $p=0.13$, partial $\eta^2=0.04$	$F(1,45)=1.90$, $p=0.17$, partial $\eta^2=0.04$
Average speed	$F(1,45)=6.94$, $p=0.01$, partial $\eta^2=0.13$	$F(1,45)=3.18$, $p=0.08$, partial $\eta^2=0.06$	$F(1,45)=1.60$, $p=0.21$, partial $\eta^2=0.03$
Number of entries	$F(1,45)=1.72$, $p=0.19$, partial $\eta^2=0.03$	$F(1,45)=7.16$, $p=0.01$, partial $\eta^2=0.13$	$F(1,45)=0.005$, $p=0.94$, partial $\eta^2=0.0001$
Attentional set-shifting task – Test 1			
Number of trials	$F(1,45)=5.21$, $p=0.02$, partial $\eta^2=0.10$	$F(1,45)=1.31$, $p=0.25$, partial $\eta^2=0.02$	$F(1,45)=10.79$, $p=0.001$, partial $\eta^2=0.19$
Number of errors	$F(1,45)=1.07$, $p=0.30$, partial $\eta^2=0.02$	$F(1,45)=3.06$, $p=0.08$, partial $\eta^2=0.06$	$F(1,45)=10.05$, $p=0.002$, partial $\eta^2=0.18$
Time to complete the test	$F(1,45)=2.14$, $p=0.15$, partial $\eta^2=0.04$	$F(1,45)=0.23$, $p=0.63$, partial $\eta^2=0.005$	$F(1,45)=7.11$, $p=0.01$, partial $\eta^2=0.13$
Probe trial	$F(1,45)=0.34$, $p=0.56$, partial $\eta^2=0.008$	$F(1,45)=6.67$, $p=0.01$, partial $\eta^2=0.12$	$F(1,45)=0.34$, $p=0.56$, partial $\eta^2=0.008$
Attentional set-shifting task – Test 2			
Number of trials	$F(1,45)=5.06$, $p=0.02$, partial $\eta^2=0.10$	$F(1,45)=2.17$, $p=0.14$, partial $\eta^2=0.04$	$F(1,45)=6.29$, $p=0.01$, partial $\eta^2=0.12$
Number of errors	$F(1,45)=4.22$, $p=0.04$, partial $\eta^2=0.08$	$F(1,45)=1.95$, $p=0.16$, partial $\eta^2=0.04$	$F(1,45)=5.81$, $p=0.02$, partial $\eta^2=0.11$
Time to complete the test	$F(1,45)=6.48$, $p=0.01$, partial $\eta^2=0.12$	$F(1,45)=7.70$, $p=0.007$, partial $\eta^2=0.14$	$F(1,45)=5.64$, $p=0.02$, partial $\eta^2=0.11$
Probe trial	$F(1,45)=0.81$, $p=0.37$, partial $\eta^2=0.01$	$F(1,45)=0.81$, $p=0.37$, partial $\eta^2=0.01$	$F(1,45)=0.81$, $p=0.37$, partial $\eta^2=0.01$
Perseverative errors	$F(1,45)=0.17$, $p=0.68$, partial $\eta^2=0.004$	$F(1,45)=0.03$, $p=0.85$, partial $\eta^2=0.001$	$F(1,45)=1.11$, $p=0.29$, partial $\eta^2=0.02$

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Regressive errors	$F(1,45)=1.35$, $p=0.25$, partial $\eta^2=0.02$	$F(1,45)=3.22$, $p=0.07$, partial $\eta^2=0.06$	$F(1,45)=2.03$, $p=0.16$, partial $\eta^2=0.04$
Never-reinforced errors	$F(1,45)=2.80$, $p=0.10$, partial $\eta^2=0.05$	$F(1,45)=1.66$, $p=0.20$, partial $\eta^2=0.03$	$F(1,45)=0.60$, $p=0.44$, partial $\eta^2=0.01$
Protein quantification – ipsilateral side			
D1 receptor	$F(1,22)=1.25$, $p=0.27$, partial $\eta^2=0.05$	$F(1,22)=0.29$, $p=0.59$, partial $\eta^2=0.01$	$F(1,22)=0.07$, $p=0.78$, partial $\eta^2=0.003$
D2 receptor	$F(1,22)=0.95$, $p=0.33$, partial $\eta^2=0.04$	$F(1,22)=1.22$, $p=0.28$, partial $\eta^2=0.05$	$F(1,22)=0.69$, $p=0.41$, partial $\eta^2=0.03$
DAT	$F(1,22)=1.21$, $p=0.28$, partial $\eta^2=0.05$	$F(1,22)=0.01$, $p=0.90$, partial $\eta^2=0.001$	$F(1,22)=0.01$, $p=0.91$, partial $\eta^2=0.001$
TH	$F(1,24)=0.009$, $p=0.92$, partial $\eta^2=0.0003$	$F(1,24)=0.89$, $p=0.35$, partial $\eta^2=0.03$	$F(1,24)=0.41$, $p=0.52$, partial $\eta^2=0.01$
pTH	$F(1,24)=10.85$, $p=0.003$, partial $\eta^2=0.31$	$F(1,24)=0.67$, $p=0.42$, partial $\eta^2=0.02$	$F(1,24)=0.26$, $p=0.60$, partial $\eta^2=0.01$
Protein quantification – contralateral side			
D1 receptor	$F(1,16)=1.26$, $p=0.27$, partial $\eta^2=0.07$	$F(1,16)=0.001$, $p=0.97$, partial $\eta^2=0.00008$	$F(1,16)=0.03$, $p=0.86$, partial $\eta^2=0.002$
D2 receptor	$F(1,21)=6.26$, $p=0.02$, partial $\eta^2=0.23$	$F(1,21)=1.19$, $p=0.28$, partial $\eta^2=0.05$	$F(1,21)=0.82$, $p=0.37$, partial $\eta^2=0.03$
DAT	$F(1,21)=4.90$, $p=0.03$, partial $\eta^2=0.21$	$F(1,21)=1.43$, $p=0.24$, partial $\eta^2=0.07$	$F(1,21)=0.39$, $p=0.53$, partial $\eta^2=0.02$
TH	$F(1,22)=0.006$, $p=0.94$, partial $\eta^2=0.0002$	$F(1,22)=0.04$, $p=0.83$, partial $\eta^2=0.002$	$F(1,22)=0.43$, $p=0.51$, partial $\eta^2=0.02$
pTH	$F(1,22)=5.20$, $p=0.03$, partial $\eta^2=0.19$	$F(1,22)=0.13$, $p=0.71$, partial $\eta^2=0.006$	$F(1,22)=0.18$, $p=0.67$, partial $\eta^2=0.008$

All analyses were performed using Two-way ANOVA.

Table 2: Central and peripheral exploration in the Open field task.

	Central exploration				Peripheral exploration	
	Distance travelled (m)	Average speed (m/s)	Latency to first entry (s)	Number of entries	Distance travelled (m)	Average speed (m/s)
CTS	1.59±0.24	0.11±0.012	26.58±6.88	11.66±1.56	15.20±1.03	0.053±0.003
CTMPH	1.81±0.24	0.091±0.006	39.11±8.73	15.16±1.63	15.32±0.81	0.055±0.003
HIS	1.95±0.29	0.11±0.013	40.42±9.75	13.46±1.55	16.17±0.67	0.057±0.002
HIMPH	2.51±0.26	0.13±0.011	11.66±3.82	17.25±1.89	18.70±0.95*	0.067±0.003*

Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. *HIMPH different from CTS and CTMPH groups. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=12-13/group.

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Figures:

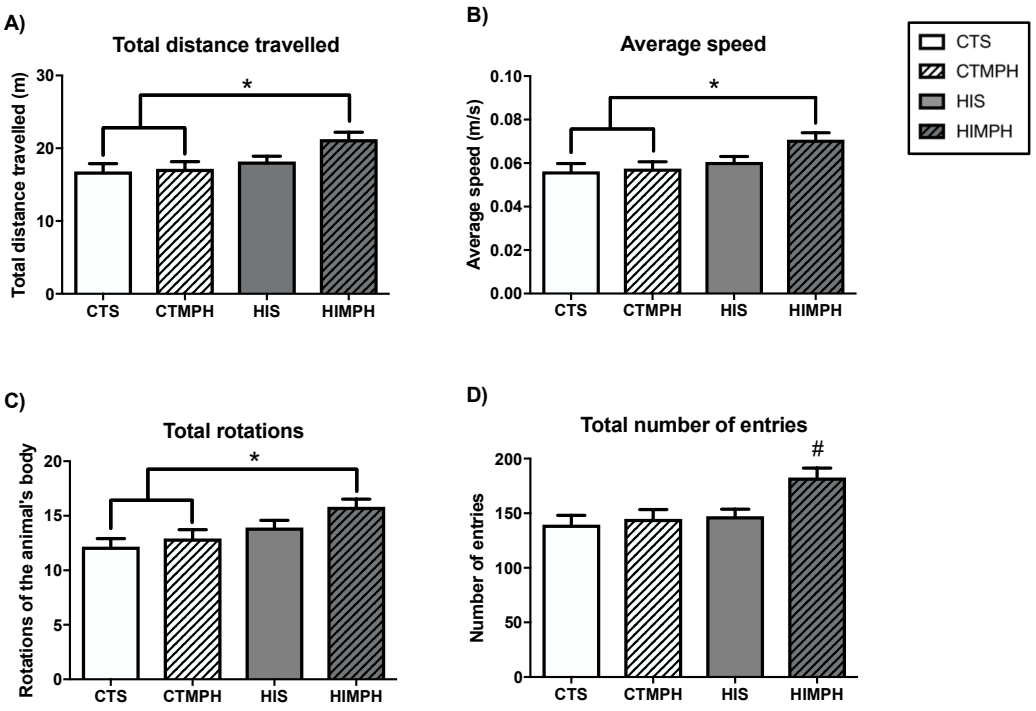

Figure 1: Total exploratory behavior during a 5-min Open field test session. The following parameters are shown: (A) Total distance travelled, (B) Average speed, (C) Total rotations of the animal's body and (D) Total number of entries in the central and periphery areas. Results are expressed as mean ± S.E.M. *Different from CTS and CTMPH. #Different from all other groups. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=12-13/group.

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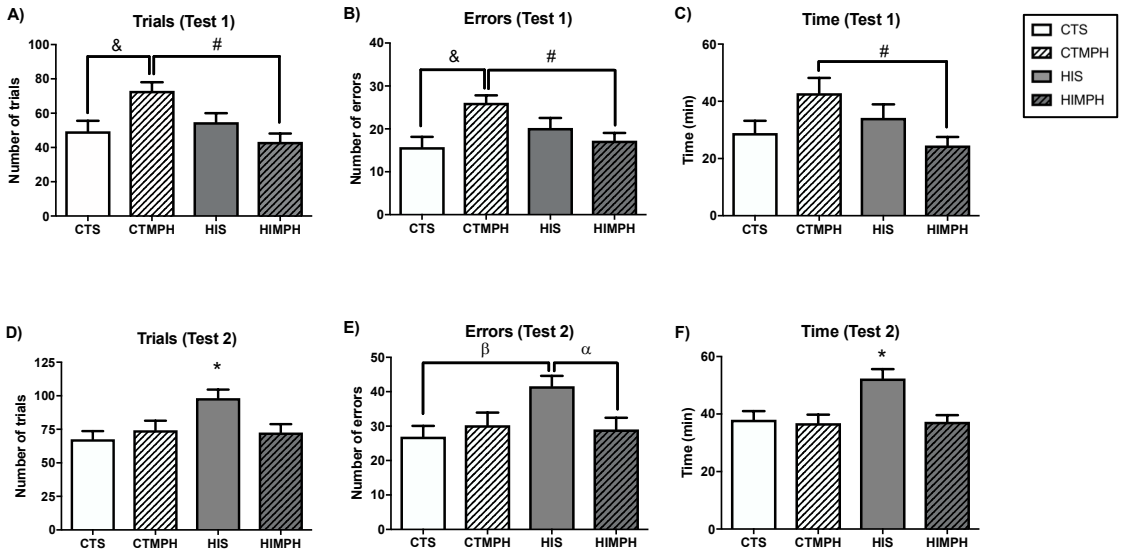

Figure 2: Performance in the attentional set-shifting task. Measures of the egocentric response acquisition (Test 1) are demonstrated by (A) Number of trials to reach the criterion, (B) Number of errors and (C) Time to complete the test. The same variables are depicted for the Test 2 (D-F), in which a shift to visual-cue discrimination occurs, requiring animal's behavioral flexibility. Results are expressed as mean ± S.E.M. &CTMPH different from CTS; #CTMPH different from HIMPH; *HIS different from all other groups; ßHIS different from CTS; αHIS different from HIMPH. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=12-13/group.

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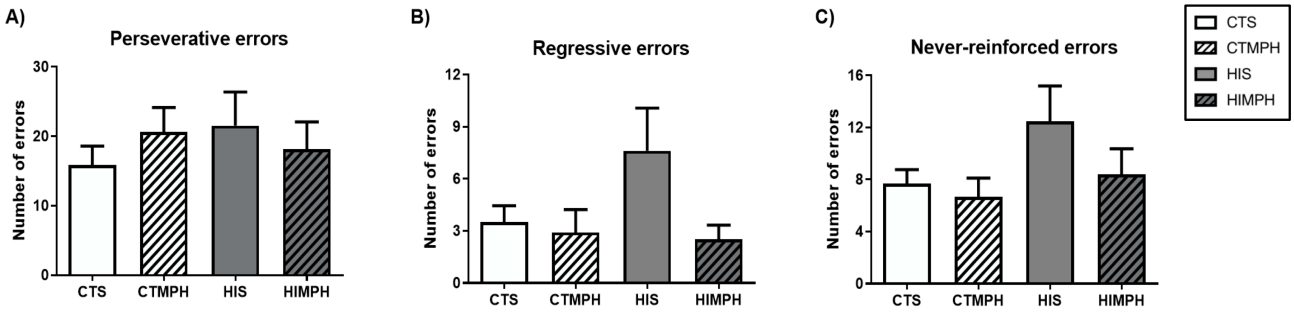

Figure 3: Types of errors performed in the visual-cue discrimination (Test 2) of the attentional set-shifting task: (A) Perseverative, (B) Regressive and (C) Never-reinforced errors. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=12-13/group.

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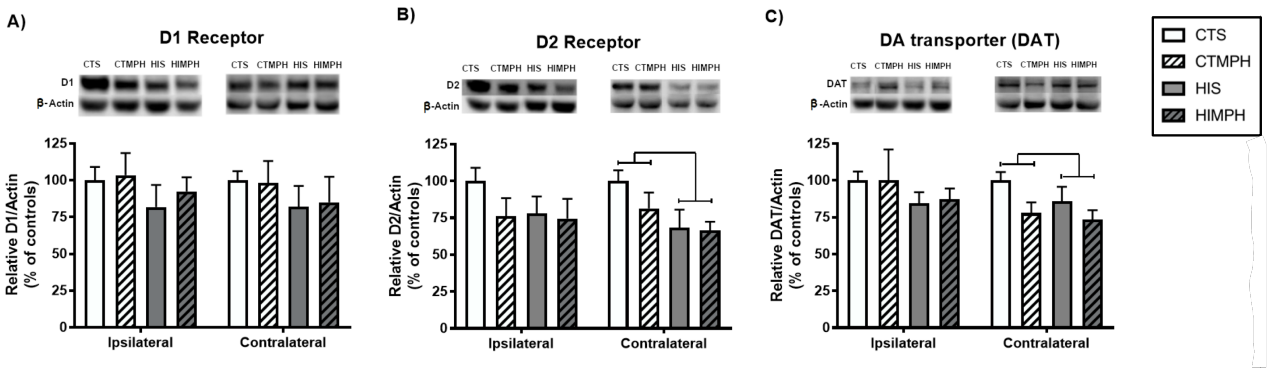

Figure 4: Protein expression of DA receptors D1 (A) and D2 (B) and DA transporter (C) in the prefrontal cortex (PFC). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. Lesion effect was observed for D2 and DAT in the contralateral hemisphere. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=5-8/group.

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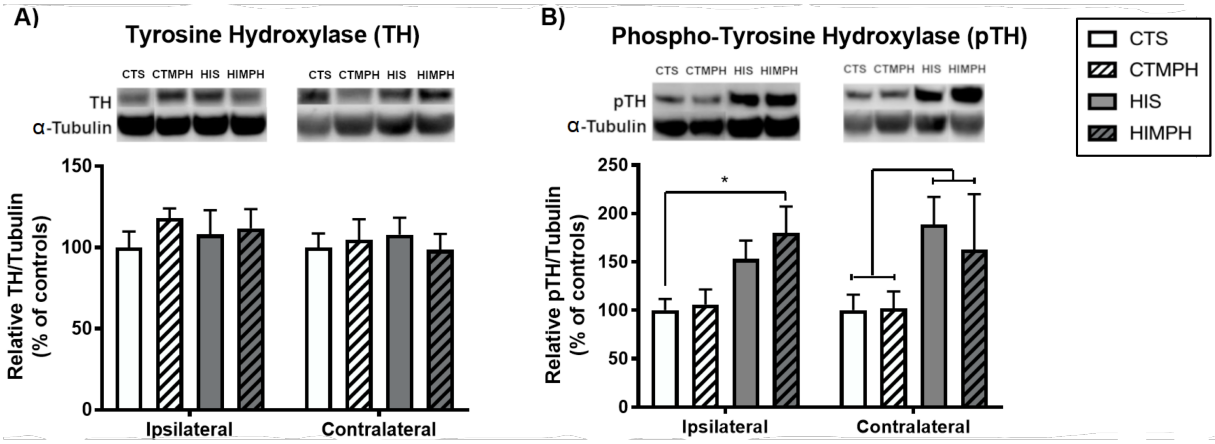

Figure 5: Protein expression of tyrosine hydroxylase (TH) and phosphorylated-tyrosine hydroxylase (pTH) enzymes in the prefrontal cortex (PFC). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<0.05$. Lesion effect was observed for pTH in both hemisphere. *HIMPH different from CTS group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=6-7/group.

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