Patrícia Maidana Miguel et al, Methylphenidate treatment increases hippocampal BDNF levels but does not improve memory deficits in hypoxic-ischemic rats, Journal of Psychopharmacology (34, 7) pp. . Copyright © 2020. DOI: 10.1177/0269881120913153.

Methylphenidate treatment increases hippocampal BDNF levels but does not improve memory deficits in hypoxic-ischemic rats

Running head: MPH does not improve memory deficits in HI rats

Patrícia Maidana Miguel[a,b], Bruna Ferrary Deniz[a,b], Heloísa Deola Confortim[a,b], Wellington de Almeida[a,b], Loise Peres Bronauth[b], Milene Cardoso Vieira[b], Karine Bertoldi[d], Ionara Rodrigues Siqueira[c,d], Patrícia Pelufo Silveira[e,f,g], Lenir Orlandi Pereira[a,b]

a) Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
b) Departamento de Ciências Morfológicas, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
c) Programa de Pós-Graduação em Ciências Biológicas, Fisiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
d) Departamento de Farmacologia, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
e) Ludmer Centre for Neuroinformatics and Mental Health, Douglas Mental Health University Institute, McGill University, Montreal, QC, Canada
f) Department of Psychiatry, Faculty of Medicine, McGill University, Montreal, QC, Canada.
g) Sackler Program for Epigenetics & Psychobiology at McGill University

*Corresponding author:
Patrícia Maidana Miguel (e-mail: patymiguel@msn.com)
Departamento de Ciências Morfológicas, ICBS, Universidade Federal do Rio Grande do Sul
Rua Sarmento Leite, 500, 90050-170, Porto Alegre, RS, Brazil

Abstract

Background: Methylphenidate (MPH) is a stimulant drug mainly prescribed to treat cognitive impairments in Attention-deficit/hyperactivity disorder (ADHD). We demonstrated that neonatal hypoxia-ischemia (HI) induced attentional deficits in rats and MPH administration reversed these deficits. However, MPH effects on memory deficits after the HI procedure have not been evaluated yet. **Aims:** We aimed at analyzing learning and memory performance of young HI rats after MPH administration and associate their performance with BDNF levels in the prefrontal cortex and hippocampus. **Methods:** Male Wistar rats were divided into four groups (n=11-13/group): control saline (CTS), control MPH (CTMPH), HI saline (HIS) and HIMPH. The HI procedure was conducted at postnatal day (PND) 7 and memory tasks between PND 30-45. MPH administration (2.5mg/kg, i.p.) occurred 30min prior to each behavioral session and daily, for 15 days, for the BDNF assay (n=5-7/group). **Results:** As expected, HI animals demonstrated learning and memory deficits in the Novel-object recognition (NOR) and Morris water maze (MWM) tasks. However, MPH treatment did not improve learning and memory deficits of these animals in the MWM - and even disrupted the animals' performance in the NOR task. Increased BDNF levels were found in the hippocampus of HIMPH animals, which seem to have been insufficient to improve memory deficits observed in this group. **Conclusions:** The MPH treatment was not able to improve memory deficits resulting from the HI procedure considering a dose of 2.5 mg/kg. Further studies investigating different MPH doses would be necessary to determine a dose-response relationship in this model.

Funding: CAPES, CNPq.

Keywords: hypoxia-ischemia; attention-deficit/hyperactivity disorder; brain-derived neurotrophic factor; water maze; novel-object recognition

Introduction

Methylphenidate (MPH) is the first choice drug for the treatment of children and adolescents with Attention-deficit/hyperactivity disorder (ADHD) (National Institute for Health and Care Excellence, 2018). Even though inattention and hyperactivity have been considered the main dysfunctions in ADHD patients, learning and memory deficits are frequently described, and are considered important co-morbidities (Andersen et al., 2013; Mangina and Beuzeron-Mangina, 2009).

Although the MPH´s precise neurochemical mechanism of action is under debate, it is recognized to block both dopamine (DA) and norepinephrine (NE) transporters (DAT and NET, respectively), reducing the clearance of these neurotransmitters from the synaptic cleft. It was shown that low doses of MPH preferentially increased NE and DA extracellular concentration within the prefrontal cortex (PFC) (Berridge et al., 2006), while more potent effects were observed on hippocampal NE than on striatal DA (Kuczenski and Segal, 2001). Therefore, MPH treatment in ADHD patients has been associated to improvements in both executive functions impairments (mainly related to PFC function) (Kramer et al., 2001; Sunohara et al., 1999), and memory deficits dependent on hippocampal activity (Bedard and Tannock, 2008; Rhodes et al., 2006; Verster et al., 2010). Based on the potential cognitive enhancement associated with this drug, it has been increasingly used by healthy students (Guthrie et al., 2003) but inconsistent findings are observed across individuals diagnosed or not with ADHD (for review (Cools and D'Esposito, 2011). For example, our group demonstrated that MPH administration in control rats affects learning during an attentional

set-shifting task (Miguel et al., 2019a) and this finding agrees with the current literature proposing that excessive DA and NE activity in the PFC (which may occur by MPH administration) could disturb cognition (Arnsten, 2011; Floresco, 2013).

DA stimulation regulates the expression of neurotrophic factors in the brain, such as the brain-derived neurotrophic factor (BDNF) (Iwakura et al., 2008; Kuppers and Beyer, 2001; Williams and Undieh, 2009). For example, experimental findings demonstrated that DAT-knockout (DAT-KO) rodents have dysregulated BDNF expression in both frontal cortex and striatum (Fumagalli et al., 2003; Leo et al., 2018). This neurotrophin is involved in neuronal growth and survival, neurotransmitter modulation and neuronal plasticity – crucial for learning and memory (Bathina and Das, 2015). For this reason, decreased BDNF activity is hypothesized to be associated with ADHD pathophysiology (Tsai, 2007; Tsai, 2017). Lower serum BDNF levels were observed in boys with ADHD-inattentive subtype when compared to healthy controls, and these levels increase to a higher extent in the inattentive group after 8 weeks of MPH treatment (Akay et al., 2018). Amiri and colleagues also reported increased BDNF levels in ADHD subjects after 6 weeks of MPH treatment (Amiri et al., 2013), reinforcing the relationship between DA signaling and BDNF production.

Pregnancy and birth complications, such as perinatal hypoxia-ischemia (HI), are environmental conditions associated with an increased risk for ADHD (Millichap, 2008; Zhu et al., 2016). Recently, we demonstrated that children exposed to several mild perinatal hypoxic-ischemic events and expressing a differential genotype associated with DA signaling in the PFC have impaired executive function, demonstrating the interaction between genetic and environmental factors for this phenotype (Miguel et al., 2019b). Neonatal HI has been

extensively modeled in rodents aiming at determining underlying mechanisms and effectiveness of therapeutic interventions (Yager and Ashwal, 2009). We have shown that HI induction using the well-recognized model of Levine-Vannucci (Vannucci and Vannucci, 2005) is able to induce ADHD-related phenotypes in adult rats, such as attentional and executive function impairments (Miguel et al., 2018; Miguel et al., 2015). Recently, we also demonstrated that acute MPH administration reverses executive function impairments in adolescent rats submitted to neonatal HI (Miguel et al., 2019a). However, MPH effects concerning memory deficits dependent on the hippocampus have not been studied yet in the HI model.

Thus, we aimed to analyze the effects of MPH in control and hypoxic-ischemic rats using two different memory tasks, the novel-object recognition (NOR) and the Morris Water maze (MWM). The NOR task measures episodic, non-spatial memory, and does not involve primary reinforcement such as food or electric shocks (Ennaceur and Delacour, 1988) while the MWM evaluates spatial learning and memory using an aversive condition (Vorhees and Williams, 2006). Additionally, BDNF levels were analyzed in both groups after MPH treatment - in the PFC and hippocampus - considering their importance in ADHD pathophysiology and neuronal plasticity. We hypothesized that MPH administration improves cognitive deficits resulting from neonatal HI via an increase in brain BDNF levels.

Materials and Methods

Animals

Male Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences (Universidade Federal do Rio Grande do Sul) and maintained in a controlled room temperature (22–24°C) on a 12:12h light/dark cycle, with food and water available *ad libitum*. On the 7th PND, pups were randomly distributed into control and HI groups and then subdivided into saline and MPH treatment, resulting in four experimental groups: control treated with saline (CTS, n=11), control treated with MPH (CTMPH, n=13), HI treated with saline (HIS, n=13) and HI treated with MPH (HIMPH, n=12). Female pups of the litters were used for another research project. Animals were maintained with their dams until PND 21 when they were weaned and housed in 3-4 per cage (Plexiglas cages), with one treatment assigned to each cage that contained animals from both control and hypoxia-ischemia groups mixed up. Another set of animals (n=5-6/group), that did not undergo behavioral tasks, was used for BDNF immunoassay. 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 (HI)

The HI procedure was induced based on the protocol developed by Levine (Levine, 1960) and modified by Rice-Vannucci (Rice et al., 1981; Vannucci and Vannucci, 2005). At PND 7, rats 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. After isolation of

the artery from other surrounding anatomical structures, it was permanently occluded with a surgical silk thread. Following a 2-h interval with their dams to recover, the pups were placed in chambers (5 pups per chamber of 1500 ml) where they were exposed to a hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90 min. The external bottom of the chamber was partially immersed in a 37°C water bath and pups did not have any contact with the water and were kept dry inside the chamber. The animals returned immediately to maternal care after hypoxia. Control animals were submitted to sham surgery, i.e., animals received only anesthesia and neck incision (Miguel et al., 2019a; Miguel et al., 2018; Miguel et al., 2015).

MPH administration

Methylphenidate hydrochloride (MPH) (Novartis, Brazil) was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (dose of 2.5mg/kg, volume of 1 ml/kg) 30 minutes prior to each behavioral session (from PND30 to PND45). Control animals received an equivalent volume of saline solution. The other set of animals (n=5-7/group) received the same daily treatment but did not undergo behavioral tasks – they were designated exclusively to biochemical analysis. The MPH dose of 2.5mg/kg, adopted in this study, corresponds to a medium dose (Dafny and Yang, 2006) and improved attentional deficits of HI animals in our previous study (Miguel et al., 2019a).

Behavioral Analysis

The novel-object recognition test was performed on PND 31, and the Morris water maze tests from PND 32 to 37 (reference memory) and from PND 42 to 45 (working memory). All behavioral tasks were conducted from 1 p.m. to 5 p.m., one animal at a time – i.e. the animals were submitted to the same arena for the NOR and the same tank for the MWM task. Two batches of animals were used for the behavioral analysis and we had animals from all experimental groups in both batches. All animals were transferred at the same time from the animal room to the testing room for acclimatization for at least 30 minutes prior to testing. All behavioral analyses were performed by a blind experimenter.

Novel-object Recognition (NOR)

The novel-object recognition task is widely used to evaluate learning and memory based on the natural tendency of rodents to interact with a novel object over a familiar one (Ennaceur and Delacour, 1988). On the day previous the NOR task (PND 30), animals were habituated to the apparatus (wood square arena: 54cm length, 38cm width and 45cm height) for 5 minutes. In the following day (PND 31), during the first session, the rats were placed in the apparatus with two similar toys (A and A', that were LEGO blocks) and the time exploring each object was recorded for a total of 5 minutes. The second session (test phase) was conducted after a 5 minutes interval, aiming to evaluate the short-term memory (Deniz et al., 2018; Pereira et al., 2008). Rats were replaced in the apparatus with a familiar (A) and a novel object (B, that was a ship toy) and the time exploring each object was recorded for 5 minutes. Object exploration was defined when the animal sniffed or touched the object with the paws; climbing onto the object without sniffing was not considered exploration (Klein et

al., 2018; Pereira et al., 2008). Both objects and the apparatus were always cleaned with alcohol 30% between trials. An object preference index was calculated using the test session data, consisting of the difference between the exploration of the new object and the familiar object, divided by the total time exploring both objects (B − A / B + A, where B is the new object and A is the familiar object) (Deniz et al., 2018; Pereira et al., 2008).

Morris Water maze (MWM)

The Morris Water Maze task was used to evaluate spatial learning, long-term reference memory, perseveration in the previous target (reversal learning) and working memory (Vorhees and Williams, 2006). Training in the MWM task started the day after the NOR task (PND 32). The maze was composed by a circular tank (117 cm diameter) virtually divided into 4 quadrants and filled up with water at 22 ± 1°C. A transparent escape platform was 2 cm submerged beneath the water surface and the rats had to learn the platform position based on visual distal cues placed on the walls of the testing room. In each trial (maximum of 60 sec), the rat was placed in the water (facing the tank wall) in an established random position that changed daily and was the same for all animals.

In the spatial learning training, the submerged platform remained at the same position in all daily sessions, and the latency to reach the platform was measured throughout the sessions - 5 sessions with 4 trials/session, 20min inter-trial interval (ITI). In this training, we used a blocking design in which we conducted all the 4 trials in 2-3 cages at a time, respecting the 20min ITI for each rat. If a rat failed to find the platform in 60 sec, it was gently guided through the water and placed on the platform for 10 sec. At the end of each trial, the rat was

towel dried and placed back into its housing cage. After training, on the sixth day, the probe trial to assess long-term reference memory was conducted. The platform was removed, and each rat was placed into the water on the opposite quadrant of the platform target area. The following parameters were measured during this probe trial (60 sec): the latency to reach the first target area crossover, the number of crossings on the target area, the time spent on the target and in the opposite quadrant (Deniz et al., 2018; Klein et al., 2018; Pereira et al., 2008).

The reversal learning phase was conducted four days after the probe trial. The location of the platform was switched to the opposite quadrant, measuring the animals' ability to extinguish their initial learning of the platform's position and search for a new goal position. It is known that rats rapidly switch their search strategies to the new goal on the first day of reversal testing – and perseverations to the old platform position may be seen on individual trials within this day (Vorhees and Williams, 2006). Therefore, we analyzed the time spent in the previous platform quadrant in 4 trials (5min ITI) on a single day. This testing day was the first session of the working memory protocol.

In the working memory protocol, the platform was reallocated daily, and the rats were subjected to 4 trials/day (with an ITI of 5min), during four consecutive days. The mean latencies to find the platform on each trial were calculated for all testing days (Carletti et al., 2016; Pereira et al., 2008). In both the reversal learning and working memory protocols, we conducted the 4 trials in one cage at a time since the required ITI was 5 minutes.

Mature BDNF assay

For mature BDNF analysis we used a different set of animals that did not perform any behavioral task. This procedure was conducted considering that behavioral tasks with long period of training or exercise can quickly alter BDNF levels (Hall et al., 2000; Silhol et al., 2007). At PND 30, animals (5-7/group) started the daily MPH or saline injections that were administered for over the same period of treatment from the animals that performed the behavioral tasks (15 days). Animals were euthanized by decapitation 30min after the last drug injection. The hippocampus and prefrontal cortex were quickly dissected out bilaterally, placed on liquid nitrogen and stored at -80°C until the biochemical assay.

Mature BDNF concentration was measured through the E-Max ELISA kit (Promega, USA), according to the manufacturer's instructions. Briefly, the samples of each rat were individually homogenized in lysis buffer (137mM NaCl, 20mM Tris-HCl (pH 8.0), Igepal (1%), glycerol (10%), 1mM phenylmethanesulfonyl fluoride (PMSF), 0.5mM sodium vanadate, 0.1mM EDTA, and 0.1mM EGTA) and centrifuged for 3min at 14,000 rpm at 4°C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated in 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody, and blocked with Block & Sample buffer. After blocking, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Colorimetric reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm; the standard BDNF curve ranged from 0 to 500 pg/mL (Klein et al., 2018; Pereira et al., 2009). All experimental groups were equally distributed in the same plate to avoid technical differences between groups and the BDNF calculation was performed by a blind experimenter.

Statistical analysis

Two-way ANOVA, with lesion and treatment as factors, was used to analyze the NOR task, long-term and working memories in the MWM and the BDNF concentration. Reference training and reversal learning in the MWM were evaluated by repeated-measures ANOVA. Log10 transformation was performed for the variables latency to cross the target, trial 3 in working memory and right hippocampus BDNF concentration to ensure homogeneity of sample variance. For the repeated-measures data, we tested the assumption of sphericity using the Mauchly's Test of Sphericity and if the assumption was violated, we applied the Greenhouse-Geisser correction. When required, analyses were followed by the post hoc Tukey's test for multiple comparisons. All variables were expressed as mean±standard error of the mean (SEM), and the results were considered significant when $p<.05$. Effect size (partial η^2) were also reported. Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Novel-object recognition test

Two-way ANOVA demonstrated significant main effects for *lesion* ($F(1,45)=11.19$, $p<.01$, partial $\eta^2=0.19$) and *treatment* ($F(1,45)=6.99$, $p<.05$, partial $\eta^2=0.13$) and a trend towards a *lesion*treatment* interaction effect ($F(1,45)=3.6$, $p=.06$, partial $\eta^2=0.07$) for the novel-object preference index. Tukey's post hoc indicated that the CTS group had higher index when compared to all other groups (Figure 1). Then, the findings demonstrated that

253 both the neonatal hypoxia-ischemia and the MPH administration impaired the animal's
254 ability to discriminate the novel object.
255
256 [Please insert Figure 1 here]
257
258 **Morris Water Maze**
259 *Spatial learning*
260 Spatial learning was assessed in the water maze for five consecutive days, with the
261 submerged platform in the same position. Repeated-measures ANOVA showed significant
262 main effects for *lesion* ($F(1,45)=12.96$, $p<.01$, partial $\eta^2=0.22$) and *session* factors
263 ($F(3.32,149.72)=26.86$, $p<.0001$, partial $\eta^2=0.37$) considering the latency to find the
264 platform. No *treatment* or *lesion*treatment*session* interaction effects were observed,
265 indicating spatial learning impairment in hypoxic-ischemic rats that was not recovered by
266 MPH administration.
267
268 [Please insert Figure 2 here]
269
270 *Long-term reference memory*
271 In the probe trial, without the platform, two-way ANOVA demonstrated a *lesion*
272 effect for the variables latency to cross the target ($F(1,45)=7.57$, $p<.01$, partial $\eta^2=0.14$) and
273 number of crossings on the target ($F(1,45)=6.58$, $p<.05$, partial $\eta^2=0.12$), with no effect of
274 *treatment* or *lesion*treatment* interaction effect. These findings showed higher latency to
275 cross the target and fewer number of crossings on the target in hypoxic-ischemic rats,

indicating deficits in long-term reference memory that were not improved by the MPH treatment (Figure 3A and B). No effect was observed for the time spent on the target area nor the time spent in the opposite quadrant (Fig. 3C-D).

[Please insert Figure 3 here]

Perseveration in the previous target (reversal learning)

The platform was relocated to the opposite quadrant aiming to investigate the behavioral flexibility of the animals to extinguish their initial learning of the platform's position. Within the first day of reversal learning (4 trials, 5min ITI), repeated-measures ANOVA demonstrated *lesion* ($F(1,45)=4.09$, $p<.05$, partial $\eta^2=0.08$) and *trial* ($F(2.03, 91.37)=17.86$, $p<.0001$, partial $\eta^2=0.28$) effects, indicating that hypoxic-ischemic animals perseverate more in the previous platform location over the trials. Comparing the performance within the 4 trials, we observed that the CTS group decreased their time spent on the previous platform quadrant from the second trial and the CTMPH group from the third trial onwards (Figure 4). On the contrary, both HIS and HIMPH groups did not decrease the latency on the previous platform quadrant throughout the trials (Figure 4A), confirming their difficulty to abandon the previous platform position.

Working memory

To measure working memory capacity, the platform was relocated daily, and the latency to find the new location within 4 successive trials was assessed. Two-way ANOVA performed for each trial demonstrated *lesion* effect for the third ($F(1,45)=5.39$, $p<.05$, partial

299 η^2=0.10) and fourth trials (F(1,45)=8.10, p<.01, partial η^2=0.15) but no *treatment* or
300 *lesion*treatment* interaction effects were observed. These findings showed working memory
301 impairments in HI animals that were not improved by the MPH administration (Figure 4B).
302
303 [Please insert Figure 4 here]
304
305 **Mature BDNF assay**
306
307 BDNF analyses were conducted in the PFC and hippocampus from both hemispheres
308 – ipsilateral and contralateral to the ischemic lesion. Two-way ANOVA did not demonstrate
309 any significant effect for the PFC in both hemispheres (Figure 5A). In the ipsilateral
310 hippocampus, *lesion* (F(1,20)=11.12, p<.01, partial η^2=0.35) and *treatment* main effects were
311 seen (F(1,20)=6.22, p<.05, partial η^2=0.23) and the Tukey post hoc revealed that the HIMPH
312 group had an increase in BDNF levels in comparison to the CTS group (Figure 5B). In the
313 contralateral hippocampus, only a trend towards the *lesion* effect was observed
314 (F(1,23)=3.48, p=.07, partial η^2=0.13) (Figure 5B).
315
316 [Please insert Figure 5 here]
317
318 **Discussion**
319 The current study was proposed to examine the effects of MPH administration on
320 learning and memory tasks performance and mature BDNF levels in the PFC and
321 hippocampus of young animals that underwent neonatal HI. The findings showed learning

and memory deficits in HI animals in all evaluated parameters, but MPH administration was not able to reverse these deficits in HI animals despite an increase in BDNF levels observed in the ipsilateral hippocampus of the HIMPH group. Additionally, MPH treatment disturbed the animals´ performance in the novel object recognition task.

Both hypoxia-ischemia and MPH administration disturbed the animals' performance in the NOR task

Findings in the NOR task demonstrated an impairment to discriminate novel and familiar objects by adolescent rats submitted to neonatal HI, as already observed in numerous studies (Deniz et al., 2018; Pereira et al., 2008; Rojas et al., 2013). Considering that we previously demonstrate alterations in DA signaling in the PFC of hypoxic-ischemic rats (Miguel et al., 2019a; Miguel et al., 2018) and that there is evidence that optimal DA levels in this structure are required for object recognition (Hotte et al., 2005; Nagai et al., 2007), we can suggest that the impairment observed in the object recognition may be associated with deregulated DA transmission. Then, we also hypothesized that administration of MPH (that increases extracellular DA levels) could improve object recognition in HI animals. Contrarily to the hypothesis, MPH administration impaired the animals´ recognition of the novel object, surprisingly inducing control animals to have a performance comparable to HI groups. The MPH effects could be justified by the psychostimulant and locomotor effects of this drug (Askenasy et al., 2007). Corroborating this idea, the same MPH dose used in the current study increased the number of entries in the central and peripheral zones of an open field in adolescent rats (Miguel et al., 2019a). We believe that hyperactivity associated with MPH

345 use may be responsible for an unfocused object exploration and consequently impaired object
346 recognition memory. Disruptive object recognition following MPH administration in control
347 animals was already observed by several authors (Bouchatta et al., 2018; Chuhan and
348 Taukulis, 2006; Heyser et al., 2013; Heyser et al., 2004). In our previous study, we also
349 observed that MPH disturbed learning acquisition in a task involving food reward,
350 corroborating data reporting cognitive impairments related to excessive PFC DA stimulation
351 (as by the MPH administration) (Arnsten, 2011; Floresco, 2013). Thus, considering these
352 unexpected findings, MPH effects under distinct doses and contexts should be carefully
353 assessed and interpreted when evaluating the different studies.
354
355 *MPH administration did not alleviate cognitive impairments of HI rats in the water maze*
356 *task*
357
358 Differently from the NOR task, learning in the MWM involves an aversive condition
359 in which animals must find a hidden platform (using spatial cues) to escape from the water.
360 Our results demonstrated impairments resulting from the neonatal HI in all stages of the task:
361 acquisition, probe trial to investigate the retention of long-term memory, working memory
362 and behavioral flexibility. MPH administration had no impact in any of these measures, in
363 both HI and CT animals. It is interesting to note that the detrimental effect of MPH treatment
364 observed in the NOR task was not observed in the MWM task. We believe that MPH-induced
365 hyperactivity, while disturbing object recognition, can favor the search for the escape
366 platform in the MWM – involving higher motivation and focus. However, no significant
367 treatment effect was observed for the latency to find the platform. Further studies should

368 analyze the effects of different MPH doses in the performance of HI animals using distinctive
369 memory protocols.

370 Another interesting point to be discussed is that the same MPH dose used in the
371 current study (2.5mg/kg) was shown to reverse the behavioral inflexibility of adolescent HI
372 animals observed in the attentional set-shifting task (Miguel et al., 2019a). Divergent findings
373 may be a result of the design of the tasks: the attentional set-shifting was created to capture
374 specifically this type of PFC-dependent behavior while the MWM is a task mainly designed
375 to capture hippocampal-dependent memory functions. As the hippocampus is one of the most
376 affected brain regions by the HI procedure and has been linked to learning and memory
377 deficits in these animals (Miguel et al., 2015; Pereira et al., 2007), we postulate that MPH
378 benefits were not sufficient to counterbalance the learning impairments related to this
379 extensive atrophy in HI animals. This point can be seen as a disadvantage of the HI model
380 towards other suggested ADHD animal models, since extensive brain lesions are not common
381 in ADHD individuals. However, to confirm that MPH is ineffective in this model, additional
382 investigations using different MPH doses should be tested.

383 Although pharmacological treatments are relevant for several outcomes, we need to
384 consider that the hypoxic-ischemic lesion is known to be progressive (Diaz et al., 2016;
385 Mishima et al., 2004) and early interventions aiming to diminish its progression and
386 consequent brain atrophy are best options. For example, hypothermia is the standard
387 treatment in infants with moderate to severe hypoxic ischemic encephalopathy (HIE) but it
388 is often not offered to infants with mild HIE because of a perceived good prognosis (Finder
389 et al., 2019; Miguel & Silveira, 2020). However, we have recently demonstrated that even
390 among healthy preschool children, a history of variations in oxygenation levels at birth is

associated with poorer cognitive flexibility (Miguel et al., 2019b). In experimental studies, researchers have been combining hypothermia together with pharmacological interventions and exciting results were observed (Huang and Jia, 2019; Huun et al., 2018; Rocha-Ferreira et al., 2018). We suppose that MPH treatment, that has a short half-life (approximately 3h) and is taken acutely to improve cognitive demands is not the most appropriate treatment for subjects with brain lesions. In these cases, early interventions combined with pharmacological treatment could be a more reasonable option, but studies should be carried out to confirm this hypothesis.

Increased BDNF levels in the ipsilateral hippocampus were observed following MPH administration in animals submitted to neonatal HI

Our findings demonstrate that systemic MPH administration (for 15 days) increased BDNF levels in the lesioned hippocampus of HI animals. Higher BDNF levels induced by MPH seem to be an attempt to recover the hippocampal dysfunction widely reported in HI animals (Miguel et al., 2015; Rojas et al., 2013). This dysfunction is not necessarily related to lower BDNF levels, since HI-untreated animals have similar BDNF levels than control rats, but can be associated to other neurotrophic factors, such as NGF and NT-3 (Fantacci et al., 2013).

It is recognized that MPH administration increases DA and NE signaling in the brain (Berridge et al., 2006; Kuczenski and Segal, 2001) and both neurotransmitters seem to regulate BDNF expression in the hippocampus (Mello-Carpes et al., 2016; Williams and Undieh, 2009). In this structure, BDNF is known to induce long-term potentiation (Pang et

al., 2004; Pastalkova et al., 2006), a form of synaptic plasticity that underlies long-term memory formation (Izquierdo and Medina, 1997; Morris, 2003). Although we observe this increase in BDNF levels in the lesioned hippocampus following MPH treatment, the findings in relation to memory formation were not improved by the MPH treatment. We need to consider that the BDNF analysis was performed in animals that did not perform the behavioral tasks, and maybe the stress related to extensive training or physical activity may have interfered with BDNF expression in these animals. Additionally, even though biochemical alteration could be observed following treatment, we believe that the large macroscopic lesion prevents the modifications to affect behavioral function.

Increased BDNF levels, but also other synaptic plasticity parameters, such as ultrastructural changes (longer active zone, thicker postsynaptic density and larger synaptic curvature), were also observed following systemic MPH administration in both control mice (Lee et al., 2012) or in the SHR strain, a recognized ADHD animal model (Kim et al., 2011; Tian et al., 2009). Another interesting point to be mentioned is that BDNF activity is hypothesized to be associated with ADHD pathophysiology (Tsai, 2007; Tsai, 2017) and that 6 or 8 weeks of MPH treatment increased BDNF levels in ADHD patients (Akay et al., 2018; Amiri et al., 2013).

In conclusion, our findings demonstrated that MPH administration in a dose of 2.5mg/kg was not able to improve HI-induced memory deficits, despite the increased BDNF levels observed in the lesioned hippocampus of HIMPH animals. Further studies investigating different MPH doses are needed to determine the dose-response relationship in this model.

Acknowledgment

We would like to thank Professor Matilde Achaval, main responsible for the laboratory's structure used in this project, and Chris Danilevicz for the technical support. This work was supported by the Brazilian funding agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

Figures:

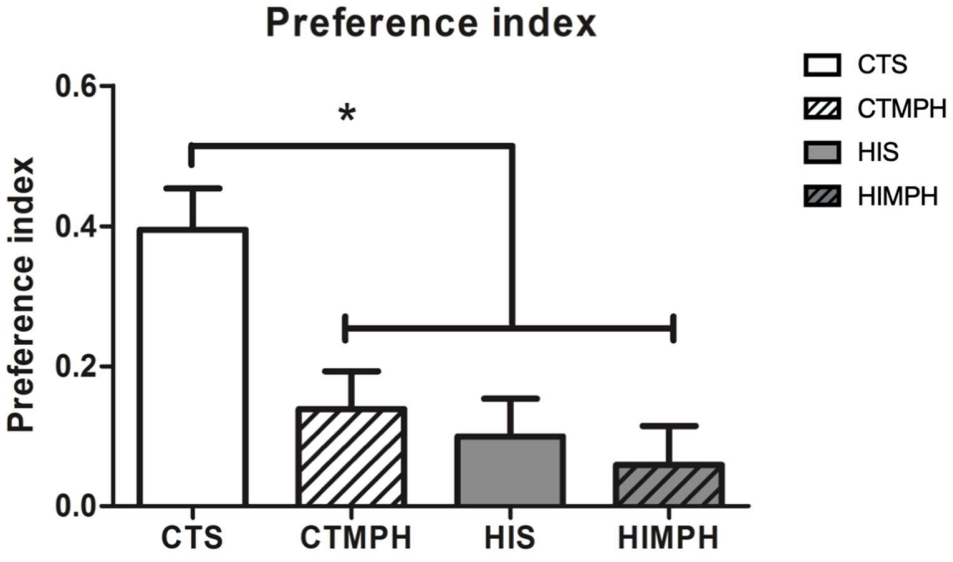


Figure 1: Preference index for the novel object in the Novel-object recognition (NOR) task. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc,

p<.05. *CTS different from all other groups. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.

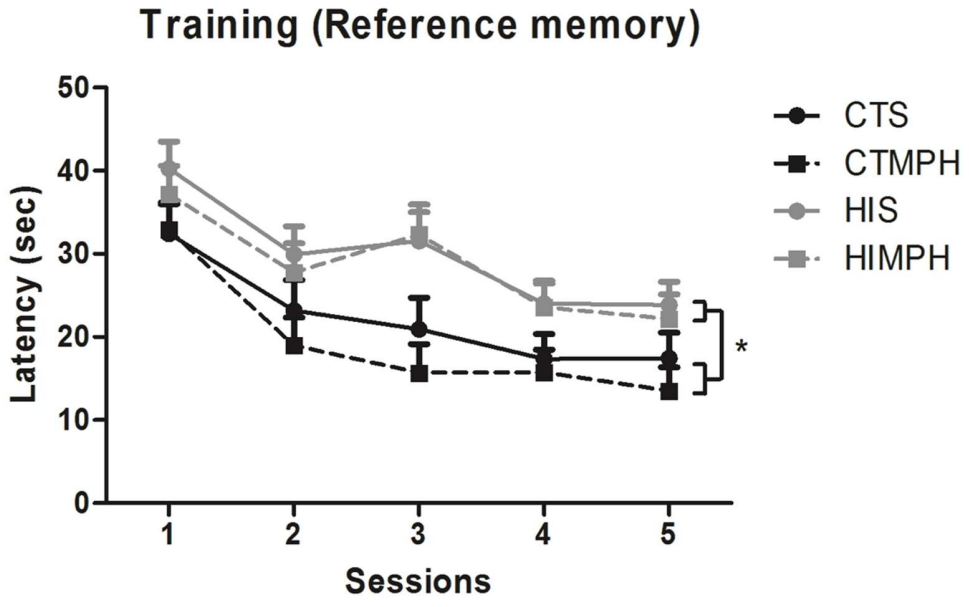


Figure 2: Spatial learning training in the Morris water maze (MWM) task. Results are expressed as mean ± S.E.M. Repeated-measure ANOVA, p<.05. *Difference between HI

and CT groups over the sessions. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.

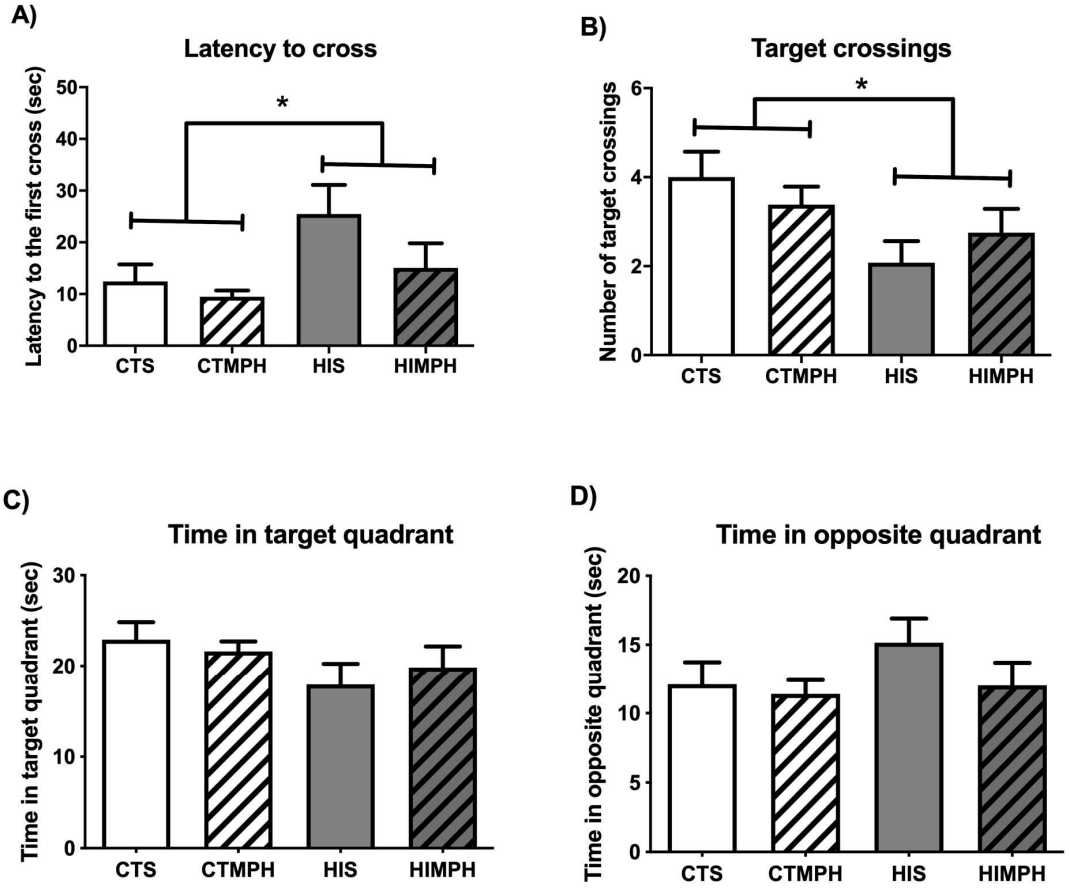
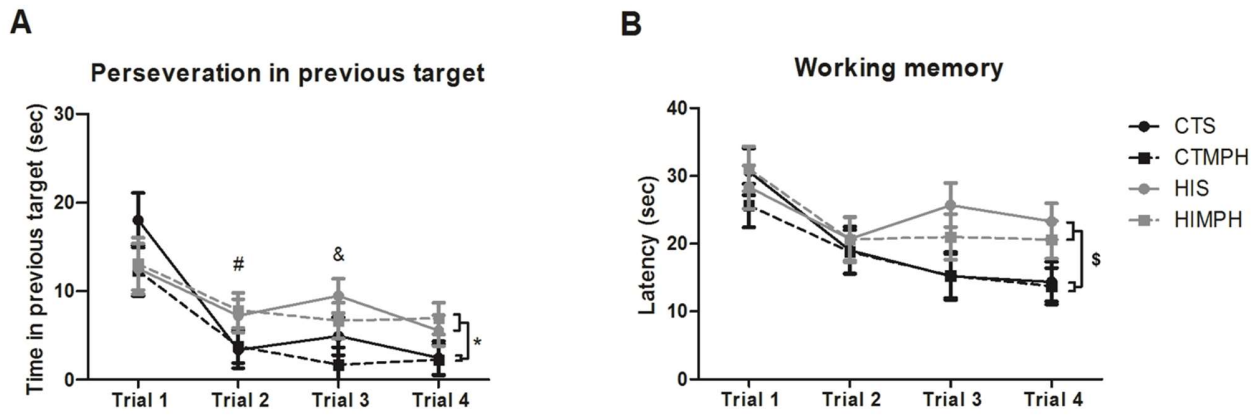


Figure 3: Performance during the probe trial to measure long-term reference memory in the MWM task. The following parameters are shown: A) Latency to the first cross on the target, B) Number of target crossings, C) Time spent on the target quadrant, and D) Time spent in opposite quadrant. Results are expressed as mean ± S.E.M. Two-way ANOVA, $p<.05$. *Difference between HI and CT groups. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.



479

480 **Figure 4:** Reversal learning (A) and working memory (B) performance in the MWM task.
481 Results are expressed as mean ± S.E.M. Repeated-measures ANOVA (reversal learning) and
482 Two-way ANOVA (working memory), followed by Tukey's post hoc, $p<.05$. *Difference
483 between HI and CT groups over the trials. #CTS group decreased time on the previous target
484 from the second trial and CTMPH group decreased latency from the third trial onwards (&).
485 $Difference between HI and CT groups on trials 3 and 4. CTS: control treated with saline;
486 CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline;
487 HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.

488

489

490

491

492

493

494

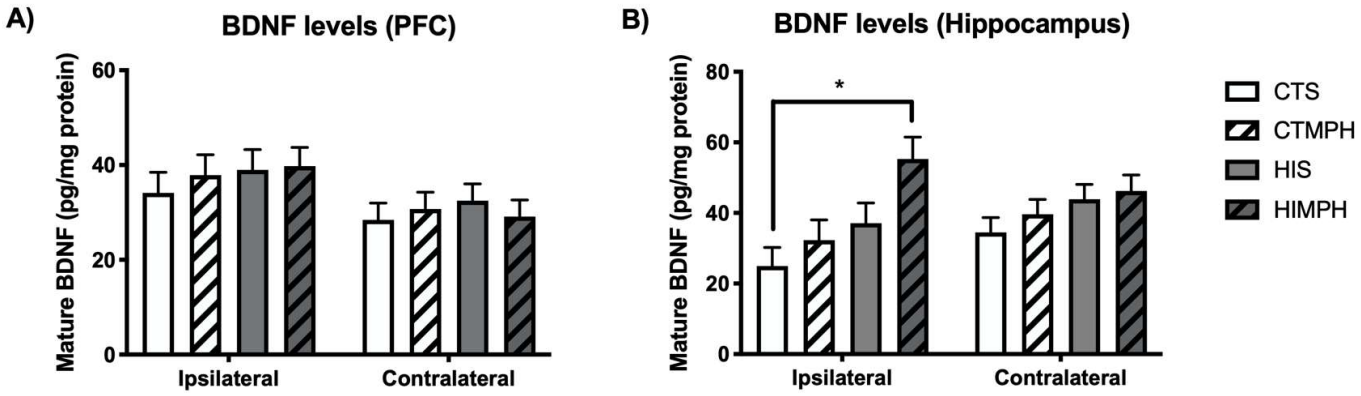


Figure 5: Brain-derived neurotrophic factor (BDNF) levels in prefrontal cortex (PFC) (A) and hippocampus (B). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<.05$. *HIMPH different from the CTS group in the right hippocampus (ipsilateral to the lesion). CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=5-7/group.

References

Akay AP, Resmi H, Guney SA, et al. (2018) Serum brain-derived neurotrophic factor levels in treatment-naive boys with attention-deficit/hyperactivity disorder treated with methylphenidate: an 8-week, observational pretest-posttest study. *Eur Child Adolesc Psychiatry* 27: 127-135.

Amiri A, Torabi Parizi G, Kousha M, et al. (2013) Changes in plasma Brain-derived neurotrophic factor (BDNF) levels induced by methylphenidate in children with Attention deficit-hyperactivity disorder (ADHD). *Prog Neuropsychopharmacol Biol Psychiatry* 47: 20-24.

Andersen PN, Egeland J and Oie M. (2013) Learning and memory impairments in children and adolescents with attention-deficit/hyperactivity disorder. *J Learn Disabil* 46: 453-460.

Arnsten AF. (2011) Catecholamine influences on dorsolateral prefrontal cortical networks. *Biol Psychiatry* 69: e89-99.

Askenasy EP, Taber KH, Yang PB, et al. (2007) Methylphenidate (Ritalin): behavioral studies in the rat. *Int J Neurosci* 117: 757-794.

Bathina S and Das UN. (2015) Brain-derived neurotrophic factor and its clinical implications. *Arch Med Sci* 11: 1164-1178.

Bedard AC and Tannock R. (2008) Anxiety, methylphenidate response, and working memory in children with ADHD. *J Atten Disord* 11: 546-557.

Berridge CW, Devilbiss DM, Andrzejewski ME, et al. (2006) Methylphenidate preferentially increases catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function. *Biol Psychiatry* 60: 1111-1120.

Bouchatta O, Manouze H, Bouali-Benazzouz R, et al. (2018) Neonatal 6-OHDA lesion model in mouse induces Attention-Deficit/ Hyperactivity Disorder (ADHD)-like behaviour. *Sci Rep* 8: 15349.

Carletti JV, Deniz BF, Rojas JJ, et al. (2016) Folic Acid Can Contribute to Memory Deficit and Na+, K+- ATPase Failure in the Hippocampus of Adolescent Rats Submitted to Hypoxia- Ischemia. *CNS Neurol Disord Drug Targets* 15: 64-72.

Chuhan YS and Taukulis HK. (2006) Impairment of single-trial memory formation by oral methylphenidate in the rat. *Neurobiol Learn Mem* 85: 125-131.

Cools R and D'Esposito M. (2011) Inverted-U-shaped dopamine actions on human working memory and cognitive control. *Biol Psychiatry* 69: e113-125.

Dafny N and Yang PB. (2006) The role of age, genotype, sex, and route of acute and chronic administration of methylphenidate: a review of its locomotor effects. *Brain Res Bull* 68: 393-405.

Deniz BF, Confortim HD, Deckmann I, et al. (2018) Folic acid supplementation during pregnancy prevents cognitive impairments and BDNF imbalance in the hippocampus of the offspring after neonatal hypoxia-ischemia. *J Nutr Biochem* 60: 35-46.

Diaz R, Miguel PM, Deniz BF, et al. (2016) Environmental enrichment attenuates the blood brain barrier dysfunction induced by the neonatal hypoxia-ischemia. *Int J Dev Neurosci* 53: 35-45.

Ennaceur A and Delacour J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav Brain Res* 31: 47-59.

Fantacci C, Capozzi D, Ferrara P, et al. (2013) Neuroprotective role of nerve growth factor in hypoxic-ischemic brain injury. *Brain Sci* 3: 1013-1022.
Finder M, Boylan GB, Twomey D, et al. (2019) Two-Year Neurodevelopmental Outcomes After Mild Hypoxic Ischemic Encephalopathy in the Era of Therapeutic Hypothermia. *JAMA Pediatr*.
Floresco SB. (2013) Prefrontal dopamine and behavioral flexibility: shifting from an "inverted-U" toward a family of functions. *Frontiers in Neuroscience* 7.
Fumagalli F, Racagni G, Colombo E, et al. (2003) BDNF gene expression is reduced in the frontal cortex of dopamine transporter knockout mice. *Mol Psychiatry* 8: 898-899.
Guthrie SK, Teter CJ, McCabe SE, et al. (2003) Illicit Methylphenidate Use in an Undergraduate Student Sample: Prevalence and Risk Factors. *Pharmacotherapy* 23: 609-617.
Hall J, Thomas KL and Everitt BJ. (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat Neurosci* 3: 533-535.
Heyser CJ, McNaughton CH, Vishnevetsky D, et al. (2013) Methylphenidate restores novel object recognition in DARPP-32 knockout mice. *Behav Brain Res* 253: 266-273.
Heyser CJ, Pelletier M and Ferris JS. (2004) The effects of methylphenidate on novel object exploration in weanling and periadolescent rats. *Ann N Y Acad Sci* 1021: 465-469.
Hotte M, Naudon L and Jay TM. (2005) Modulation of recognition and temporal order memory retrieval by dopamine D1 receptor in rats. *Neurobiol Learn Mem* 84: 85-92.
Huang A and Jia L. (2019) Crocin enhances hypothermia therapy in hypoxic ischemia-induced brain injury in mice. *Acta Neurol Belg*.
Huun MU, Garberg H, Loberg EM, et al. (2018) DHA and therapeutic hypothermia in a short-term follow-up piglet model of hypoxia-ischemia: Effects on H+MRS biomarkers. *PLoS One* 13: e0201895.
Iwakura Y, Nawa H, Sora I, et al. (2008) Dopamine D1 receptor-induced signaling through TrkB receptors in striatal neurons. *J Biol Chem* 283: 15799-15806.
Izquierdo I and Medina JH. (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol Learn Mem* 68: 285-316.
Kim H, Heo HI, Kim DH, et al. (2011) Treadmill exercise and methylphenidate ameliorate symptoms of attention deficit/hyperactivity disorder through enhancing dopamine synthesis and brain-derived neurotrophic factor expression in spontaneous hypertensive rats. *Neurosci Lett* 504: 35-39.
Klein CP, Hoppe JB, Saccomori AB, et al. (2018) Physical Exercise During Pregnancy Prevents Cognitive Impairment Induced by Amyloid-beta in Adult Offspring Rats. *Mol Neurobiol*.
Kramer AF, Cepeda NJ and Cepeda ML. (2001) Methylphenidate effects on task-switching performance in attention-deficit/hyperactivity disorder. *J Am Acad Child Adolesc Psychiatry* 40: 1277-1284.
Kuczenski R and Segal DS. (2001) Locomotor effects of acute and repeated threshold doses of amphetamine and methylphenidate: relative roles of dopamine and norepinephrine. *J Pharmacol Exp Ther* 296: 876-883.
Kuppers E and Beyer C. (2001) Dopamine regulates brain-derived neurotrophic factor (BDNF) expression in cultured embryonic mouse striatal cells. *Neuroreport* 12: 1175-1179.

Lee TH, Lee CH, Kim IH, et al. (2012) Effects of ADHD therapeutic agents, methylphenidate and atomoxetine, on hippocampal neurogenesis in the adolescent mouse dentate gyrus. *Neurosci Lett* 524: 84-88.

Leo D, Sukhanov I, Zoratto F, et al. (2018) Pronounced Hyperactivity, Cognitive Dysfunctions, and BDNF Dysregulation in Dopamine Transporter Knock-out Rats. *J Neurosci* 38: 1959-1972.

Levine S. (1960) Anoxic-ischemic encephalopathy in rats. *Am J Pathol* 36: 1-17.

Mangina CA and Beuzeron-Mangina H. (2009) Similarities and differences between learning abilities, "pure" learning disabilities, "pure" ADHD and comorbid ADHD with learning disabilities. *Int J Psychophysiol* 73: 170-177.

Mello-Carpes PB, da Silva de Vargas L, Gayer MC, et al. (2016) Hippocampal noradrenergic activation is necessary for object recognition memory consolidation and can promote BDNF increase and memory persistence. *Neurobiol Learn Mem* 127: 84-92.

Miguel PM, Deniz BF, Confortim HD, et al. (2019a) Methylphenidate administration reverts attentional inflexibility in adolescent rats submitted to a model of neonatal hypoxia-ischemia: Predictive validity for ADHD study. *Exp Neurol*.

Miguel PM, Deniz BF, Deckmann I, et al. (2018) Prefrontal cortex dysfunction in hypoxic-ischaemic encephalopathy contributes to executive function impairments in rats: Potential contribution for attention-deficit/hyperactivity disorder. *World J Biol Psychiatry* 19: 547-560.

Miguel PM, Pereira LO, Barth B, et al. (2019b) Prefrontal Cortex Dopamine Transporter Gene Network Moderates the Effect of Perinatal Hypoxic-Ischemic Conditions on Cognitive Flexibility and Brain Gray Matter Density in Children. *Biol Psychiatry*.

Miguel PM, Schuch CP, Rojas JJ, et al. (2015) Neonatal hypoxia-ischemia induces attention-deficit hyperactivity disorder-like behavior in rats. *Behav Neurosci* 129: 309-320.

Miguel PM and Silveira PP. (2020). Neonatal Hypoxia Ischaemia and individual differences in neurodevelopmental outcomes. *JAMA Peds. Accepted.*

Millichap JG. (2008) Etiologic classification of attention-deficit/hyperactivity disorder. *Pediatrics* 121: e358-365.

Mishima K, Ikeda T, Yoshikawa T, et al. (2004) Effects of hypothermia and hyperthermia on attentional and spatial learning deficits following neonatal hypoxia-ischemic insult in rats. *Behavioural Brain Research* 151: 209-217.

Morris RG. (2003) Long-term potentiation and memory. *Philos Trans R Soc Lond B Biol Sci* 358: 643-647.

Nagai T, Takuma K, Kamei H, et al. (2007) Dopamine D1 receptors regulate protein synthesis-dependent long-term recognition memory via extracellular signal-regulated kinase 1/2 in the prefrontal cortex. *Learn Mem* 14: 117-125.

National Institute for Health and Care Excellence. (2018) *Attention deficit hyperactivity disorder: diagnosis and management*. Available at: https://www.nice.org.uk/guidance/ng87/chapter/Recommendations#medication.

Pang PT, Teng HK, Zaitsev E, et al. (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* 306: 487-491.

Pastalkova E, Serrano P, Pinkhasova D, et al. (2006) Storage of spatial information by the maintenance mechanism of LTP. *Science* 313: 1141-1144.

Pereira LO, Arteni NS, Petersen RC, et al. (2007) Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat. *Neurobiol Learn Mem* 87: 101-108.
Pereira LO, Nabinger PM, Strapasson AC, et al. (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. *Brain Res* 1247: 188-195.
Pereira LO, Strapasson AC, Nabinger PM, et al. (2008) Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. *Brain Res* 1218: 257-266.
Rhodes SM, Coghill DR and Matthews K. (2006) Acute neuropsychological effects of methylphenidate in stimulant drug-naïve boys with ADHD II - broader executive and non-executive domains. *Journal of Child Psychology and Psychiatry* 47: 1184-1194.
Rice JE, 3rd, Vannucci RC and Brierley JB. (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* 9: 131-141.
Rocha-Ferreira E, Poupon L, Zelco A, et al. (2018) Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy. *Brain* 141: 2925-2942.
Rojas JJ, Deniz BF, Miguel PM, et al. (2013) Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat. *Exp Neurol* 241: 25-33.
Silhol M, Arancibia S, Maurice T, et al. (2007) Spatial memory training modifies the expression of brain-derived neurotrophic factor tyrosine kinase receptors in young and aged rats. *Neuroscience* 146: 962-973.
Sunohara GA, Malone MA, Rovet J, et al. (1999) Effect of methylphenidate on attention in children with attention deficit hyperactivity disorder (ADHD): ERP evidence. *Neuropsychopharmacology* 21: 218-228.
Tian Y, Wang Y, Deng Y, et al. (2009) Methylphenidate improves spatial memory of spontaneously hypertensive rats: evidence in behavioral and ultrastructural changes. *Neurosci Lett* 461: 106-109.
Tsai SJ. (2007) Attention-deficit hyperactivity disorder may be associated with decreased central brain-derived neurotrophic factor activity: clinical and therapeutic implications. *Med Hypotheses* 68: 896-899.
Tsai SJ. (2017) Role of neurotrophic factors in attention deficit hyperactivity disorder. *Cytokine Growth Factor Rev* 34: 35-41.
Vannucci RC and Vannucci SJ. (2005) Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci* 27: 81-86.
Verster JC, Bekker EM, Kooij JJ, et al. (2010) Methylphenidate significantly improves declarative memory functioning of adults with ADHD. *Psychopharmacology (Berl)* 212: 277-281.
Vorhees CV and Williams MT. (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc* 1: 848-858.
Williams SN and Undieh AS. (2009) Dopamine D1-like receptor activation induces brain-derived neurotrophic factor protein expression. *Neuroreport* 20: 606-610.
Yager JY and Ashwal S. (2009) Animal models of perinatal hypoxic-ischemic brain damage. *Pediatr Neurol* 40: 156-167.

Zhu T, Gan J, Huang J, et al. (2016) Association Between Perinatal Hypoxic-Ischemic Conditions and Attention-Deficit/Hyperactivity Disorder: A Meta-Analysis. *J Child Neurol* 31: 1235-1244.