Neonatal hypoxia-ischemia induces dysregulated feeding patterns and ethanol consumption that are alleviated by methylphenidate administration in rats

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Abstract

Impulsivity, as observed in patients diagnosed with Attention-deficit/hyperactivity disorder (ADHD), can induce dysregulated behaviors such as binge eating and drug addiction. We previously demonstrated that neonatal hypoxia-ischemia (HI) resulted in ADHD-like behaviors in rats and that methylphenidate (MPH) administration (the first therapeutic option for ADHD) reversed these deficits. Here, we aimed at investigating addictive-like behaviors, such as the reward-based feeding behavior (using the BioDAQ monitor) and ethanol consumption (using the IA2BC procedure) in adult animals subjected to neonatal HI and treated with or without MPH. Male Wistar rats were divided into four groups (n=10-12/group): control saline (CTS), CTMPH, HI saline (HIS) and HIMPH. The HI procedure was conducted at postnatal day (PND) 7 and behavioral analyses between PND 60–90, in which MPH (2.5 mg/kg, i.p.) was administered 30 min prior to each behavioral evaluation (6 sessions in BioDAQ and 12 sessions in the IA2BC protocol). HI animals had a dysregulated feeding intake shortly after eating a small piece of the palatable diet, and MPH reversed this dysregulated pattern. However, when the palatable diet was freely available, MPH stimulated a higher intake of this diet in the first exposure day, and this effect was potentialized in HIMPH rats. Increased ethanol intake was observed in HI rats, and MPH administration alleviated this behavior; contrarily, MPH treatment in control rats induced an increase in ethanol consumption. The present findings give additional support to the relationship between neonatal HI and ADHD but the differential response to MPH in control or HI animals highlights the importance of avoiding indiscriminate use of MPH by healthy individuals.

Keywords: ADHD; Attention-deficit/hyperactivity disorder; BioDAQ; IA2BC; intermittent access 2-bottle choice; perinatal complication

Introduction

Impulsivity, which is broadly defined as the tendency to act prematurely without foresight [1], can induce dysregulated behaviors such as binge eating and drug addiction [2]. As impulsivity is a core feature in patients diagnosed with Attention-deficit/hyperactivity disorder (ADHD), it is not surprising that ADHD and obesity are associated [3]. Moreover, it was demonstrated that this relation was driven by food addiction and binge eating, especially in adults [4]. Concerning substance abuse disorders, prospective studies have shown that children with ADHD were more likely to develop disorders of substance abuse/dependence (including nicotine, alcohol, marijuana, cocaine, and other substances) during adolescence or adulthood [5]. Besides, elevated levels of impulsivity were reported to mediate the association between childhood ADHD and alcohol problems in adulthood [6].

Both impulsivity and ADHD neurobiology have been associated with lower levels of brain dopamine (DA) signaling [7,8]; contrarily, drug and palatable food intake are known to increase DA neurotransmission in the reward pathways, especially in the nucleus accumbens (NAc) [9]. Thus, it is suggested that abnormal food intake or drug abuse would be an attempt to compensate for the decreased activation of the brain reward system, as a form of self-medication [10,11]. The first-line of pharmacological treatment for ADHD, the methylphenidate (MPH) stimulant (commercially known as Ritalin), confirms this dopaminergic principle. It blocks the dopamine transporter (DAT), highly expressed in the striatum (a region that includes the NAc) [12], and enhances DA availability on the synaptic cleft. In fact, MPH treatment has been associated with lower rates of alcohol and drug use in ADHD youth when compared to ADHD-untreated or healthy controls, possibly via increased DA signaling [13]. Despite this positive effect of MPH, there is

some research that implicates its use in childhood as the causal link to later substance abuse disorder [14], probably through modifications in sensitivity to reward induced by the drug.

In relation to feeding behavior, there is a consensus that MPH treatment induces loss of appetite and growth stunting in humans [15]. However, the characteristics of the population should be considered in this type of analysis. Davis and colleagues demonstrated that food-related behaviors (appetite, cravings and snack-food intake) have diminished in response to MPH in normal weight individuals. Contrarily, a small increase in all the parameters were seen in obese males after MPH challenge [16]. Besides, MPH increased the desire for food in food-deprived humans [17] and such effect was also observed in rats [18]. Taken together, these results indicate that MPH effects in relation to addictive behaviors could vary depending on the characteristics of the population as well as their physiological state.

ADHD etiology has a strong genetic component, but environmental conditions, especially those occurring during the perinatal period, have an important role on the disorder. For example, the association between perinatal hypoxia-ischemia (HI) and later ADHD diagnosis was evident in the meta-analysis conducted by Zhu et al. [19]. We have confirmed this relationship in experimental studies, using a rat model of neonatal HI proposed by Levine [20] and modified by Rice and colleagues [21]. Rats that underwent neonatal HI had attentional deficits, impulsive action and disturbances in the DA system [22,23]. MPH treatment was able to improve the attentional deficits in this model and also upregulate phosphorylated-tyrosine hydroxylase in the prefrontal cortex, the rate-limiting enzyme for DA synthesis [24]. However, the analysis of addictive-like behaviors following the HI procedure was not explored in the literature, and this could provide a platform to the understanding of the ADHD-like characteristics observed in this model. Thus, we aimed to analyze feeding behaviors (facing standard or highly palatable chow)

and alcohol consumption in hypoxic-ischemic or control rats treated or not with MPH in adulthood. Considering the impulsivity trait and DA disturbances observed in hypoxic-ischemic animals, we hypothesized that these animals would have higher consumption of the palatable diet and alcohol, and these behaviors would be reversed with MPH treatment.

Materials and Methods

Animals

Pregnant Wistar rats at the end of gestation were obtained from the institutional breeding facility (CREAL, ICBS, UFRGS) and maintained at the university hospital animal research facility (UEA, CPE-HCPA) under standard conditions: controlled room temperature (22±2◦C), 12:12h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.) and food and water available *ad libitum.* The day of birth was considered postnatal day 0 (PND 0) and on the 7th PND, male pups were randomly distributed into control (CT) and HI groups (the HI protocol is described below). Female pups of the litters were also assigned to CT and HI groups, but they were designated to another research project. After HI procedure, the pups were maintained with their dams in a minimum number of six and maximum of eight per litter, until the weaning (PND 21), when they were housed in 2-3 per cage (Plexiglas cages: 49x34x16cm). From PND 60, CT and HI groups were subdivided in saline and MPH treatment, resulting in four experimental groups (n=12/group): control treated with saline (CTS), control treated with MPH (CTMPH), HI treated with saline (HIS) and HI treated with MPH (HIMPH). The behavioral analyses were conducted between PND

60-90 and animals were euthanized twenty-four hours after the final behavioral session. The timeline of experimental procedures is shown in Fig.1.

All procedures were approved by the Institutional Ethics Committee on Animal Use (UFRGS 29750; GPPG/HCPA 15-0566) 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 conducted on the 7th PND, using the protocol developed by Levine [20] and modified by Rice and colleagues [21]. Rats were anesthetized with isoflurane (4-5% for induction and 1.5-2% for maintenance) 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. The neck incision was sutured and bloodstains due to surgery were removed to minimize the refusal of the mother to feed or take care of the pup. Control animals were submitted to sham surgery, i.e., animals received only anesthesia, neck incision, and skin suture. 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. The animals returned immediately to maternal care after hypoxia [22-24].

MPH administration

Methylphenidate hydrochloride (MPH) (Novartis, Brazil) treatment started on PND 60, concomitantly with the beginning of the behavioral analysis. The MPH dose of 2.5mg/kg, adopted in this study, corresponds to a medium dose [25] and was able to improve attentional deficits of HI animals in our previous study [24]. MPH was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (dose 2.5mg/kg, volume 1 ml/kg), once a day, at the end of the light cycle. It is important to note that the MPH administration occurred only before the behavioral analyses, in 6 consecutive days for the BioDAQ and 12 intermittent sessions for the IA2BC protocol. Control animals received an equivalent volume of saline solution on the same days.

Feeding behavior (BioDAQ® Food Intake Monitor)

After reaching 60 days of life, rats were transferred into cages equipped with a BioDAQ® food intake monitoring system (Research Diets, USA), which can provide detailed feeding behavior data, such as total food intake and meal patterns. The BioDAQ® uses a food hopper mounted on an electronic strain gauge-based load cell connected to a computer for data transmission. The food hopper is weighed 50 times per sec (accurate to 0.01 g) and the mean and standard deviation (S.D.) of food consumption over approximately 1 sec is calculated by the computer software. Feeding is signaled by a change in the food hopper weight (defined as S.D. > 2000 mg), caused by the animal eating. The BioDAQ® system can record two kinds of events: feeding bouts and meals. A bout is an episode of uninterrupted feeding, in which the end happens when the hopper is left undisturbed for 5 s (defined as a S.D. <2000 mg). A meal is defined as a group of bouts with a difference in hopper weight of >0.1 g, separated from other feeding episode

within a range of 15 min [26,27]. The duration of the feeding event, its start date and time and the amount eaten is recorded and exported to the computer [28,29].

Two days prior to their transference to the BioDAQ system, animals received a portion of the palatable diet (4.82 kcal/g, 14% protein, 34% fat, 30% carbohydrate in each kg, whose 20% were sucrose; Prag Soluções Biociências®) in their home cage to avoid neophobia during the experiment. Rats were individually housed for the feeding behavior analysis, which lasted 6 days. In the habituation phase (days 1 to 4), rats were given access to standard rat chow (2.95 kcal/g, 22% protein, 4% fat, 45.5% carbohydrate in each kg; NUVILAB®) on both food hoppers from the cage. Specifically, on days 3 and 4, a small piece of the palatable diet was given to the animals in their BioDAQ cage before the feeding analyses – to familiarize the animals with the diet that would be present the following days.

The food preference analyses occurred during the fifth and sixth days on the BioDAQ, considered days 1 and 2 of exposure to the highly palatable diet. In this assessment, one of the food hoppers was fully provided with palatable diet while the other hopper remained filled with standard chow. A schematic presentation of the BioDAQ protocol is depicted in Fig.1. The palatable diet position was swapped between days to avoid side preference. For the analyses of the standard chow intake, the sum of both hoppers was used, and for the subsequent analysis of food preferences, the food hoppers were analyzed separately.

Animals were weighed daily before MPH or saline administration, that occurred at the end of the light cycle. This period was chosen to capture the drug effect in the most active phase of the rats. At the time that animals were removed from the cage, the diets were replenished and BioDAQ® system were cleaned for maintenance. The feeding data was analyzed at 2h and 20h after drug administration to evaluate both acute and protracted effects of the drug. The following

variables were analyzed: 1) total consumption relative to body weight (g/kg), 2) number of bouts, 3) number of meals, 4) bout size (amount consumed (g/kg)/number of bouts) and 5) meal size (amount consumed (g/kg)/number of meals), 6) total caloric consumption in kilocalories (palatable diet plus standard diet) and 7) preference index for the palatable diet (caloric intake from the palatable diet/total calorie intake)[28,29].

Ethanol preference (Intermittent access to ethanol in 2-bottle choice procedure; IA2BC)

At the end of the feeding behavior analysis, animals were transferred from the BioDAQ® to standard rat cages for the analysis of ethanol consumption, which started two days later to acclimatize the animals to the new no-drip water bottles. We used the intermittent access to ethanol 20% in 2-bottle choice procedure (IA2BC), as described in Carnicella et al. [30]. Each IA2BC drinking session occurred on alternate days, in which animals were isolated and had 24h-concurrent access to two bottles, one with ethanolic solution (20% in tap water, v/v) and another with tap water. The MPH or saline administration occurred only in the drinking sessions; in the withdrawal period, animals were regrouped (2-3/cage) with their familiar animals to avoid prolonged social isolation, a condition known to increase ethanol intake in the IA2BC protocol [31]. The drinking sessions begun right after drug administration, at the end of the light cycle. Rat chow was available during the sessions and both bottles, chow and animals were weighted before and after drinking sessions to calculate the consumptions and body weight gain. In each session, the position of the bottles was alternated to control for side preferences. A total of 12 sessions were carried out and in the last three sessions (sessions 10-12), the water and ethanol consumption were

also measured 2h after drug administration to capture possible MPH acute effects. This short measurement occurred only in the last sessions to avoid fluid spillage due to bottle handling.

The following variables were analyzed throughout the 12 sessions: 1) ethanol intake (ml/kg), 2) water intake (ml/kg), 3) total fluid intake (ml/kg), 4) ethanol preference (%), 5) food intake (g/kg), and 6) body weight gain (g). Considering the higher intake variability in the first sessions, the mean of the last 6 sessions (sessions 7-12) of each variable was also analyzed.

Statistical analysis

Repeated-measures ANOVA followed by Tukey's post hoc, with lesion and treatment as factors, was used to analyze the consumption during the habituation to the BioDAQ, food preference and ethanol parameters throughout the 12 sessions (IA2BC protocol). Two-way ANOVA, followed by Tukey's post hoc, was conducted to analyze feeding behavior at each habituation day, the mean of all measures in the last 6 sessions of the IA2BC protocol, and the mean ethanol and water consumption in the first 2h of exposure (only in the last 3 sessions). All variables were expressed as mean ± standard error of the mean (SEM), and the results were considered statistically significant when $p \leq .05$. Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Feeding behavior

Habituation to the BioDAQ (days 1 to 4)

One outlier in the CTS group was excluded from the final analyses, resulting in 11 animals in this group and 12 in the remaining groups. In relation to the 2h analyses, a *day* main effect was observed in all feeding parameters. Tukey's post hoc demonstrated that only the HIS group significantly increased the total consumption and meal size on days 3 and 4 (in comparison to day 1) and the number of bouts and meals on day 4 (Fig.2). All other groups had no significant increase in consumption over the days. When analyzing each day separately, a significant *treatment* main effect on day 3 was observed for bout size ($F(1,43)= 4.03$, $p=0.05$), as well as a trend for treatment effect for number of meals ($F(1,43)=3.73$, $p=0.06$), suggesting a decrease in the number of meals in animals treated with MPH (Fig.2C) as a consequence of increased bout size (CTS: 1.06±0.19, CTMPH: 1.43±0.19, HIS:1.1±0.19, HIMPH: 1.5±0.16). On day 4, significant *lesion* effects were observed for total consumption ($F(1,43)=4.5$, $p<0.05$) and number of bouts ($F(1,43)=5.18$, $p<0.05$). The post hoc analysis indicated that the HIS group consumed more rat chow compared to both CTS and CTMPH groups; in relation to the HIMPH group, the difference did not reach statistical significance ($p=0.06$) (Fig.2A). Additionally, HIS rats had higher number of bouts in relation to the CTMPH group (Fig.2B).

Analysis of the consumption over 20h also captured the *day* effect for all variables, confirming that animals increased the consumption throughout the days. Moreover, *day*lesion* ($F(3,129)=3.52$, $p<0.05$) and *day*lesion*treatment* ($F(3,129)=2.78$, $p<0.05$) were observed for total chow consumption; and *day*lesion* interaction effect ($F(3,129)=5$, $p<0.01$) identified for number of bouts. The Tukey's post hoc indicated that both hypoxic-ischemic and the CTMPH groups increased chow intake, number of bouts and meal size on days 3 and 4, when compared to day 1. This difference was only observed on day 4 in the CTS group, suggesting a more stable

pattern of eating in this group (Supplementary Fig.S1). Two-way ANOVA was also performed within each day to investigate possible punctual group differences. For number of bouts, a *lesion* effect was observed on day 4 ($F(1,43)= 4.23$, $p<0.05$), in which HI animals had more bouts when compared to controls (Supplementary Fig.S1B). In relation to meal size, a *lesion*treatment* interaction effect was observed on day 2 ($F(1,43)= 5.45$, $p<0.05$), and the post hoc indicated a trend for a larger meal size in the HIMPH group compared to the HIS group ($p=0.07$; Supplementary Fig.S1D).

Food preference (days 1 and 2 of exposure to palatable chow)

Data analyses revealed no differences between groups in any of the standard chow intake measures as animals ate very little from this diet. Analyzing the palatable diet consumption between days 1 and 2 of exposure (sessions of 2h), repeated-measures ANOVA showed a *day* main effect and *day*treatment* interaction effect for the measures: total consumption in grams (*day* $F(1,43)=4.8$, $p<0.05$; *day*treatment* $F(1,43)=12.24$, $p<0.01$), number of bouts (*day* $F(1,43)=21.57$, $p<0.001$; *day*treatment* $F(1,43)=6.4$, $p<0.05$), meal size (*day* $F(1,43)=10.44$, $p<0.01$; *day*treatment* $F(1,43)=17.82$, $p<0.001$), and total caloric consumption (*day* $F(1,43)=7.24$, $p<0.05$; *day*treatment* $F(1,43)=12.24$, $p<0.01$). In the first 2h following MPH injection and diet exposure, the HIMPH group consumed more palatable diet than CTS rats (Fig.3A); and comparing the difference between day 1 and 2 within groups, HIMPH was the only group that significantly had decreased diet consumption (Fig.3A), number of bouts (Fig.3B) and total caloric consumption (Fig.3D) on day 2 compared to day 1. For meal size, both CTMPH and HIMPH groups decreased meal size on day 2 compared to day 1, and the CTMPH group also had a larger meal portion in relation to the CTS group on day 1 (Fig.3C).

A *day* main effect was observed for palatable diet preference ($F(1,43)=11.97$, $p<0.01$) and the post hoc showed a trend ($p=0.06$) for an increase in preference on day 2 only for the CTS group, indicating that all other groups had higher preference since day 1 (Day 1: CTS 0.88±0.03, CTMPH 0.92±0.03, HIS 0.96±0.03, HIMPH 0.95±0.03; Day 2: CTS 0.97±0.01, CTMPH 0.98±0.01, HIS 0.99±0.01, HIMPH 0.97±0.01).

The feeding behavior was also analyzed over 20h after the drug administration. Here, repeated-measures ANOVA demonstrated *day*treatment* interaction effects for total consumption of the palatable diet in grams ($F(1,43)=4.19$, $p<0.05$), as well as meal size ($F(1,43)=9.63$, $p<0.01$). Tukey's post hoc indicated that HIMPH animals ate larger meals in relation to CTS group on day 1 (Supplementary Fig.S2C). A *Day*treatment* interaction effect ($F(1,43)=4.76$, $p=0.03$), as well as *lesion* ($F(1,43)=4.66$, $p<0.05$) and *day* main effects ($F(1,43)=5.07$, $p<0.05$) were also statistically significant for total caloric consumption. The post hoc pointed out a higher caloric intake in the HIMPH group in relation to the CTS group on day 1 (Supplementary Fig.S2D).

Ethanol preference

Three outliers were excluded from the analyses, 1 CTMPH rat and 2 from the HIS group. For water intake, repeated-measures ANOVA demonstrated significant *lesion* main effect ($F(1,41)= 5.42$, $p<0.05$) and *session*lesion*treatment* interaction effect ($F(6.68,274.26)=2.49$, $p<0.05$), but no difference was observed on the post hoc (Fig.4A). The mean intake in the last 6 sessions (sessions 7-12) had *lesion* main effect ($F(1,41)=4.33$, $p=0.04$) and *lesion*treatment* interaction effects ($F(1,41)=3.86$, $p=0.05$); the Tukey's post hoc test indicated that the HIS group ingested less water than CTS rats (Fig.4B). For ethanol consumption (Fig.4C), *session*

(F(5.85,240.07)=3.83, p<0.01) and *lesion* main effects were observed over the sessions (F(1,41)=3.93, p=0.05). In the mean ethanol intake, *lesion* main effect was again observed (F(1,41)=4.56, p<0.05), but the post hoc indicated only a trend for the HIS group consuming more ethanol than the CTS group (p=0.09; Fig.4D). Analyzing preference for ethanol, i.e., alcohol solution consumed in relation to the total fluid intake, significant main effects for *session* (F(6.44,264.04)=3.20, p<0.01), *lesion* (F(1,41)=6.83, p<0.05) and a trend for *lesion*treatment* interaction effect were seen (F(1,41)= 3.57, p=0.06) (Fig.4E). *Lesion* main effect (F(1,41)=7.19, p<0.05) and *lesion*treatment* interaction effect (F(1,41)=5.04, p<0.05) were confirmed in the mean preference analysis, and the post hoc pointed out that the HIS group had a higher preference for ethanol in relation to the CTS group (Fig.4F). Overall, we can observe that MPH had different effects in control vs. hypoxic-ischemic animals, increasing water intake and decreasing alcohol preference in the HIMPH group, but having the opposite effect on CTMPH rats (Fig.4B and 4F).

Session and *session*lesion*treatment* effects were statistically significant for total fluid intake (session: F(6.16,252.68)=2.17, p<0.05; *session*lesion*treatment:* F(6.16,252.68)=2.53, p<0.05) and rat chow intake (session: F(5.3,217.38)=6.25 p<0.001; *session*lesion*treatment:* F(5.3,217.38)=2.14, p=0.05) (Fig.5A and 5C). The post hoc showed that the HIMPH group increased total fluid intake in sessions 3 and 5 in comparison to session 1 (Fig.5A), and less chow intake was also observed in this group on session 1 compared to sessions 3 to 12 (Fig.5C). This suggests that HIMPH animals had lower intake in general in the first sessions. When averaging sessions 7-12, no statistically significant effect was observed for fluid and chow intake, suggesting that MPH effects in HI animals were only observed at the beginning of the protocol (Fig.5B and 5D). Body weight was affected by *session* (F(3.24,132.85)=79.61, p<0.001) and *lesion* factors (F(1,41)=19.66, p<0.001), showing that animals gained weight throughout sessions, but HI

animals always had lower body weight than controls (Fig.5E). Although no statistical significance was detected in the post hoc, we observe that the HIMPH was the only group with decreasing body weight in the first sessions (Fig.5E), probably as a consequence from their decreased fluid and chow intake (Fig.5A and B, respectively). In the average of sessions 7-12, a *lesion* main effect was observed for body weight ($F(1,41)=19.85$, $p<0.001$). Tukey's test showed that the CTMPH group had higher body weight in relation to both HI groups; contrarily, HIMPH rats had lower body weight in relation to both CT groups, once more demonstrating that the MPH treatment affected CT and HI animals differently (Fig.5F).

Ethanol and water consumption were also evaluated 2h after drug administration in sessions 10-12 (last 3 sessions). Mean consumption was analyzed by two-way ANOVA, that showed no statistically significant differences between the groups for water intake (Fig.6A). However, a *lesion* main effect was observed for ethanol consumption ($F(1,41)=4.72$, $p<0.05$), indicating that HI animals consumed more ethanol in the first 2h of alcohol exposure (Fig.6B), in a very similar pattern to that observed in 24h-sessions (Fig.4D). For total fluid intake and ethanol preference, no statistically significant differences were found, although we can observe that HIS animals have already a tendency to increase their preference for ethanol in the first 2h of exposure (Fig.6C).

Discussion

The current study was delineated to investigate ADHD-related outcomes, such as addictive-like behaviors (reward-based feeding behavior and ethanol consumption), and the possible MPH effect in adult rats submitted to neonatal hypoxia-ischemia. Our main findings

showed that HI animals had a dysregulated feeding pattern in relation to standard chow shortly after eating a small piece of a palatable diet, and MPH administration was able to revert this behavior. When palatable food was freely available, MPH treatment induced an increase in palatable food intake in the first exposure day, having a higher effect in hypoxic-ischemic animals. Increased ethanol intake was observed in HI-untreated rats, and MPH administration decreased ethanol intake in the HI group. Contrarily, MPH treatment induced an increase in ethanol consumption in control rats.

HI animals showed a dysregulated feeding pattern shortly after eating a highly palatable food sample, and MPH administration reversed this behavior

During the habituation phase on the BioDAQ apparatus, on days 1 and 2, the animals had only standard chow in the food hoppers. However, on days 3 and 4, they received a small piece of the highly palatable food for habituation to the new diet. Intriguingly, after eating this small piece of palatable food, HIS animals increased their standard chow intake, specifically in the subsequent 2h (Fig.2). This finding suggests that HIS rats had a differential response to the pleasurable sensation associated with highly palatable food intake, hence increasing general food intake. In fact, some of our previous studies indicate that HI animals have a higher incentive salience for rewarding stimuli. For example, higher perseverative responses to receive a sweet pellet in adult HI animals were observed [23]. We have also described normal cognitive learning when tasks involved sweet reward, despite the substantial cognitive deficits associated with this HI model [22,24]. Additionally, we cannot exclude the possibility that these animals persisted eating the standard diet by a failure in inhibitory control processes or as a way to maintain a higher level of

DA stimulation [32]. Both inhibitory control failure as well as disruption in parameters of DA transmission were already observed in this model, supporting these hypotheses [22-24,33]. Considering that dysregulated eating patterns are frequently associated with ADHD in humans [3,4], the current findings support our hypothesis that neonatal HI results in ADHD-phenotypes in adult rats, corroborating our range of studies indicating the association between these two conditions [22-24].

Our findings further revealed that MPH administration was able to repair the dysregulated behavior of HI rats, since the HIMPH group did not have altered feeding pattern over the days, behaving similarly to controls. Moreover, we found that MPH administration showed a tendency to induce an increase in standard chow bout size after animals had eaten a small portion of palatable diet on day 3, consequently decreasing the number of meals. As day 3 was the animals' first contact with the palatable food, we infer that MPH increased the animal's focus on feeding at that given episode, increasing the bout size with uninterrupted bites. However, differently from the pattern observed in the HIS group, animals treated with MPH did not increase the total amount of food consumed, inducing in fact a lower total intake in treated animals on this day (Fig.2A). This indicates that MPH administration increased the focus on feeding behavior, but when animals realized that the food available was only the standard one, they interrupted the consumption. Some of the findings observed in the 2h-sessions were maintained over the 20h-sessions, such as the effect of MPH increasing focus on feeding and leading to larger meal portions in HIMPH animals on day 3. However, higher number of bouts in hypoxic-ischemic animals, independent of the treatment, were observed on day 4, suggesting that the MPH effect is more restricted to the drug half-life (approximately 3h).

MPH administration increased palatable food intake and this effect was higher in hypoxic-ischemic rats

In the food preference analyses, one of the food hoppers was filled up with a palatable diet and the feeding activity was analyzed over 2 or 20h after drug administration (in 2 exposure days). MPH administration induced an increase in the consumption of the palatable diet on day 1, and this behavior was observed especially in the first two hours of diet exposure (Fig.3). This finding corroborates our previous interpretation that MPH treatment increased the focus on feeding behavior. But contrarily to the observed with the standard diet, the animals remained focused on eating when palatable diet is available, eating larger amounts of this food. It has been suggested that stimulation of the mesolimbic pathway, as it occurs with MPH treatment, affects incentive salience properties of the rewarding stimulus [34]. Peciña and colleagues demonstrated that knockout rats for the dopamine transporter (DAT) - and consequently with higher levels of synaptic DA - attributed greater value to a sweet reward, increasing its consumption [35]. As the MPH increases DA transmission mainly by inactivating DAT function, our findings could be considered as in line with those reported by Peciña.

Although MPH increased palatable food consumption in all treated groups, this effect was higher in HI animals. Considering that alterations in dopaminergic signaling parameters in HI animals were already observed [22,24], we propose that these modifications make these animals more responsive to the effects of MPH. In fact, we previously demonstrated that MPH has a potentiation effect in HI rats, increasing locomotion and phosphorylated-tyrosine hydroxylase (the rate-limiting enzyme for DA synthesis) only in this group [24] as well as brain-derived neurotrophic factor (BDNF) levels in the hippocampus [36]. It is important to note that, contrary

to our hypothesis, HI itself did not increase palatable food intake when the diet was freely available. The lack of differences was intriguing considering that this group was more responsive to the presentation of palatable food during the habituation phase. However, in the analysis of preference for the palatable diet (in comparison to standard chow), we observed that only the CTS group had a trend ($p=0.06$) to increase the preference for palatable diet between the first and second days, suggesting that MPH groups and also the HIS group had already a higher preference for the palatable diet on the first day. This finding was an indicative that the HIS group may have a different behavior when facing the highly palatable diet. The lesion effect itself was seemingly not captured in other feeding parameters because of the short period of exposure to the freely available palatable diet (2 days). In this protocol, only MPH had the ability to substantially increase the consumption beyond the animals' natural capacity. Possibly a prolonged exposure to the palatable diet could inform better about feeding behavior over the days. Another point that should be mentioned is that homeostatic signals are also important, and interact with hedonic signals to induce feeding [37]. For example, we observed that HIMPH rats significantly increased the consumption in the first day, and consequently ate less in the second day. This indicates that the caloric intake at one point in time interferes with subsequent feeding behavior. In the same way, HIS animals had a higher standard chow intake in the last habituation day (day 4), and this difference may influence the subsequent food intake measures.

At first, the observed findings of MPH increasing consumption seem intriguing considering that the MPH is known to suppress appetite and has been suggested for obesity treatment [38,39]. However, a very interesting review on this topic suggests that "the effect that stimulants have for enhancing reward could lead to inappropriate use, or potentiate addictive behavior or compulsions such as binge eating" [40], which is one of the reasons why stimulants are not appropriate for

obesity treatment and supporting our findings. Some research already observed that MPH increases the desire for food in food-deprived humans [17] and rats [18], and more fascinating, although MPH decreased appetite, cravings and snack-food intake in normal weight individuals, it increases these behaviors in obese individuals [16]. Thus, these findings inform us about the importance to study the MPH effects in distinct populations, physiological states and environment conditions.

Methylphenidate attenuated the higher ethanol intake in hypoxic-ischemic animals, but stimulated the intake in control animals

Ethanol intake was measured over 12 intercalate sessions, and our results demonstrated that adult animals submitted to neonatal HI increased their ethanol intake and preference for ethanol compared to control rats treated with saline. There is currently no population-level risk estimates data investigating addictive-like behaviors following hypoxic-ischemic encephalopathy (HIE) as the majority of the studies assessed the outcomes in infancy or early childhood. However, as discussed above, it is known that perinatal exposure to HI increases the risk of subsequent ADHD diagnosis [19], that in turn can induce substance abuse disorders [5]. Although no populational studies are available, a case report described a male patient who suffered perinatal asphyxia, was diagnosed with ADHD at age of 3-4 years and initiated recreational use of ecstasy and cannabis at age of 21 years. This patient progressed to an additional intake of LSD at age 23 years and was later referred for psychiatric hospitalization [41].

Considering that substance abuse disorders are a common co-morbidity in ADHD patients, we reinforce that the neonatal HI model induces ADHD-related outcomes in rats, as indicated previously [22-24]. Impulsivity has been linked to drug addiction in humans, and higher levels of

impulsivity were also found in adult rats that underwent neonatal HI [23], suggesting that this behavioral phenotype could be associated with higher ethanol intake in these animals. Impairments in mesolimbic DA signaling induce substance abuse disorders, to compensate this deficit [42]. In HI animals, dysregulated DA parameters were observed in the striatum [33,43], a region constituted by the NAc which receives DA projections coming from the ventral tegmental area (VTA). The VTA DA neurons also innervate the PFC [44], a structure that displays impairments in DA signaling as a consequence of HI exposure [22,24]. Thus, we suggest that a reduction in DA transmission in HI animals may cause an increased ethanol intake in this group. Supporting this idea, increased DA signaling induced by MPH administration was able to decrease the ethanol consumption in the HIMPH group. In agreement with our findings, a clinical trial conducted by Hammerness and colleagues [13] showed that MPH treatment in ADHD adolescent patients significantly reduced the rates of alcohol and drug use in comparison with ADHD-untreated or healthy controls.

Another point that should be mentioned is that excessive use of alcohol and other substances is frequently considered a habitual behavior, resulting from a loss of flexible control over drug use [45,46]. Cognitive flexibility is part of the executive functions, i.e., higher order brain functions highly dependent on DA transmission in the PFC [47]. Interestingly, we have shown that children exposed to perinatal hypoxic-ischemic conditions and presenting a genetic background reflecting higher PFC activity of the DAT machinery demonstrated cognitive inflexibility [48]. In preclinical studies, we showed cognitive inflexibility associated with PFC DA dysregulation in adolescent HI rats, that was reversed by MPH administration [24]. We can suggest that lower cognitive flexibility in HI rats may induce a higher ethanol intake, and MPH-induced improved cognitive flexibility may explain the lower ethanol intake in HIMPH rats. Giving support

to our findings, Shnitko and colleagues demonstrated that pre-existing low cognitive flexibility in rhesus monkey was predictive of future classification as a heavy alcohol drinker [49].

Contrarily to the effects observed in HI rats, MPH administration in control animals resulted in increased ethanol consumption. This detrimental effect caused by MPH was not an unexpected result, since behavioral impairments, such as learning and memory deficits, were already observed in CT rats when administered with this same MPH dose [24,36]. Excessive DA transmission in the PFC, as well as lower DA levels, have been linked to cognitive impairments, and this has been recognized as the "inverted-U" curve relationship between PFC DA levels and cognition [50,51]. Thus, our findings showing a differential response to MPH in control or hypoxic-ischemic animals highlight the importance of avoiding indiscriminate use of MPH by healthy individuals.

Hypoxic-ischemic animals under the MPH effect had lower fluid and food intake in the first sessions of the IA2BC protocol and consequently this group had decreased weight gain in the first sessions (Fig.5). This finding demonstrates a different habituation to the novel environment in the HIMPH group, which could be associated with higher locomotor activity following the MPH administration [24]. In the fourth session, this group normalized their behavior, being similar to the other groups, but they always had lower body weight than their control (HIS), a group which had higher caloric intake from the ethanol. Contrarily, CTMPH had the highest body weight and this group consumed higher amounts of ethanol than their control (CTS). Overall, these results sustain the assumption that MPH treatment can affect CT or HI animals differently.

In conclusion, the findings demonstrated that neonatal HI induces ADHD-related outcomes in rats, such as dysregulated feeding activity and ethanol intake in adulthood, providing additional support to the face validity of the HI model as a possible ADHD experimental model. MPH

administration was able to alleviate these behaviors in HI animals, confirming also the predictive validity of this model. Additionally, MPH administration induced higher palatable diet intake in both CT and HI groups, but this effect was higher in HI animals, probably by a higher attributed value to the palatable diet in this group. Then, the current results added important new findings to the ADHD field, both to the experimental and clinical aspects, supporting that perinatal hypoxia-ischemia may substantially disrupt the developing brain.

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Authors contribution

PMM, PPS and LOP were responsible for the study concept and design. PMM, LPB, BFD, HDC, BCdeO and RDM contributed to the acquisition of animal data. PMM, PPS and LOP assisted with data analysis and interpretation of findings. PMM drafted the manuscript and PPS and LOP provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

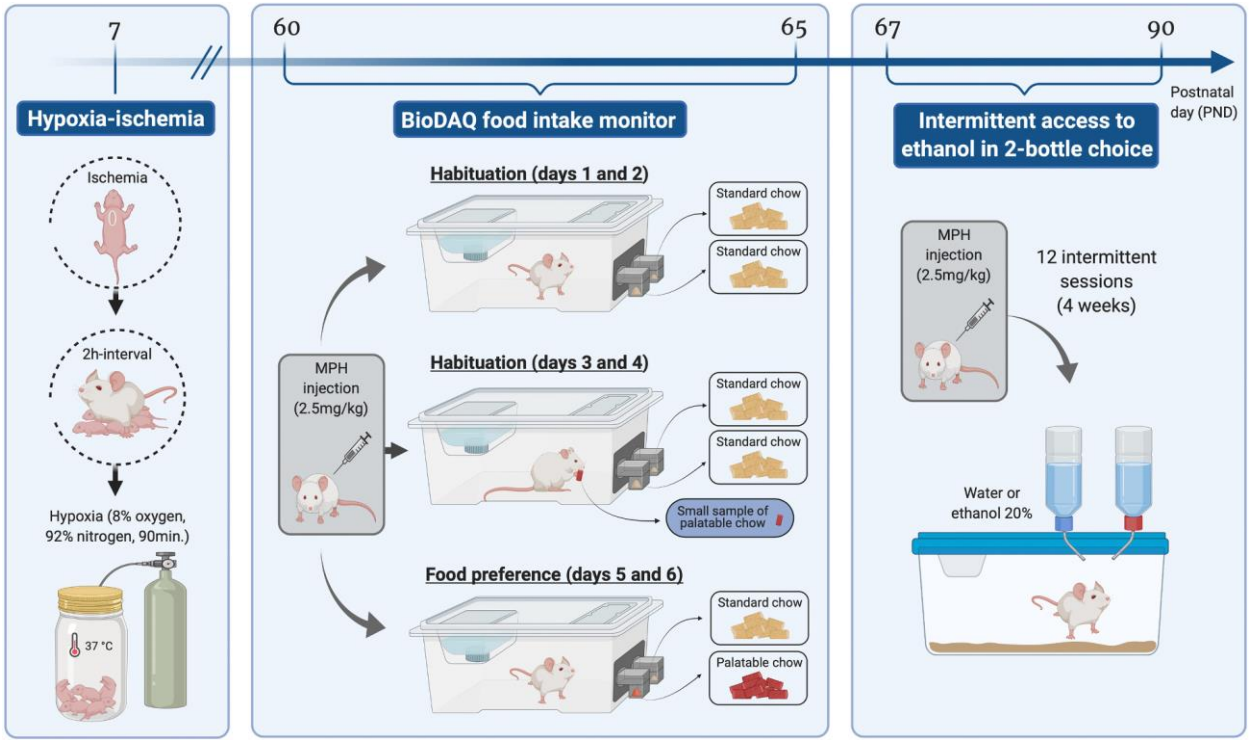


Figure 1: Timeline of experimental procedures. The hypoxia-ischemia (HI) procedure was conducted at postnatal day (PND) 7 and the behavioral analyses between PND 60–90, in which MPH (2.5 mg/kg, i.p.) was administered 30 min prior to each behavioral session. MPH: methylphenidate. *Created with BioRender.com*

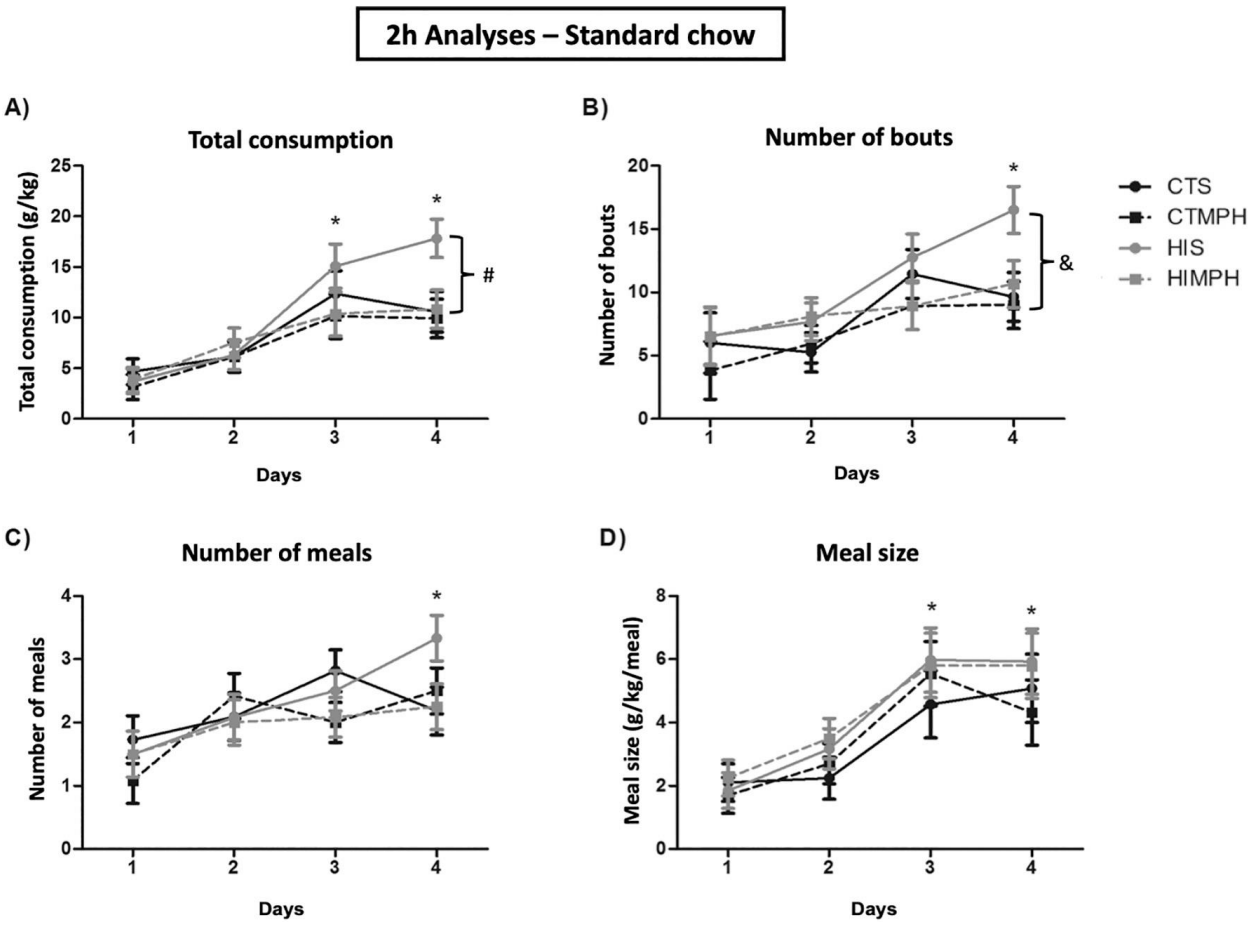
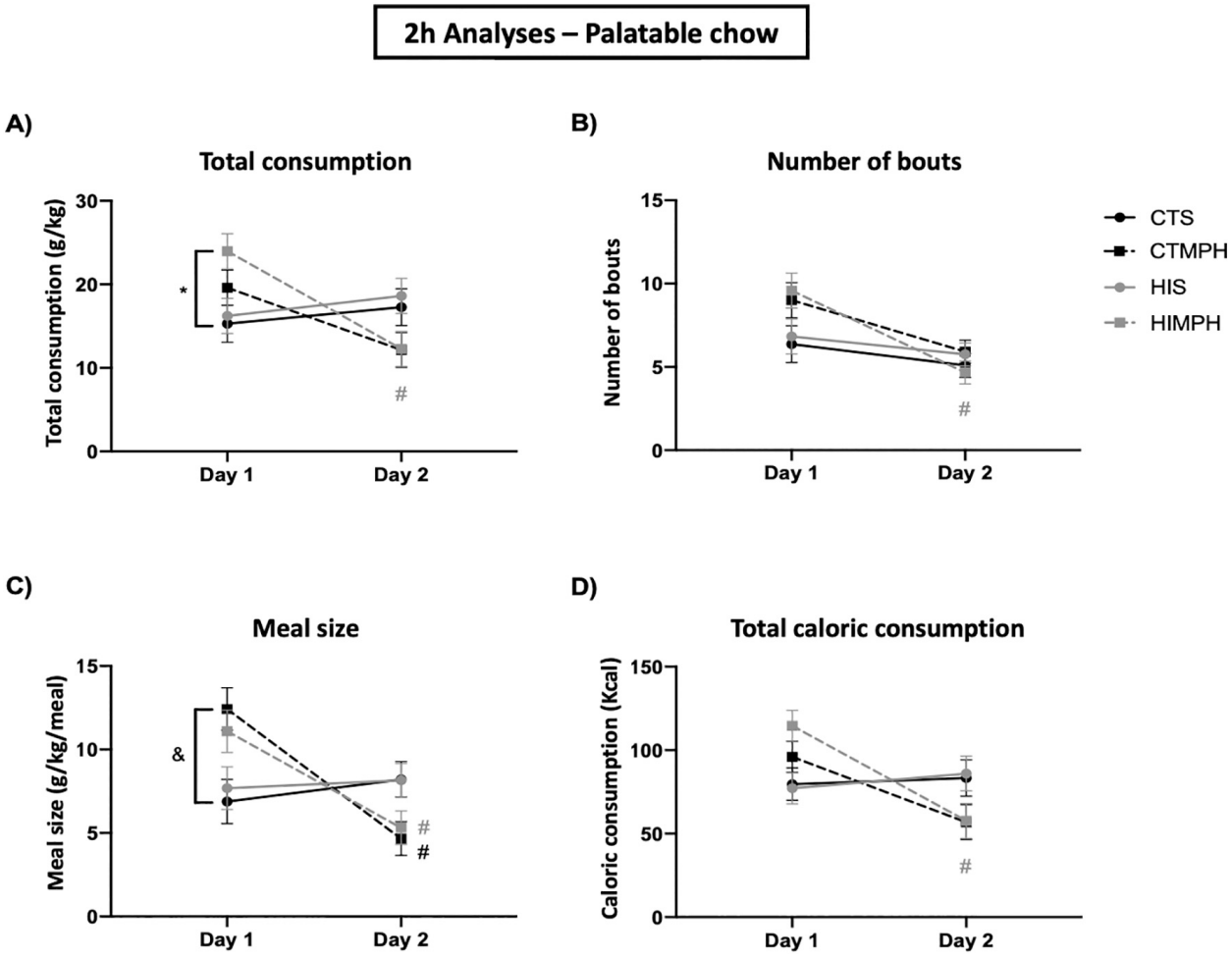


Figure 2. Feeding activity parameters in relation to standard rat chow in sessions of 2h in the first 4 days in the BioDAQ. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA throughout the days and two-way ANOVA within each day, followed by Tukey's post hoc, $p<.05$. *difference in relation to the first day in the HIS group, #HIS different from both CT groups, &HIS different from CTMPH group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.



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565 Figure 3. Feeding activity parameters in relation to the palatable diet in sessions of 2h in 2 days of
566 exposure. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA followed by
567 Tukey's post hoc, $p<.05$. *HIMPH different from the CTS group in the first day, #(gray) difference
568 between days in the HIMPH group, #(black) difference between days in the CTMPH group,
569 &CTMPH different from the CTS in the first day. CTS: control treated with saline; CTMPH:
570 control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH:
571 hypoxia-ischemia treated with methylphenidate. n=11-12/group.

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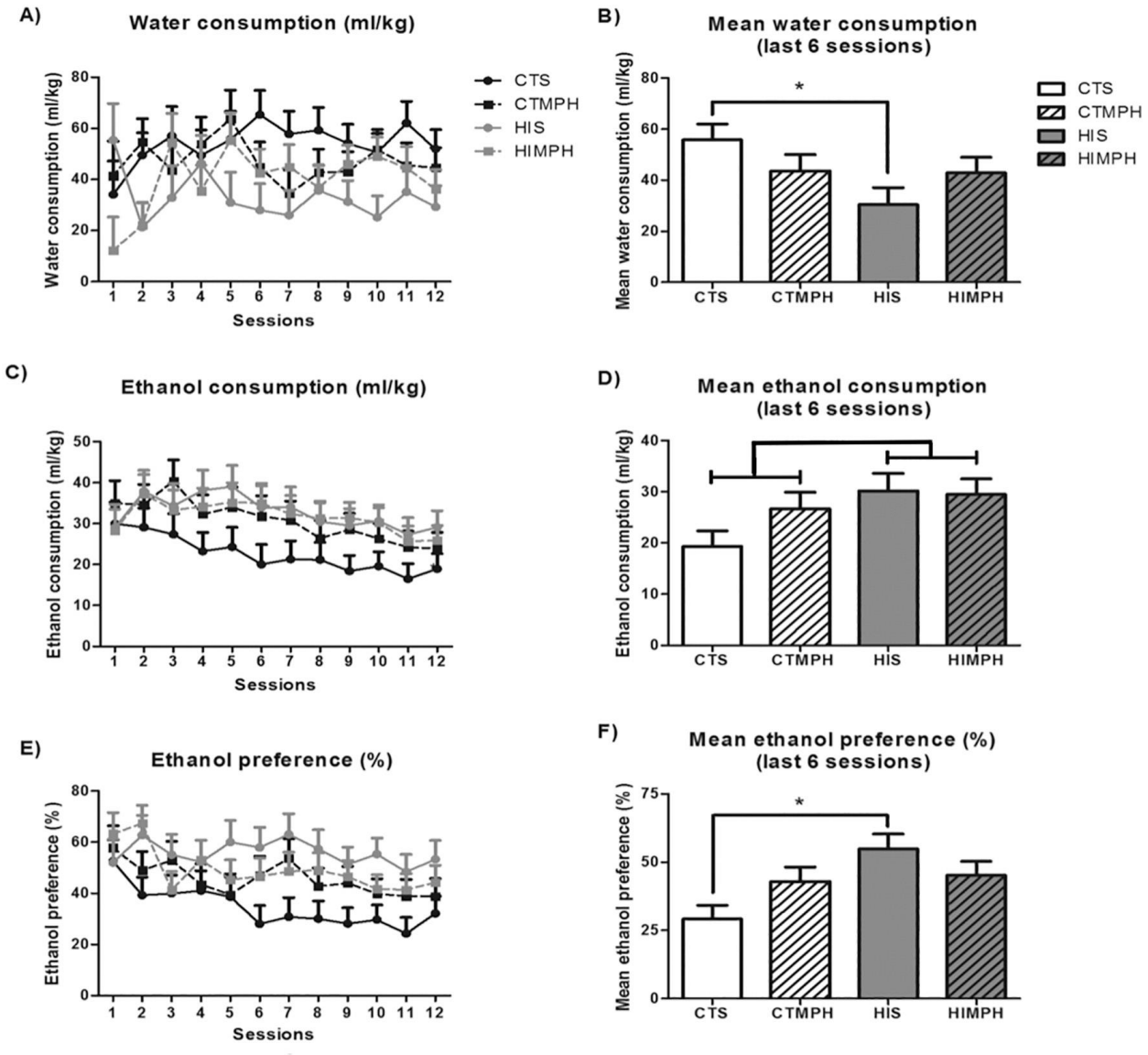

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575 Figure 4. Water and ethanol consumption, as well as the ethanol preference, over the 12 sessions
576 of the IA2BC procedure (A, C, E) or the mean of the last 6 sessions of each measure (B, D, F).
577 Results are expressed as mean ± S.E.M. Repeated-measures ANOVA or two-way ANOVA,
578 followed by Tukey's post hoc, $p<.05$. *HIS different from the CTS group. Lesion effect was
579 observed for mean ethanol consumption. CTS: control treated with saline; CTMPH: control treated
580 with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia
581 treated with methylphenidate. n=10-12/group.

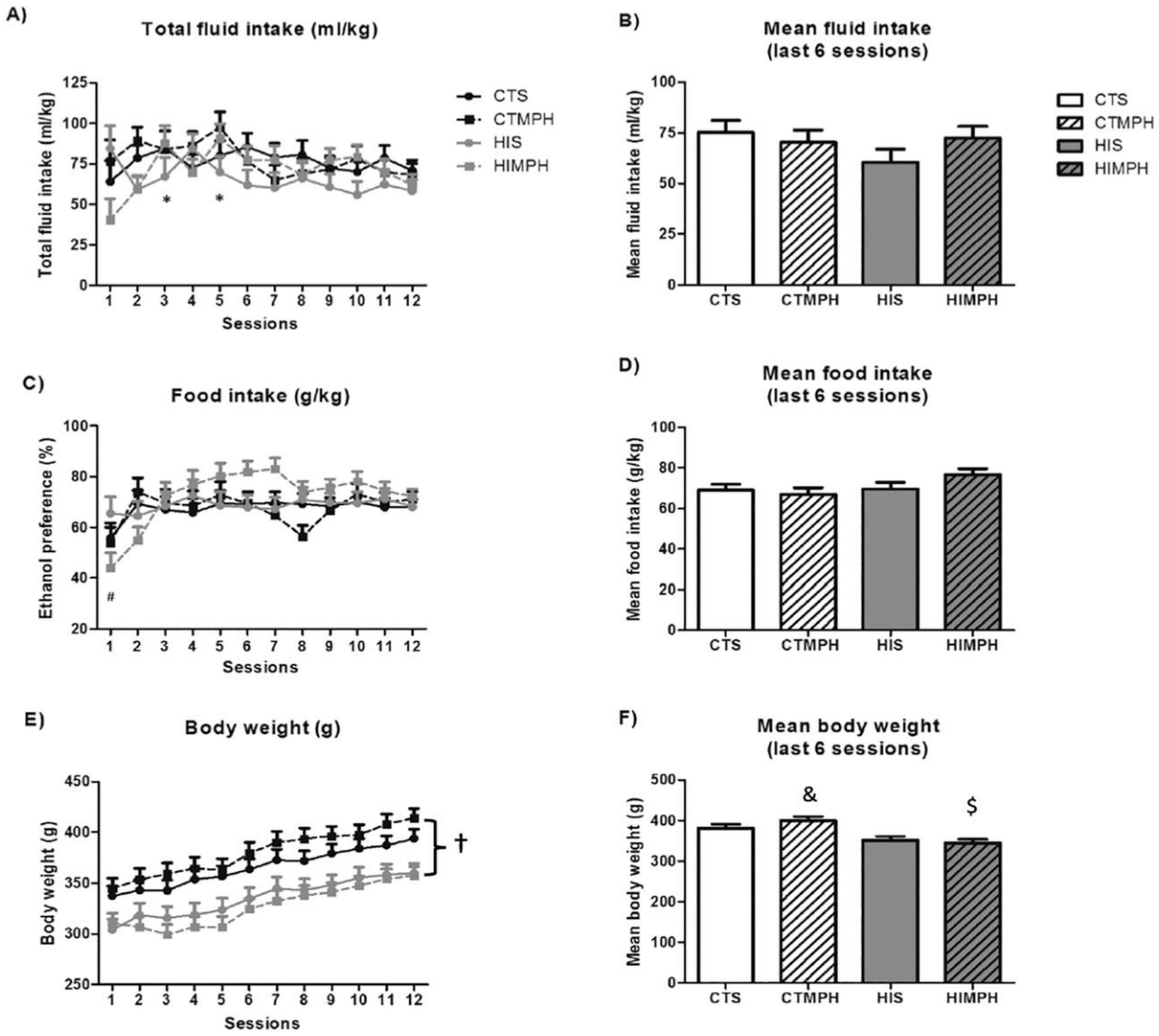

Figure 5. Total fluid and food intake, as well as the body weight measurement, over the 12 sessions of the IA2BC procedure (A, C, E) or the mean of the last 6 sessions of each measure (B, D, F). Results are expressed as mean ± S.E.M. Repeated-measures ANOVA or two-way ANOVA, followed by Tukey's post hoc, $p<.05$. *Difference in relation to the first session, in the CTMPH group; #difference in relation to sessions 3 to 12, in the CTMPH group; †difference between HI and CT animals over the sessions (Lesion effect); &CTMPH different from HIS and HIMPH groups; $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=10-12/group.

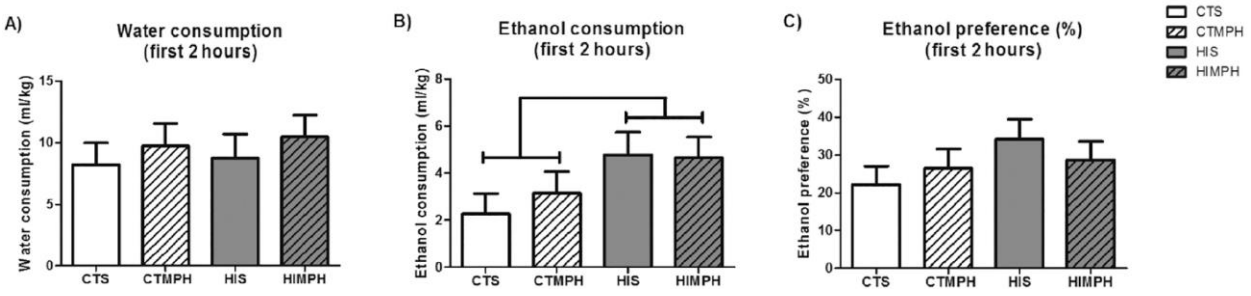

Figure 6. Mean of the water (A) and ethanol consumption (B), as well as the ethanol preference (C) in the first 2 hours after drug administration in the last 3 sessions (sessions 10-12). Results are expressed as mean ± S.E.M. Two-way ANOVA, followed by Tukey's post hoc, $p<.05$. Lesion effect was observed for ethanol consumption. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=10-12/group.

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