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Research Article

RESEARCH ARTICLE

Prenatal Exposure to Methylphenidate Affects the Dopamine System and the Reactivity to Natural Reward in Adulthood in Rats

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Abstract

Background: Methylphenidate (MPH) is a commonly-used medication for the treatment of children with Attention-Deficit/Hyperactivity Disorders (ADHD). However, its prescription to adults with ADHD and narcolepsy raises the question of how the brain is impacted by MPH exposure during pregnancy. The goal of this study was to elucidate the long-term neurobiological consequences of prenatal exposure to MPH using a rat model.
Methods: We focused on the effects of such treatment on the adult dopamine (DA) system and on the reactivity of animals to natural rewards.
Results: This study shows that adult male rats prenatally exposed to MPH display elevated expression of presynaptic DA markers in the DA cell bodies and the striatum. Our results also suggest that MPH-treated animals could exhibit increased tonic DA activity in the mesolimbic pathway, altered signal-to-noise ratio after a pharmacological stimulation, and decreased reactivity to the locomotor effects of cocaine. Finally, we demonstrated that MPH rats display a decreased preference and motivation for sucrose.
Conclusions: This is the first preclinical study reporting long-lasting neurobiological alterations of DA networks as well as alterations in motivational behaviors for natural rewards after a prenatal exposure to MPH. These results raise concerns about the possible neurobiological consequences of MPH treatment during pregnancy.

Keywords: dopamine, methylphenidate, motivation, prenatal, rat, sucrose

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Introduction

Methylphenidate (MPH) is a psychostimulant medication that acts on the dopamine (DA) and noradrenergic neurotransmissions by blocking the DA and norepinephrine transporters (DAT and NET) in the striatum and the prefrontal cortex (Wilens, 2008). It is commonly prescribed for treating children with Attention-Deficit/Hyperactivity Disorders (ADHD; Fone and Nutt, 2005), who are characterized by impairing levels of hyperactivity, impulsivity, and/or inattention (American Psychiatric Association, 2013). ADHD has a worldwide pooled prevalence of ~5% in school-aged children with boys being 3 to 6 times more affected than girls (Polanczyc et al., 2007). About 65% of ADHD cases with childhood onset persist until adulthood, with a pooled prevalence of adult ADHD estimated at ~2.5% (Faraone et al., 2000; Simon et al., 2009), albeit with fewer gender differences (Murphy, 1996; Simon et al., 2009; DeZwaan et al., 2012; Dideriksen et al., 2013). Because MPH has a favorable efficacy/tolerance ratio (Faraone and Buitelaar, 2010), it has been used in children but also in adults with ADHD over the recent years (Banaschewski et al., 2006). In addition, MPH is also prescribed in adults with narcolepsy (Billiard, 2008). Although the prescription of MPH is not recommended during pregnancy, the potential benefits can outweigh the risks in some cases (Dideriksen et al., 2013; Bolea-Alamanac et al., 2014). This raises questions about MPH effects on the early developing brain and stresses the importance of evaluating the long-term consequences of prenatal exposure to MPH on the brain fetus.

Clinical studies, although limited by concurrent use of other psychostimulants, have suggested that such treatment does not increase the risk of congenital malformations (Dideriksen et al., 2013). Few preclinical studies have investigated the long-term behavioral consequences of *in utero* exposure to MPH in murine models and those few have reported decreased anxiety-related behaviors and altered executive functions in adulthood (McFadyen-Leussis et al., 2004; Lloyd et al., 2013). However, the long-term neurobiological modifications underlying these abnormalities are still unknown.

Studies have suggested that MPH may cross the placental barrier, like other amphetamine derivatives (Shah and Yates, 1978; Burchfield et al., 1991; Bolea-Alamanac et al., 2014). Moreover, MPH has a higher affinity for the DAT than for the NET (Gatley et al., 1996), and has been suggested to mainly target DA neurotransmission (Wilens, 2008), which is critical for brain development and plays a role in the adult brain (Levitt et al., 1997). Thus, we investigated whether prenatal exposure to MPH early in the gestation (during the development of the DA system) could impact DA neurobiology in adult animals. To this aim, we used a rat model of prenatal exposure to MPH during the last week of gestation in order to cover the period in which DA neurons are formed and developed in the rat brain (Olson and Seiger, 1972). The neuroanatomical consequences of a prenatal MPH exposure on the DA system were evaluated in the adult animals by quantifying tyrosine hydroxylase (TH) expression, as well as DAT- and DA-receptor density in DA brain regions. The functional consequences of such exposure has also been investigated by measuring the metabolic activity of DA-related areas using micro positron emission tomography (microPET) imaging, and by quantifying the basal function and reactivity of the mesolimbic DA pathway to cocaine stimulations using *in vivo* microdialysis, c-Fos immunohistochemistry, and locomotor activity measurements. Since MPH is a psychostimulant medication, we also examined the behavioral reactivity of these animals to a natural reward using the operant responding for sucrose and sucrose preference paradigms that depend on mesolimbic DA transmission (Hajnal et al., 2004).

Materials and Methods

Animal Model

This study was performed using 48 pregnant Wistar rats (CERJ) that arrived in the laboratory at gestational day (GD) 6 (with an inferred conception at GD0). The pregnant females were individually housed in a room with a 12-hour light/dark cycle (lights on from 07:00 to 19:00 hours) under steady temperature (21 ± 1°C) and humidity (55 ± 5%) with access to food and water *ad libitum*. The animals were treated in accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and with Regional Ethical Committee authorizations.

Each pregnant dam was randomly assigned to the experimental or control group and injected subcutaneously (s.c.) with the D-threo enantiomer of MPH (10 mg/kg/day; Sigma-Aldrich) or saline from GD13 to GD20, because the DAT and NET targeted by MPH appear during the second gestational week in the rat (Herlenius and Lagercrantz, 2004). The s.c. pathway was chosen to minimize the stress and ensure MPH injections to the pregnant females. Only male progeny were selected, and were grouped by a maximum of 3 animals per cage. The experiments were performed at postnatal day 70, considering the litter as the statistical unit, i.e., only one male per litter was used for an experiment. Six additional pregnant Wistar dams were used to assess the pharmacokinetic profile of an acute subcutaneous injection of MPH (10 mg/kg) during the second gestational week. The protocol of this experiment is depicted in the Supplementary Materials.

Scintigraphic Imaging

Metabolic scintigraphic imaging using 2-deoxy-2-(^{18}F)fluoro-d-glucose (^{18}FDG; Cyclopharma Tours) was performed on control and MPH animals in basal conditions (n = 7 and 6, respectively). Because the local uptake of ^{18}FDG reflects the cerebral metabolic rate of glucose (CMRglc), such *in vivo* analysis provides a regional analysis of the brain metabolic status (Sokoloff et al., 1977; Phelps et al., 1979). Each rat was anesthetized using isoflurane (Baxter) and catheterized in the tail vein for ^{18}FDG injection. Each animal was placed on a thermo-regulated bed (Minerve) and centered in the field of view of the Explore VISTA-CT microPET camera (GE Healthcare). A CT-scan was performed for attenuation correction of the PET images and a list-mode PET acquisition of 60 minutes started after the bolus injection of ^{18}FDG (18.5 MBq/100 g) followed by a saline flush. After data reconstruction using a 2-D OSEM algorithm, all the images were coregistered and normalized for tissue activity in the whole brain. Quantitative results were expressed as mean ± standard deviation (SD) and were presented on z-score maps. The values of ^{18}FDG uptake in the regions containing DA cell bodies and projection areas (frontal cortex, dorsal and ventral striatum, amygdala, hippocampus, thalamus, and hypothalamus) were derived from these images using a set of regions of interests (ROI) already defined by Schiffer and colleagues (Schiffer et al., 2006) in PMOD v3.2 software (PMOD Technologies Ltd, Zurich, Switzerland). More details related to image processing and data analysis are available in the Supplementary Materials section.

Microdialysis

DA levels were investigated in control and MPH rats (n = 7 for each group) using *in vivo* microdialysis in the shell part of the nucleus accumbens (NacSh). Based on the number of animals, these experiments were performed under anesthesia to reduce inter-animal variability. Each rat was anesthetized using

isoflurane and placed in a stereotactic apparatus (Stoelting) and its body temperature was maintained at 37.5 ± 1°C throughout the experimentation using a thermostated bench (CMA150, CMA/Microdialysis). A hole was drilled to place a 2 mm microdialysis probe (CMA11, CMA/Microdialysis) at the site of implantation according to the Paxinos and Watson atlas (AP +1.6, ML +0.75, DV -7.6; Paxinos and Watson, 2008). The probe was perfused at a flow rate of 2.2 µL/min with artificial cerebral spinal fluid (Na_2HPO_4 2 mM, NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.2 mM) using a CMA syringe pump (CMA/Microdialysis). DA levels were quantified at basal conditions and after acute cocaine injection (2 mg/kg, i.v.; Sigma-Aldrich) to stimulate DA release. Details related to DA quantification are available in the Supplementary Materials section.

c-Fos Immunohistochemistry

These experiments were performed on control and MPH rats after i.v. saline (n = 8 for controls and n = 5 for MPH rats), or cocaine injections (n = 8 for controls and n = 12 for MPH rats; acute condition; 2 mg/kg i.v.; Sigma-Aldrich). Briefly, each rat was anesthetized (with 4% isoflurane), injected with saline or cocaine, and perfused with heparin-NaCl solution followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline solution 90 minutes after treatments (Graybiel et al., 1990; Brown et al., 1992). Next, the protocol detailed for TH immunohistochemistry was performed using a rabbit polyclonal anti-c-Fos antibody (1:10000; sc-52, Santa Cruz Biotechnology). Details related to data analysis are available in the Supplementary Materials section.

TH Immunohistochemistry

Brain slices obtained for the c-Fos experiments in basal conditions were used for TH quantification (n = 8 and 5 for control and MPH rats, respectively). Free-floating sections were incubated for 24 hours at 4°C with rabbit polyclonal anti-TH antibody (1:500; AB152, Santa Millipore), and for 2 hours at room temperature with a biotinylated goat anti-rabbit IgG antibody (1:400; Vector Laboratories). Tissue sections were further processed using the ABC Vectastain Elite kit (Vector Laboratories) and 3.3'-diaminobenzidine detection. The analyses of TH immunohistochemistry are detailed the Supplementary Materials section.

Autoradiography

The DAT and D2 receptors (D2R) were quantified in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (DAT: n = 6 per group; D2R: n = 5 and 6 for control and MPH rats, respectively) and in the striatum (DAT: n = 6 and 8; D2R: n = 5 and 8 for control and MPH rats, respectively) using [^{125}I]-PE2I (prepared according to Chalon et al., 1999) and [^{3}H]-YM-09151-2 (PerkinElmer), and the D1 receptors (D1R) were quantified in the striatum (n = 5 and 8 for control and MPH rats) using [^{3}H]-SCH-23390 (PerkinElmer) using the methodology previously detailed by Chalon et al. (1999) and Bouchez et al. (2008). For more details, see the Supplementary Materials section.

Cocaine-Induced Locomotor Activity

The locomotor activity of adult control and MPH rats (n = 9 and 8, respectively) was assessed using a circular corridor (50 cm diameter) equipped with a digital camera and was quantified using the Ethovision XT70 Software (Noldus) during 10-minute sessions. All rats were habituated to the apparatus for one week and then tested for their locomotor activity in basal conditions, and 10 minutes after an acute cocaine injection (15 mg/kg i.p.).

Operant Responding for Sucrose

This was performed in control and MPH rats (n = 8 and 7, respectively) to evaluate motivation for sucrose. Animals were food-restricted (15 g/day/rat) and placed in Coulbourn experimental chambers controlled by Graphic State interfaces and software and equipped with 2 levers and a pellet dispenser. First, 10 daily sessions of 30 minutes each were conducted using a fixed ratio 1 (FR1) schedule of reinforcement. One press on the active lever resulted in the delivery of a sucrose pellet followed by a 5-second refractory period and a 10-second time-out period. Next, rats were tested under a progressive ratio (PR) schedule for 4 daily sessions (Solinas and Goldberg, 2005). Each session lasted 180 minutes, or ended after 30 minutes if the rat did not activate the lever.

Sucrose Preference

Control and MPH rats were also tested for their preference for sucrose (n = 6 for both control and MPH rats). The week before each experiment, rats were housed individually and handled daily, with access to a 2% sucrose solution (Sigma) in 10 mL pipettes during 1 hour for 5 consecutive days, 1.5 hour after the lights turned off and 1.5 hour before the lights turned on. The preference between sucrose and water was assessed during 30 minutes for 3 consecutive days at the beginning and at the end of the dark cycle. The position of the pipettes was alternated at each session to prevent position-biased drinking. Results were expressed as percentages of preference for sucrose: (sucrose intake/total intake) x 100.

Pharmacokinetic Experiments

Pharmacokinetic experiments were conducted on 6 pregnant rats exposed to an acute s.c. injection of D-threo-MPH (10 mg/kg). Pregnant dams were anesthesized using isoflurane and a catheter was inserted into the femoral artery for blood sampling. Blood samples of approximately 50 µL were collected just before and 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min after MPH injection. Each blood sample was immediately centrifuged to harvest the plasma, which was then frozen at -80°C.

Analyses of plasma samples were performed on a UPLC Ultimate® 3000 system (Dionex), coupled to a Q-Exactive Mass Spectrometer (Thermo Scientific), operated in the positive electrospray ionization mode. For the detailed methodology, see the Supplementary Materials section.

Statistical Analyses

The statistical analysis of MicroPET data is detailed in the Supplementary Material section. The effects of prenatal treatment, cocaine injections, and their interactions on DA release in the NacSh were assessed using a two-way ANOVA in addition to a one-way ANOVA with Dunnett's adjustment for intra-group effects of cocaine injections. In addition, the comparison of basal DA levels in control and MPH rats was performed using an bilateral unpaired t-test. Intergroup comparisons between averaged control and MPH values of TH/c-Fos expression, DAT, D2R and D1R densities were also performed using a bilateral unpaired t-test. Intragroup comparisons to evaluate the effects of cocaine injections on c-Fos expression were examine using a bilateral paired t-test. A two-way ANOVA was performed to examine the effects of prenatal treatment, cocaine injections, and their interactions on the locomotor effects of cocaine. In addition, unpaired and paired *t*-tests were

performed to assess the intergroup differences in locomotor activity in basal conditions, and the intragroup effect of cocaine, respectively. A two-way ANOVA was used to assess the effects of prenatal treatment, cocaine injections, and their interactions on sucrose consumption during operant responding.

Results

Prenatal MPH Treatment Alters the Expression of TH, DAT, D1R and D2R in Adulthood

Using TH immunohistochemistry, we observed that adult rats prenatally treated with MPH displayed an increased number of tyrosine hydroxylase positive (TH+) cells in the DA cell bodies that was only significant in the SNc [$t(10) = 3.04$, $p = 0.012$ and $t(10) = 1.99$, $p = 0.075$ in the SNc and VTA, respectively; Figure 1A]. In addition, increased TH expression was detected in terminal DA regions such as the striatum and in the Nac [$t(11) = 2.76$, $p = 0.019$ and $t(11) = 3.50$, $p = 0.005$ in the medio and latero-dorsal striatum, respectively; $t(11) = 5.36$, $p < 0.001$ and $t(11) = 4.90$, $p < 0.001$ in the shell and core parts of the Nac, respectively; Figure 1B].

The autoradiographic data showed that MPH and control rats had similar DAT densities in the SNc/VTA [$t(10) = 0.50$, $p = 0.627$ and $t(10) = 1.11$, $p = 0.295$ in the SNc and VTA, respectively], and that MPH rats had higher DAT density in the striatum compared to controls [$t(12) = 3.49$, $p = 0.045$ and $t(12) = 3{,}94$, $p = 0.002$ in the dorsal and ventral striatum, respectively; Figure 2A]. MPH rats also displayed elevated D2R density in the SNc/VTA compared to controls [$t(9) = 2.73$, $p = 0.023$ and $t(9) = 2.34$, $p = 0.044$ in the SNc and VTA, respectively], and similar density in the striatum [$t(11) = 1.81$, $p = 0.097$ and $t(11) = 0.55$, $p = 0.590$ in the dorsal and ventral striatum, respectively; Figure 2B]. Prenatal MPH treatment did not led to modifications in the D1R density in the striatum [$t(11) = 0.48$, $p = 0.644$ and $t(11) = 0.70$, $p = 0.499$ for the dorsal and ventral striatum, respectively; Figure 2C].

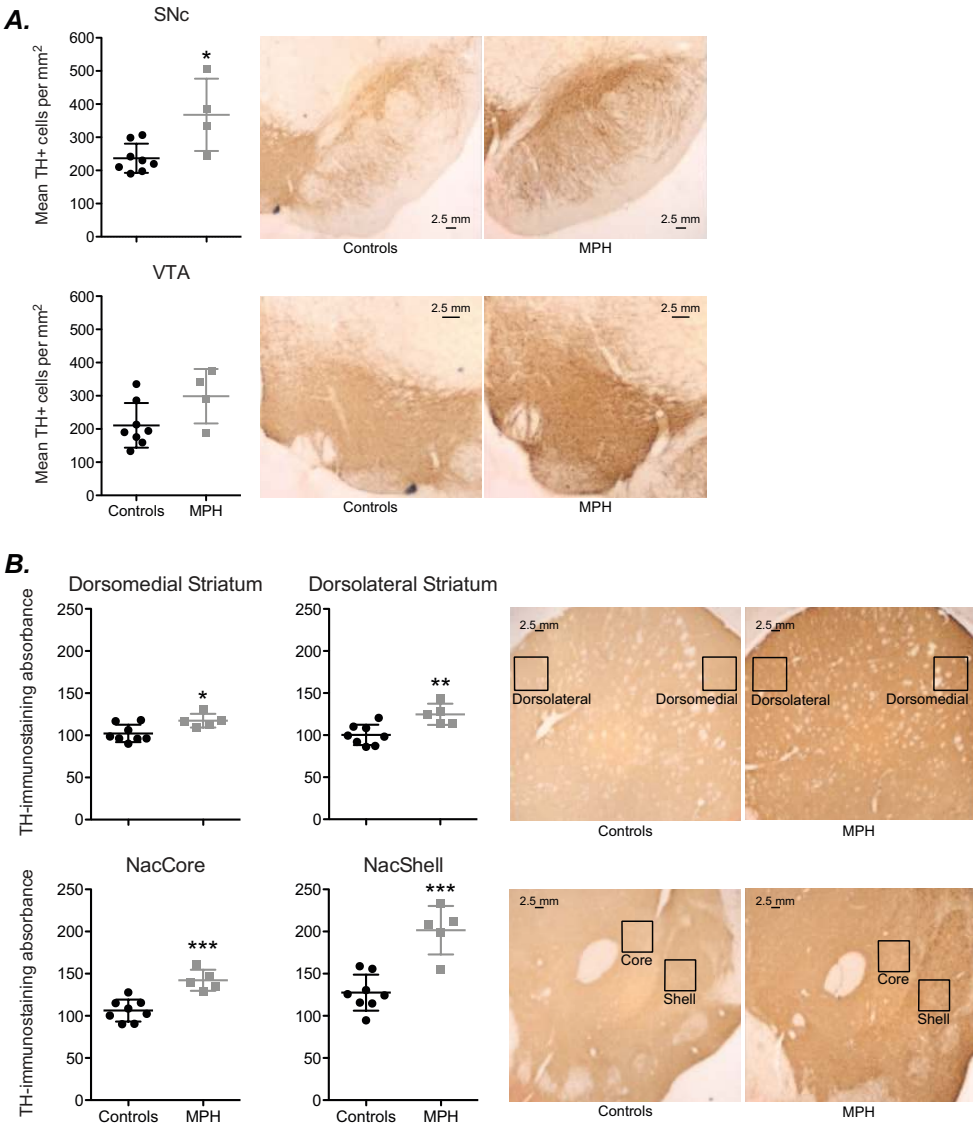

Figure 1. Quantification of tyrosine hydroxylase positive (TH+) cells in the dopamine cell bodies and tyrosine hydroxylase (TH) immunostaining in the striatum. (A) Mean number of TH+ cells/mm^2 ± standard deviation (SD) in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (n = 8 and 4 for control and methylphenidate [MPH] rats, respectively), and representative examples of immunostaining. (B) Mean absorbance of TH immunostaining ± SD in the dorsomedial striatum, dorsolateral striatum, core, and shell of the nucleus accumbens (n = 8 and 5 for control and MPH rats, respectively), and representative examples of immunostaining. Statistical analyses were performed using a bilateral unpaired *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

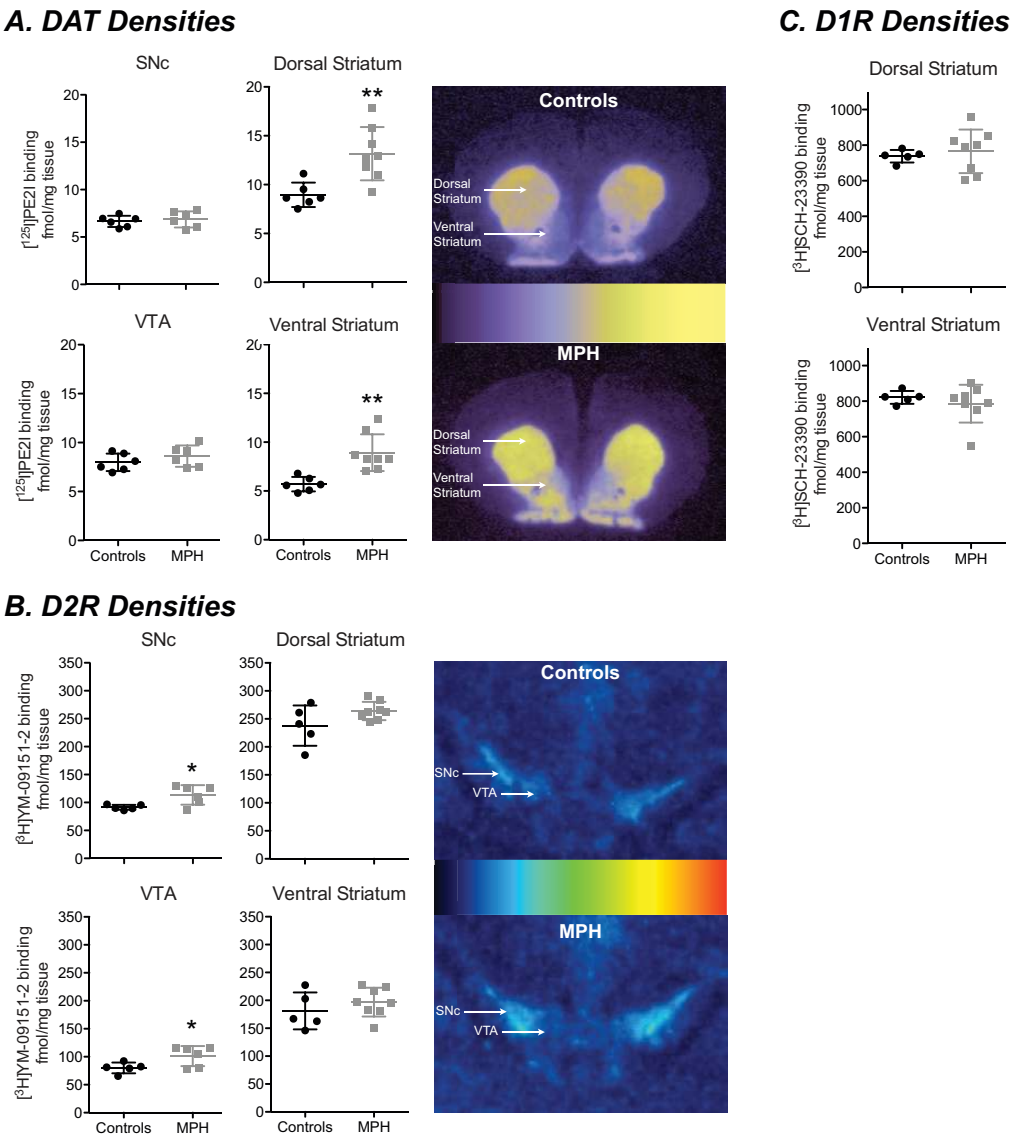

Figure 2. Quantification of the dopamine transporters (DAT), D1 receptors (D1R), and D2 receptors (D2R) in the dopamine cell bodies and striatum and striatum. (A) DAT density in the substantia nigra pars compacta (SNc), ventral tegmental area (VTA), dorsal striatum, and ventral striatum were quantified using [^{125}I]-PE2I. The averaged [^{125}I]-PE2I binding in each area is presented for control (n = 6) and methylphenidate (MPH) rats (n = 6 and 8 in the SNc, VTA and striatum, respectively), in addition to representative autoradiograms. (B) D2R density in the SNc, VTA, dorsal striatum, and ventral striatum quantitated via [^{3}H]-YM-09151-2 binding. The averaged [^{3}H]-YM-09151-2 binding in each area is presented for control (n = 5) and MPH rats (n = 6 and 8 in the SNc, VTA and striatum, respectively), in addition to representative autoradiograms. (C) D1R density in the dorsal striatum and ventral striatum quantitated via [^{3}H]-SCH-23390 binding. The averaged [^{3}H]-SCH-23390 binding in each area is presented for control (n = 5) and MPH rats (n = 8). Statistical analyses were performed using a bilateral unpaired *t*-test (*$p < 0.05$; **$p < 0.01$).

MPH-Treated Rats Show Metabolic Abnormalities in DA Regions

Figure 3 shows that MPH rats displayed increased ^{18}FDG uptake in an area containing the SN/VTA [from -4.68 to -6.36 mm from bregma; $t(11) = 4.10$, $p < 0.001$ and $t(11) = 3.40$, $p = 0.001$ in the left and right hemispheres, respectively] where DA neurons are located. MPH rats also showed a decreased ^{18}FDG uptake in DA projection areas, including the right cingulate cortex [from 3.72 to 2.76 mm from bregma: $t(11) = -3.70$, $p = 0.002$], the striatum [centro-dorsal part from 2.52 to -1.56 mm from bregma: $t(11) = -3.40$, $p = 0.004$ and $t(11) = -5.60$, $p < 0.001$ in the left and right hemispheres, respectively; right ventral part from 2.52 to -1.28 mm from bregma: $t(11) = -4.70$; $p < 0.001$], and the thalamus [from -1.08 to -4.68 mm from bregma: $t(11) = -4.40$, $p = 0.002$], with the exception of the left hypothalamus, in which we detected an increased uptake [lateral part from -2.16 to -4.20 mm from bregma: $t(11) = 3.60$, $p = 0.003$]. No difference in ^{18}FDG uptake was observed in other DA regions.

Prenatal MPH Exposure Leads to Modifications in DA Levels and c-Fos Expression in the Shell of the Nucleus Accumbens

As shown in Figure 4A, MPH-treated rats displayed higher basal extracellular DA concentrations than controls [$t(12) = 7.42$, $p < 0.001$] in the NacSh. Besides, cocaine injection and the prenatal treatment had an effect on the percentages of basal DA in control and MPH rats [cocaine effect: $F(10,120) = 73.38$, $p < 0.001$; prenatal treatment effect: $F(1,12) = 4.87$, $p = 0.012$; Figure 4B]. Indeed, the maximum percentage of basal DA after the cocaine injection in the NacSh was significantly lower in MPH-treated rats than in controls [$t(12) = 4.42$, $p < 0.001$].

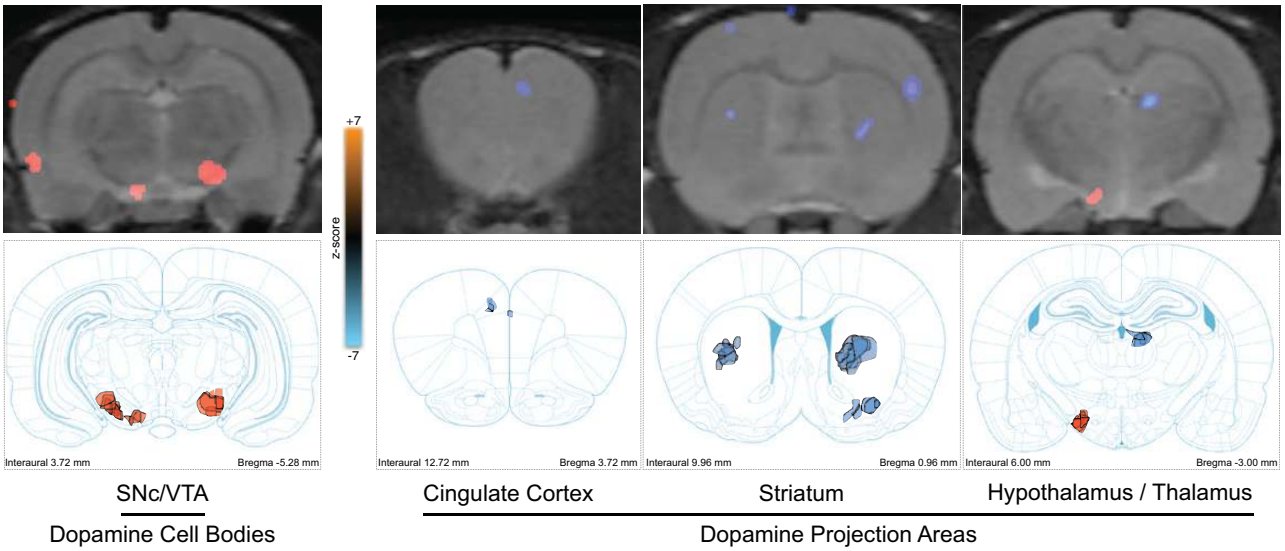

Figure 3. Representation of the modifications in 2-deoxy-2-(^{18}F)fluoro-d-glucose (^{18}FDG) uptake reflecting brain metabolic activity in the dopamine (DA) brain regions of adult male rats prenatally exposed to methylphenidate (MPH) vs. controls in basal conditions (n = 6 and 7, respectively). For each region, images of the generated z-score maps fused with an MRI template are presented (upper part), as well as a summary of the antero-posterior significant modifications on representative coronal plates of the Paxinos and Watson atlas (lower part; increases and decreases in ^{18}FDG uptake in red and blue, respectively; student's two-tailed t-test; $p < 0.01$).

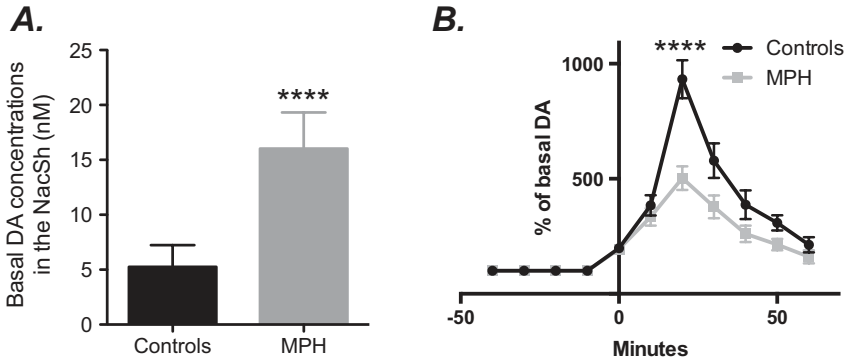

Figure 4. Quantitation of extracellular dopamine (DA) in the shell of the nucleus accumbens (NacSh) of anesthetized adult male rats prenatally exposed to methylphenidate (MPH) and controls in basal and stimulated conditions. (A) Mean ± standard deviation (SD) DA concentrations in basal conditions in MPH and control animals (n = 7 each). (B) Temporal variations of the mean percentages of basal DA ± SD in MPH and control animals before and after acute cocaine injection (2 mg/kg; i.v.). Inter-group comparisons of DA basal levels were performed using a bilateral unpaired t-test (*$p < 0.0001$), and a two-way analysis of variance (ANOVA) was performed to assess the effects of prenatal treatment, cocaine injections, and their interactions.

In addition, Figure 5 shows that a prenatal MPH treatment led to an elevated number of c-Fos+ cells in the NacSh in adulthood in basal conditions [$t(9) = 6.70$, $p < 0.001$]. Moreover, while an acute cocaine injection increased c-Fos expression in the NacSh of control rats [$t(10) = 5.47$, $p < 0.001$], no further c-Fos increase was observed in MPH rats in this condition [$t(13) = 0.02$, $p = 0.982$].

Prenatal MPH Exposure Results in Long-Lasting Impairments in Behavioral Sensitivity to Cocaine

As shown in Figure 6, similar locomotor activities are observed in basal conditions for control and MPH rats [$t(15) = 1.02$, $p = 0.323$]. Statistical analyses revealed an effect of cocaine injection on the locomotor activity [$F(1,15) = 20.53$, $p < 0.001$] but also highlighted an effect of the prenatal treatment [$F(1,15) = 6.94$, $p = 0.019$]. In fact, adult rats prenatally exposed to saline showed significantly increased locomotor activities after an acute cocaine injection [$t(8) = 4.74$, $p = 0.002$], while rats prenatally exposed to MPH did not [$t(7) = 1.79$, $p = 0.116$].

MPH Rats Showed No Preference and Less Motivation for Sucrose

For sucrose reinforcement experiments, both MPH and control rats learned the task leading to a similar amount of sucrose pellets being retrieved at the last session under an FR1 schedule [Figure 7A; prenatal treatment effect: $F(1,12) = 2.07$, $p = 0.176$; session effect: $F(9,108) = 111.98$, $p < 0.001$; interaction: $F(9,108) = 2.38$, $p = 0.017$], even though MPH rats acquired the self-administration behavior more quickly than controls [last significant difference between sessions 4 and 5 for MPH rats: $F(9) = 46.35$, $p < 0.001$; last significant difference between sessions 6 and 7 for control rats: $F(9) = 25.75$, $p < 0.001$]. However, the last ratio completed by MPH rats under a PR schedule was significantly lower than that of control rats

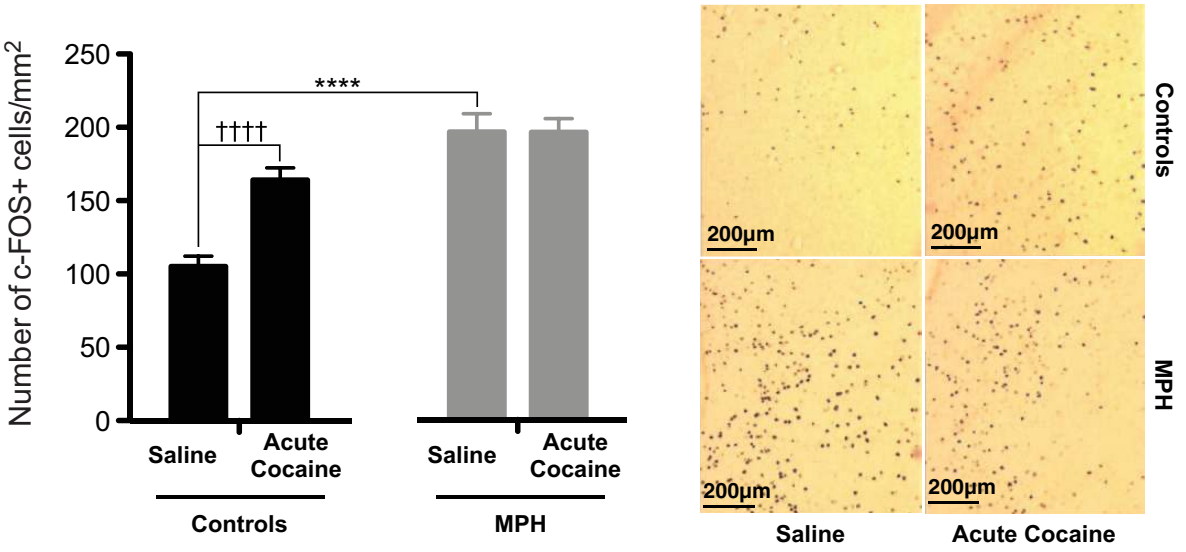

Figure 5. Quantitation of c-Fos expression in the shell of the nucleus accumbens (NacSh) of anesthetized adult male rats prenatally exposed to methylphenidate (MPH) and controls in basal and stimulated conditions. The number of c-Fos immunopositive cells per mm² in the NacSh in control and MPH animals after a saline or acute cocaine injection is presented (mean ± standard deviation; n = 8 for controls in each condition; n = 5 and 12 for MPH animals in the saline and acute cocaine conditions, respectively), in addition to representative examples of immunostaining. Inter-group comparisons were performed using a bilateral unpaired *t*-test (**p* < 0.0001), and intra-group comparisons were performed using a bilateral paired *t*-test (†*p* < 0.0001).

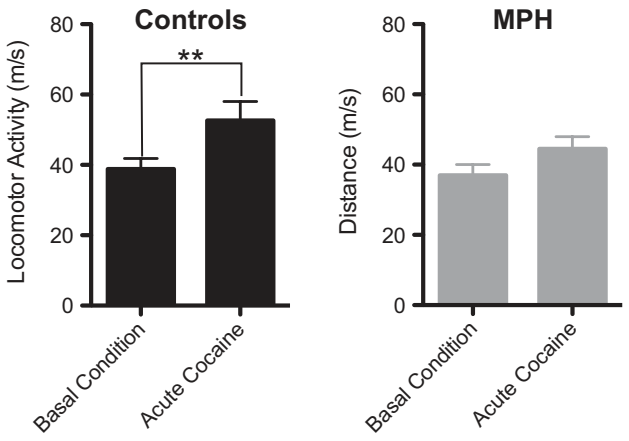

Figure 6. Quantitation of the locomotor activity of adult methylphenidate (MPH) and control rats in basal conditions and after acute or repeated cocaine injection(s) (15 mg/kg i.p.). The mean locomotor activity (meter per 10 minutes) ± standard error of the mean is presented for each group (n = 9 and 8 for control and MPH rats, respectively) in each condition. A two-way analysis of variance was performed to assess the effects of prenatal treatment, conditions, and their interactions; intra-group comparisons were performed using one-way ANOVA followed by Dunnett's post hoc analysis (**p* < 0.01).

[Figure 7B; $t(40) = 2.11$, $p = 0.041$]. No significant differences were observed between initial and final weights of MPH or control rats during these experiments [$t(12) = 1.84$, $p = 0.0911$ for initial weights; $t(12) = 1.25$, $p = 0.235$ for final weights; Figure S1].

For sucrose preference experiments, control rats exhibited a strong preference for sucrose over water, while MPH rats showed no preference [Figure 7C; $t(10) = 13.79$, $p < 0.001$]. No differences in total amount of liquid ingested were observed between the groups at each session [Figure 7D; prenatal treatment effect: $F(1,8) = 0.18$, $p = 0.681$; inter-session effect: $F(5,40) = 1.40$, $p = 0.247$].

MPH Pharmacokinetic Profile After an Acute s.c. Injection

Figure S2 shows that an acute s.c. injection of MPH in pregnant dams led to a maximum MPH plasma concentration (308 ± 68 ng/mL) 2 hours post-injection. In addition, MPH plasma levels only slowly decreased 4 hours post injection (240 ± 42 ng/mL).

Discussion

In this study, we found that adult rats prenatally exposed to MPH displayed abnormalities in the DA system, an altered reactivity to cocaine injection at the neurochemical and behavioral levels, and decreased preference and motivation for a natural reward such as sucrose. Altogether, these results suggest that *in utero* exposure to MPH may alter normal brain development and may result in long-lasting malfunctioning of the reward system.

At the presynaptic level, adult rats prenatally exposed to MPH exhibited an increased number of TH+ cells in the SN/VTA and increased TH expression in the striatum. This could be explained by the fact that these animals have an elevated number of DA neurons or that more neurons reach the detection threshold for TH. Moreover, an increased metabolic activity was observed in the SN/VTA. Although the spatial resolution of microPET imaging is limited and cannot differentiate the SN compacta from the reticulata containing DA and GABAergic neurons, respectively, these results could be due to an elevated DA activity in adult rats prenatally exposed to MPH. Increased basal levels of extracellular DA were detected by *in vivo* microdialysis in the NacSh of MPH rats. Because we did not use the no-net-flux method for DA quantification, the observed basal DA concentrations are confounded by modifications of probe efficiency between *in vitro* calibration and *in vivo* experiments. However, basal DA concentrations of control rats were in agreement with those previously reported in the NacSh (Frank et al., 2008). The theory of microdialysis suggests that a higher DA reuptake increases probe efficiency (Chefer et al., 2009). As MPH

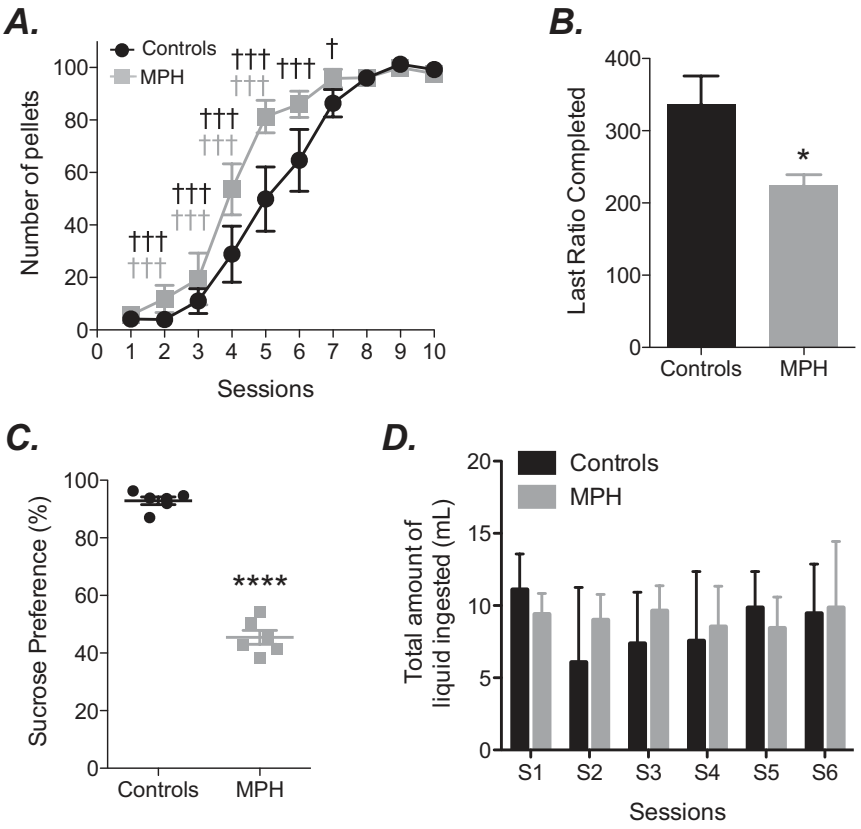

Figure 7 . (A) Changes in the mean ± standard error of the mean (SEM) number of sucrose pellets retrieved by methylphenidate (MPH) rats and controls (n = 7 and 8 for MPH and control animals, respectively) during a 2-hour fixed ratio 1 (FR1) schedule lasting 10 days. (B) Mean ± SEM break point defined as the last ratio completed for sucrose intake under a progressive ratio (PR) schedule of 4 days for MPH rats and controls (n = 7 and 8 for MPH and control rats, respectively). (C) Sucrose preference in adult male rats prenatally exposed to MPH and in controls (n = 6 for both groups). (D) Total amount of liquid ingested at each session of sucrose preference for controls and MPH rats. Statistical analyses were performed using a bilateral unpaired *t*-test for inter-group comparisons (*$p < 0.05$; **$p < 0.0001$) and one-way analysis of variance followed by Dunnett's post hoc analysis to compare the number of sucrose pellets retrieved at each session to that obtained at the last session (†$p < 0.05$; ††$p < 0.001$).

rats displayed higher DAT density, their basal DA levels should be underestimated in our study, further supporting an enhanced basal DA transmission in these animals. DATs in the striatum are exclusively localized in the axonal membranes of the DA neurons (Maiya and Mayfield, 2004) and the D2R in the SN/VTA are mainly localized in the soma and dendrites (Meador-Woodruff et al., 1989). Such elevations in these presynaptic DA markers could be due to an increased number in DA neurons, leading to a hyperDA function in MPH rats as suggested by the microPET results.

On the postsynaptic side, adult rats prenatally exposed to MPH exhibited decreased metabolic activity in the striatum with no modifications in the density of DA receptors. This could be due to an elevated post-synaptic D2 neurotransmission, as an increased D2 neurotransmission has been shown to reduce the glutamatergic activation of the striatopallidal medium spiny neurons (Surmeier et al., 2007). Tonic DA is thought to modulate D2 neurotransmission (Grace et al., 2007) due its higher affinity for D2R than for D1R (Maeno, 1982; Richfield et al., 1989), thus these data further support an increased tonic DA activity in adult rats prenatally exposed to MPH. In addition, an increased basal expression of c-Fos was observed in the NacSh of these animals. Although this may appear contradictory to the decreased metabolic activity already described, studies already show opposite results between c-Fos immunohistochemistry and ^{14}C-2-deoxyglucose experiments (Cochran et al., 2002). In addition, several studies already suggest that c-Fos expression reflects more a genomic response at the cell body level than an index of neuronal activity (Cirelli and Tononi, 2000; Gozzi et al., 2012). Moreover, D2R stimulation by selective agonists has been reported to increase c-Fos expression in the Nac (Yamada et al., 2007). Thus, the elevated basal expression of c-Fos detected in the NacSh could be a consequence of a hyperactive D2 neurotransmission in the MPH rats.

We further explored the impact of these post-synaptic adaptations on the functionality of the mesolimbic pathway by investigating the effects of DA stimulation, induced by cocaine, on the extracellular DA levels and c-Fos expression in the NacSh of these animals. Based on the hyperactivity of the DA neurons and on the increased DAT density in the striatum, we expected that cocaine stimulation would have increased effects in these rats. Interestingly, acute cocaine injection induced a lower elevation of the percentages of basal DA levels in MPH rats than in controls. This can be attributed to an increase in basal levels of DA in these animals and can be interpreted as a decreased signal-to-noise ratio of the mesolimbic DA transmission in response to external stimulations. Moreover, c-Fos expression in the NacSh was not further increased by the cocaine injection in MPH rats, contrary to what was observed in control animals. This lack of c-Fos reactivity also suggests an impacted signal-to-noise ratio of the DA transmission. Thus, these pre- and post-synaptic molecular adaptations may lead to decreased behavioral reactivity of these animals to DA stimulations. This was further supported by the lack of increased locomotor activity induced by a

cocaine injection in the adult rats prenatally exposed to MPH, in sharp contrast to what was observed in controls. As no modifications in motor development have been observed in several studies dealing with prenatal MPH exposure (McFadyen-Leussis et al., 2004; Panos et al., 2014), we assume that our behavioral data are not confounded by such developmental alterations.

The NacSh is critical for reward-related information processing (Di Chiara, 2002) and accumbal DA is clearly involved in reward-related and motivational tasks (Salamone and Correa, 2012). Therefore, we hypothesized that the neurobiological alterations previously described in MPH rats would lead to behavioral modifications in reward processes. Consistent with this hypothesis, MPH rats showed no preference for sucrose over water and had a decreased motivation to obtain sucrose pellets in a progressive-ratio operant procedure. These results were not confounded by weight modifications during this experiment between MPH and control rats, suggesting that the reactivity to natural rewards was dampened in these animals. Thus, the similar rate of response to sucrose pellets observed during the FR1 schedule for both groups may seem surprising; however, the operant response for sucrose requires food restriction to drive behavior while the sucrose preference protocol does not. Interestingly, MPH rats show higher levels of visits to the food trough in the first five days of the food reinforcement experiment than control rats (data not shown) without showing differences in the number of reinforcements obtained, which are indeed very low. Therefore, this effect cannot be explained by consumption behavior, and is more likely to be due to an increased exploratory behavior and possibly reduced anxiety, as already suggested in adult mice prenatally exposed to MPH (McFadyen-Leussis et al., 2004). Overall, these data show a lower neurobiological response to cocaine in adult rats prenatally exposed to MPH and a decreased motivation for natural rewards, as already described in studies which reported the consequences of adolescent exposure to MPH (Bolanos et al., 2003; Carlezon et al., 2003).

Interestingly, prenatal exposure to methamphetamine in rats has been shown to induce a tolerance to the effects of cocaine in adult rats (Šlamberová et al., 2012). Similarly to our results, these animals display increased basal DA levels in the Nac (Bubenikova-Valesova et al., 2009). In contrast, prenatal exposure to cocaine has not been shown to alter DA basal levels and seems to enhance the rewarding potency of cocaine in adulthood (Heyser et al., 1992; Keller et al., 1996; Lin and Kellogg, 1996; Rocha et al., 2002; Estelles et al., 2006; Malanga et al., 2007 2009). MPH is often related to cocaine in terms of mechanism of action, but it has a greater affinity for the DAT and NET than for the serotonin transporter when compared to other amphetamine derivatives, while cocaine has similar affinities for the monoamine transporters (Han and Gu, 2006). Based on the critical role of serotonin in brain development, such distinct pharmacological profiles could underlie the different results observed in these animal models of prenatal exposure to psychostimulants.

Our primary aim was to investigate how early developmental disruption of the DA neurotransmission could impact adult brain functions. Therefore, we injected a high non-toxic dose of D-threo-MPH, as L-threo-MPH has been reported to be ineffective (Ding et al., 1997). This was performed during the second week of gestation in rats, the time at which DAT starts to be expressed during rat brain development (Olson and Seiger, 1972; Levitt et al., 1997; Herlenius and Lagercrantz, 2004). From a clinical perspective, this developmental period roughly corresponds to most of the first trimester in humans (Rice and Barone, 2000). Thus, this protocol is expected to model an early prenatal exposure to MPH. The dose of MPH used in our experiments was expected to lead to higher maximum plasma levels of MPH than those observed in MPH-treated humans (Swanson and Volkow, 2003; Spencer et al., 2006; Hysek et al., 2014), as confirmed by our pharmacokinetic data. Thus, this can be viewed as a limitation for the extrapolation of our results to clinical data. Gerasimov and colleagues (2000) suggested that MPH administered per os (p.o.) at the dose of 5 mg/kg or i.p. at the dose of 2 mg/kg should model a clinical MPH treatment. However, it has been shown to lead to higher plasma peak levels (Kuczenski and Segal, 2002) that occur roughly 30 min after administration, and exert a short half-life in contrast to what is observed in humans (Wargin et al., 1983; Patrick et al., 1984; Aoyama et al., 1990; Bakhtiar and Tse, 2004). To overcome these pharmacokinetic differences, other studies used multiple daily MPH treatments (Komatsu et al., 2012; Panos et al., 2014). Interestingly, the pharmacokinetic profile we obtained after an acute s.c. MPH injection seems to be comparable to what is observed in humans (Swanson and Volkow, 2003; Spencer et al., 2006; Hysek et al., 2014), and it would be interesting to further explore this route of administration for the modeling of clinical MPH administration in rats. As suggested by others, modeling clinical MPH treatments in animals is difficult and cannot only rely on the maximum plasma levels (Kuczenski and Segal, 2002).

This study is the first reporting neurobiological alterations in adult male rats prenatally exposed to MPH. In addition to previous reports suggesting decreased anxiety and altered executive functions after such treatments (McFadyen-Leussis et al., 2004; Lloyd et al., 2013), we found that adult male rats prenatally exposed to MPH show alterations in the mesolimbic DA system that result in alterations in the reactivity to natural and pharmacological rewards. Although the extrapolation of these preclinical data to clinical conditions is limited, this work suggests that prenatal MPH exposures could represent a long-term and maybe permanent risk for the future child.

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

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Statement of Interest

Drs Galineau and Castelnau currently receive research support from Shire. In the past year, Dr Faraone received consulting income and/or research support from Shire, Akili Interactive Labs, and Alcobra, and research support from the National Institutes of Health (NIH). The Medical Genetics Research Center and Department of Psychiatry (Dr Faraone's institution) is seeking a patent for the use of sodium-hydrogen exchange inhibitors in the treatment of ADHD. In previous years, Dr Faraone received consulting fees, was on Advisory Boards, or participated in continuing medical education programs sponsored by Shire, Alcobra, Otsuka, McNeil, Janssen, Novartis, Pfizer, and Eli Lilly. Dr Faraone receives royalties from books published by Guilford Press (*Straight Talk about Your Child's Mental Health*) and by Oxford University Press (*Schizophrenia: The Facts*). Dr Cortese served as scientific consultant for Shire

Pharmaceuticals from June 2009 to December 2010. He received support to attend meetings from Eli Lilly and Co. in 2008 and from Shire in 2009–2010.

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