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Research report

Neonatal programming with testosterone propionate reduces dopamine transporter expression in nucleus accumbens and methylphenidate-induced locomotor activity in adult female rats

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ABSTRACT

Research in programming is focused on the study of stimuli that alters sensitive periods in development, such as prenatal and neonatal stages, that can produce long-term deleterious effects. These effects can occur in various organs or tissues such as the brain, affecting brain circuits and related behaviors. Our laboratory has demonstrated that neonatal programming with sex hormones affects the mesocorticolimbic circuitry, increasing the synthesis and release of dopamine (DA) in striatum and nucleus accumbens (NAcc). However, the behavioral response to psychostimulant drugs such as methylphenidate and the possible mechanism(s) involved have not been studied in adult rats exposed to sex hormones during the first hours of life. Thus, the aim of this study was to examine the locomotor activity induced by methylphenidate (5 mg/kg i.p.) and the expression of the DA transporter (DAT) in NAcc of adult rats exposed to a single dose of testosterone propionate (TP: 1 mg/50 µL s.c.) or estradiol valerate (EV: 0.1 mg/50 µL s.c.) at postnatal day 1. Our results demonstrated that adult female rats treated with TP have a lower methylphenidate-induced locomotor activity compared to control and EV-treated adult female rats. This reduction in locomotor activity is related with a lower NAcc DAT expression. However, neither methylphenidate-induced locomotor activity nor NAcc DAT expression was affected in EV or TP-treated adult male rats. Our results suggest that early exposure to sex hormones affects long-term dopaminergic brain areas involved in the response to psychostimulants, which could be a vulnerability factor to favor the escalating doses of drugs of abuse.

1. Introduction

Sex hormones modulate several physiological functions in reproductive and non-reproductive tissues, such as the brain. Sex hormone receptors expressed in some brain areas such as prefrontal cortex (PFC), amygdala, hippocampus, *locus coeruleus* and hypothalamus, are involved in the regulation of executive function, emotions, short-term memory, arousal and homeostasis, among others [1,2]. Another important brain circuitry that expresses sex hormone receptors is the mesocorticolimbic circuitry or reward system [3,4] formed by dopaminergic neurons from ventral tegmental area (VTA) that project their axons to nucleus accumbens (NAcc) and PFC [5,6]. This circuitry is activated by natural reinforcers such as sex [7] and food [8]. But it is also activated by other reinforcers such as drugs of abuse [9], which also increase dopamine (DA) release in NAcc and striatum [10]. Psychostimulant drugs such as amphetamine, methamphetamine, cocaine and methylphenidate increase extracellular NAcc DA levels, which favors addictive-like behaviors. Methylphenidate and cocaine block the DA transporter (DAT), and prevent DA uptake, increasing extracellular level of the transmitter [11].

The neuromodulatory role of sex hormones on areas of mesocorticolimbic circuitry has been studied in adult gonadectomized animals. For example, ovariectomized (OVX) rats exhibit a reduction in TH-positive neurons in VTA [12] and a reduction in NAcc DAT expression

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[13]. At the behavioral level, it has been shown that OVX rats do not produce amphetamine-induced conditioned place preference [14] and cocaine-induced locomotor activity [15,16]. In orchidectomized (OCT) rats it has been shown an increase in TH-positive neurons in VTA [17], an increase in striatal DA turnover [18] and loss of cocaine-induced locomotor sensitization [19], among other changes.

Evidence from the literature indicates that sex hormones are able to modulate the expression of key proteins in dopaminergic neurotransmission of adult animals [20]. However, the long-term effects produced by early exposure to sex hormones had not been well examined. We previously studied the long-term effects of neonatal programming with sex hormones on the physiology of midbrain dopaminergic neurons, observing an increase in VTA TH expression and DA synthesis as well as increase in NAcc DA release [21,22]. Here, we investigated the effects of neonatal programming, using a single dose of testosterone propionate (TP) or estradiol valerate (EV), on locomotor activity induced by methylphenidate in female and male adult rats. We also tested the expression of DAT in NAcc and striatum to determine whether the behavioral results are related with changes in the expression of the transporter.

2. Materials and methods

2.1. Animals and reagents

2.1.1. Animals

Fifty-three Sprague-Dawley pups (27 female and 26 male) from seven litters were used. All animals were housed in a room with controlled humidity (55 ± 5%) and temperature (21 ± 2 °C). A 12-h light cycle was used with lights on at 08:00 h, with food (Cat# 0001495, Prolab® RMH 3000, LabDiet, St. Louis, MO, USA) and water *ad libitum*. All experimental procedures were approved by the Ethics Committee of the Universidad de Valparaíso (Approval Number 001-2016) and performed in accordance with Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes) [23]. Efforts were made to minimize the number of animals used and their suffering.

2.1.2. Reagents

Testosterone propionate (TP), estradiol valerate (EV) and sesame oil were purchased from Sigma-Aldrich (Sigma-Aldrich, Inc., St. Louis, MO, USA). Methylphenidate hydrochloride was donated from Laboratorios Andrómaco S.A. (Peñalolén, Santiago, Chile). All other reagents were of analytical and molecular grade.

2.2. Experimental procedure

2.2.1. TP, EV and sesame oil administration

Animals were injected at postnatal day one (PND1) with a single dose of TP (1 mg/50 µL of sesame oil s.c.), EV (0.1 mg/50 µL of sesame oil s.c.), or vehicle (control group: 50 µL of sesame oil s.c.). The pharmacological doses of sex hormones have been previously published [21,22,24]. Pups were divided randomly into six groups: control female (n = 9), control male (n = 11), EV female (n = 10), EV male (n = 10), TP female (n = 7) and TP male (n = 6). All pups were raised with a nursing mother until weaning age (PND21). After weaning, animals were housed in groups according to sex and treatment in standard cages. At PND60, male rats were used for locomotor activity experiments. Female rats were used between PND60 – 62 to perform the experiments of locomotor activity in the diestrus stage of the estrous cycle to have comparable the experimental groups in terms of hormonal milieu. In addition, the reason for using the females in the diestrus stage is due to the fact that EV and TP are constantly arrested in this stage of the estrous cycle (by action of the neonatal administration of hormones). After behavioral experiments, rats were euthanized and brain tissue was dissected to determine NAcc DAT expression.

2.2.2. Determination of estrous cycle

The stage of estrous cycle was daily recorded from PND40 to the end of the study (PND60 − 62). The estrous cycle was assessed by analyzing the relative proportion of leukocytes, epithelial cells, and cornified cells in daily vaginal lavages, which characteristically change during the various stages of the estrous cycle [25].

2.2.3. Locomotor activity

Basal, saline- and methylphenidate-induced locomotor activities were measured in TP, EV, and control rats. During the first 30 min that each animal was placed in a test cage (44 cm long, 22 cm height and 28 cm wide), basal locomotor activity was established. At 30 min, saline solution (1 mL/Kg i.p.) was injected and locomotor activity was recorded for another 60 min. At 90 min, a single dose of methylphenidate (5 mg/Kg i.p.) was injected and locomotor activity was recorded for an additional 60 min. Locomotor activity was recorded with wireless cameras (model LX-C202, Lynx Security, China) fixed above each test cage and connected to a computer in another room. Videos were analyzed using ANY-Maze™ video tracking system (Stoelting™ Co., Wood Dale, IL, USA), measuring the total distance traveled (m) every 5 min. Test cages were wiped and cleaned with 5%$^{v}/_{v}$ ethanol solution after each trial.

2.2.4. Western blot

Once the protocol of locomotor activity was finalized, the animals were euthanized via decapitation with a guillotine for small animals (model 51330, Stoelting™ Co., Wood Dale, IL, USA) and the rat brain was extracted. NAcc and striatum were microdissected at 4 °C using a micro-punch (Harris Micro-Punch™, 2.0 mm of diameter, Ted Pella Inc., CA, USA). These tissues were weighed and stored at −80 °C for further analysis. To determine DAT expression in NAcc and striatum, both brain areas were homogenized using RIPA buffer (pH = 8.0, 150 mM NaCl, 50 mM Tris-HCl, 1%$^{v}/_{v}$ Nonidet P40, 0.1% $^{w}/_{v}$ SDS, 2 mM EDTA, 1.5 mM PMSF, and a protease inhibitor cocktail [Cat# G6521, Promega™]) using a sonicator (model XL2005, Microson Ultrasonic Cell Disruptor, Heat Systems, USA). The total protein concentration was determined using a microplate spectrophotometer (Epoch™, BioTek Instruments Inc., Winooski, VT, USA). Protein samples (30 µg) were separated by SDS-PAGE on 10% polyacrylamide gels under denaturing conditions (4% concentrator gel, 8% resolute gel). Proteins were transferred to nitrocellulose membrane (Cat# 88018, 0.45 µm pore, Thermo Scientific™, Rockford, IL, USA) at 350 mA for 1.5 h. Non-specific sites of membrane binding were blocked with 5% skim milk in T-TBS (0.1% Tween-20, 20 mM TBS, 137 mM NaCl) for 1 h at room temperature. Nitrocellulose membranes were incubated with T-TBS overnight at 4 °C and then incubated with the indicated antibody. The antibodies used in this study were: primary antibody anti-DAT developed in goat diluted 1:1000 (Cat# C-20 SC-1433, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-GAPDH developed in rabbit diluted 1:10,000 (Cat# ab9485, Abcam, Cambridge, MA, USA). Membranes were washed three times with T-TBS and then incubated in T-TBS during 1 h at room temperature with the secondary anti-goat antibody (for DAT) conjugated with horseradish peroxidase (HRP) developed in rabbit diluted 1:20,000 (Cat# 305-035-047, Jackson ImmunoResearch Inc., West Grove, PA, USA) or anti-rabbit antibody (for GAPDH) conjugated with HRP developed in donkey diluted 1:10,000 (Cat# 711-036-152, Jackson ImmunoResearch Inc., West Grove, PA, USA). For chemiluminescent detection, we used EZ-ECL kit (Cat# 20–500-500, Biological Industries Ltd, Beit Haemek, Israel) and the images of the membranes were obtained using a benchtop transilluminator (EpiChemi^{3} Darkroom, UVP, Upland, CA, USA). The images were analyzed using Image-J™ software (http://rsbweb.nih.gov/ij/).