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MATERNAL ADMINISTRATION OF FLUTAMIDE DURING LATE GESTATION AFFECTS THE BRAIN AND REPRODUCTIVE ORGANS DEVELOPMENT IN THE RAT MALE OFFSPRING

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Abstract—We have previously demonstrated that male rats exposed to stress during the last week of gestation present age-specific impairments of brain development. Since the organization of the fetal developing brain is subject to androgen exposure and prenatal stress was reported to disrupt perinatal testosterone surges, the aim of this research was to explore whether abnormal androgen concentrations during late gestation affects the morphology of the prefrontal cortex (PFC), hippocampus (HPC) and ventral tegmental area (VTA), three major areas that were shown to be affected by prenatal stress in our previous studies. We administered 10-mg/kg/day of the androgen receptor antagonist *flutamide* (4′nitro-3′-trifluoromethylsobutyranilide) or vehicle injections to pregnant rats from days 15–21 of gestation. The antiandrogenic effects of flutamide were confirmed by the analysis of androgen-dependent developmental markers: flutamide-exposed rats showed reduced anogenital distance, delay in the completion of testis descent, hypospadias, cryptorchidism and atrophied seminal vesicles. Brain morphological studies revealed that prenatal flutamide decreased the number of MAP2 (a microtubule-associated protein type 2, present almost exclusively in dendrites) immunoreactive neuronal processes in all evaluated brain areas, both in prepubertal and adult offspring, suggesting that prenatal androgen disruption induces long-term reductions of the dendritic arborization of several brain structures, affecting the normal connectivity between areas. Moreover, the number of tyrosine hydroxylase (TH)-immunopositive neurons in the VTA of prepubertal offspring was reduced in flutamide rats but reach normal values at adulthood. Our results demonstrate that the effects of prenatal flutamide on the offspring brain morphology resemble several prenatal stress effects suggesting that the mechanism of action of prenatal stress might be related to the impairment of the organizational role of androgens on brain development.

Key words: prenatal flutamide, male rat offspring, prefrontal cortex, hippocampus, ventral tegmental area, tyrosine hydroxylase.

INTRODUCTION

An efficient establishment of synaptic circuits during maturation is essential for the development of normal brain function. The majority of excitatory synapses are formed on dendritic spines and changes in spine density and morphology account for functional differences at the synaptic level (Segal, 2010). The cerebral cortex and the hippocampal formation are essential components of the neural pathways that mediate stress responses and are essential for learning and memory formation (Madeira and Lieberman, 1995). On the other hand the *mesocorticolimbic DA (dopaminergic) system*, that comprises neurons from the ventral tegmental area (VTA) projecting mainly to the hippocampus (HPC) and the prefrontal cortex (PFC) (Kuhar et al., 1999; Chinta and Andersen, 2005; Baier et al., 2012), regulates diverse behavioral and cognitive functions that are crucial for the integration of individual perception and its adaptation to the environment (Missale et al., 1998). During the last years, increasing evidences from rodent models demonstrate that exposure to different stressful events during the last week of gestation strongly impacts on structural and functional fetal central nervous system development, leading to impaired adaptation to stressful conditions, enhanced propensity to self-administer drugs, vulnerability to anxiety and learning deficits (Darnaudery and Maccari,

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Abbreviations: AcB, acetate buffer; ANOVA, analysis of variance; AR, androgen receptor; DA, dopaminergic; DHT, dihydrotestosterone; FLU, flutamide (4′nitro-3′-trifluoromethylsobutyranilide); GD, gestational days; HPC, hippocampus; MAP2, microtubule-associated protein type 2; NeuN, neuron-specific nuclear antigen; PBS, phosphate-buffered saline; PFC, prefrontal frontal cortex; PND, postnatal day; TH, tyrosine hydroxylase; VEH, vehicle; VTA, ventral tegmental área.

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2008; Huizink et al., 2004; Weinstock, 2001, 2008). In addition, the offspring display anomalies in neuronal development and brain morphology which persist into adulthood (Fride and Weinstock, 1989). Our laboratory has a long-standing interest in the effects of prenatal stress on the brain development, especially on the mesocorticolimbic DA pathway (Baier et al., 2012). We have demonstrated that several impairments induced by prenatal stress on the DA metabolism were differentially affected if assayed before or after puberty. This observation confirms the suggestion from previous investigations that perinatal events might render the DA circuitry more vulnerable to puberty variation of the hormonal circulating levels (Diaz et al., 1997). However, the reduction in dendritic arborizations induced by prenatal stress in PFC and HPC, that were reported to occur at adult stages (Barros et al., 2006), were also found prepubertally (Pallares et al., 2013b), suggesting that some plastic morphological processes might be programed prenatally but are relatively insensitive to the increase of sexual hormones during puberty.

The effect of gonadal hormones on brain maturation takes place at two different periods of life known as the classical *organizational/activational* hypothesis of gonadal steroid action (Alonso and Lopez-Coviella, 1998). During the prenatal period, gonadal steroid hormones (i.e. estrogens and androgens) *organize* the developing brain by changing the architecture of several neural substrates which later in puberty are *activated* by the gonadal steroids surge in a directed manner. The direction of adult hormonal responsiveness will dictate sex-specific behavior and physiology (Zhang et al., 2010). In males, testosterone and its 5-alpha reduced metabolite dihydrotestosterone (DHT) are the major circulating androgenic hormones. In rats, androgens-induced masculinization of the reproductive tract and brain sexual behavior takes place over a limited period of perinatal development called *the critical period of differentiation* which is initiated with testosterone peaks on gestational days (GD) 18–19 and is extended up to the first postnatal week (Corbier et al., 1978; Lee et al., 1975). During this period, androgen-dependent tissues are intensely modified (Knickmeyer and Baron-Cohen, 2006). However, the occurrence of some factors during this perinatal phase can interfere with the physiological, morphological, behavioral, and neuroanatomical differences between males and females (Scott et al., 2009). For example, it was reported that prenatal stress suppresses the surge of prenatal testosterone, affecting the male reproductive tract formation, inducing abnormal testosterone levels and feminizing the male sexual behavior (Barros et al., 2004; Gerardin et al., 2005; Shono and Suita, 2003). In our hands, we have shown that prenatal stress induced long-term imbalance of male sexual hormons concentrations in serum, advanced the spermatogenesis development and exerted an age-dependent misbalance on alpha receptor expression on PFC and HPC brain areas (Pallares et al., 2013a,b). Moreover, it was observed that physiological and behavioral damage caused by prenatal stress was prevented by replacement with neonatal testosterone (Pereira et al., 2006), corroborating the importance of neonatal testosterone surge during the sexual differentiation process of the brain. The fetal rat brain expresses androgen receptors (ARs) as early as GD 12 with a peak expression at GD 17–18 (Brannvall et al., 2005). The majority of studies examining the effects of early gonadal action on the adult male rat have focused on sexual behaviors or anatomical aspects of sexually dimorphic central nucleus. In contrast, the effects of early-life manipulation of gonadal steroids on the development of other brain regions outside the hypothalamus were poorly explored.

Androgen organizational influence over mesostriatal and mesolimbic DA system was demonstrated by Creutz and Kritzer (2004). Moreover, Yang and Shieh (2007) suggested that gonadal hormones play a regulatory role in the stimulation of cocaine and amphetamine-regulated transcript peptide in mesolimbic and nigrostriatal DA system and Johnson et al. (2010) demonstrated that testosterone play a suppressive role in midbrain DA pathways. The organizational role of androgens in HPC was explored by Zhang and collaborators (2010) who reported that neonatal androgenic surges disruption increased depression-like behaviors in prepubertal male rats as well as reduced the number of MAP2 (microtubule-associated protein type 2)-immunopositive neurons in the dentate gyrus and the density of dendritic spines of the pyramidal neurons of the CA1 hippocampal areas.

Based on the existing literature and our own results, in the present study we antagonized the AR in the prenatal period in order to examine if the morphological consequences of gestational stress on the programing of the brain neural architecture of the offspring, might be related to the disruption of the perinatal androgen surge. We exposed pregnant rats to the non-steroidal drug flutamide during the last week of gestation which is a powerful and specific antiandrogen that crosses the placental barrier (Neri et al., 1972) and blocks AR by inhibiting its translocation to the nucleus from the cytoplasm of the target cells. We hypothesize that prenatal administration of flutamide might impair sexual maturation as well as brain morphology development in the prepubertal and adult offspring in a similar manner to the exposure of stress during late gestation that was previously reported by our group.

EXPERIMENTAL PROCEDURES

Animals

Eight virgin female Wistar rats weighing 250–280 g and sexually experienced Wistar male rats weighing 400–450 g were obtained from outbred rats belonging to the animal facility at the University of Buenos Aires. A maximum of four rats were housed per cage with *ad libitum* access to standard rat chow (*Asociación de Cooperativas Argentinas* – Buenos Aires, Argentina) and water. A constant light/dark cycle, with lights on at 06:00 h and off at 18:00 h, and a room temperature of 21–25 °C were maintained. Females were individually mated with a male in a mating cage. Vaginal smears were taken on the following morning. The day on which