ABSTRACT

Introduction: Brain regions implicated in sexual behavior begin to differentiate in the last trimester of gestation. Antenatal therapy with corticosteroids is often used in clinical practice during this period to accelerate lung maturation in pre-term risk pregnancies. Clinical and animal studies highlighted major behavioral impairments induced later in life by these treatments, especially when synthetic corticosteroids are used.

Aim: To evaluate the implications of acute prenatal treatment with natural versus synthetic corticosteroids on adult male rat sexual behavior and its neurochemical correlates.

Methods: Twelve pregnant Wistar rats were injected with dexamethasone (DEX-1mg/kg), corticosterone (CORT-25mg/kg) or saline on late gestation (pregnancy days 18 and 19). Following this brief exposure to corticosteroids, we assessed the sexual behavior of the adult male progeny and subsequently correlated these behaviors with the levels of cathecolamines and mRNA of dopamine and androgen receptors (AR) in brain regions relevant for sexual behavior.

Main Outcome Measures: Sexual behavior of adult male offspring was assessed by exposure to receptive females. This was correlated with serum testosterone levels and levels of cathecolamines (determined by HPLC) and dopamine and androgen receptors mRNA expression (real-time PCR) in brain regions implicated in sexual behavior.

Results: Prenatal DEX exposure resulted in a decreased number and increased latency time to mounts and intromissions in adulthood. These findings correlated with decreased levels of

serum testosterone and increased hypothalamic expression of AR mRNA. DEX animals also displayed lower dopamine levels and higher dopamine receptor mRNA expression both in hypothalamus and nucleus accumbens (NAcc). The milder phenotype of CORT animals was correlated only with decreased dopamine levels in NAcc.

Conclusion: Antenatal corticotherapy programs adult male sexual behavior through changes in specific neuronal and endocrine mediators. Importantly, equipotent doses of corticosterone trigger less detrimental consequences than dexamethasone, emphasizing the differential impact of activation of the different corticosteroid receptors.

Keywords: Antenatal corticotherapy; Corticosteroids; Dopamine; Neurodevelopment; Sexual behavior

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INTRODUCTION

The last trimester of gestation and early postnatal period are critical for brain sexual differentiation (Segarra et al., 1991). Insults at this period, including stress and prolonged exposure to corticosteroids, have been shown to disrupt several behaviors in adulthood, namely male sexual behavior (Holson et al., 1995, Gerardin et al., 2005, Piffer et al., 2009). Interestingly, exposure to corticosteroids during late gestation has been correlated with a sustained perturbation in male steroidogenesis (Page et al., 2001) and an impoverished dopaminergic innervation of the nucleus accumbens (NAcc) (Leao et al., 2007), which is of particular relevance when considering the facilitatory role of dopamine in the different aspects of sexual behavior (Giuliano and Allard, 2001).

In clinical practice, glucocorticoids are prescribed in about 10% of pregnancies at risk of preterm delivery in order to promote fetal lung maturation (Crowley, 1995, NIH, 1995, Crane et al., 2003). Dexamethasone (DEX) and betamethasone, the preferred drugs (Jobe et al., 2003), are synthetic corticosteroids that cross the placenta with 100% efficacy and have been shown to reduce the morbidity and mortality of the preterm infant after delivery (NIH, 1995). Despite this, the safety of the exposure of the developing fetal brain to glucocorticoids has been questioned as it might have life-long effects on adult behavior and neuroendocrine function (Matthews, 2000, Welberg et al., 2001, Oliveira et al., 2006). Available data suggests that the activity of the hypothalamic-pituitary-adrenal (HPA) axis, which is vital to stress response, might

be reprogrammed by manipulations in the corticosteroid milieu during late gestation; this altered pattern of the HPA is believed to be, at least in part, responsible for the behavioral and neuroendocrine changes (Welberg and Seckl, 2001), as well as for increased risk for hypertension, type 2 diabetes (Levitt et al., 1996, Lindsay et al., 1996, Sapolsky et al., 2000) and neuropsychiatric disorders (Welberg et al., 2001). Of particular interest is the evidence suggesting a less deleterious effect on adult emotional behavior of the acute administration of endogenous corticosteroids (Oliveira et al., 2006), especially in light of evidence showing that cortisol displays similar therapeutic efficacy to DEX during pregnancy and neonatal life (Crowley, 1995).

In light of this evidence, we decided to assess the impact of short-term antenatal corticosteroid exposure in male sexual behavior and search for its neurochemical and endocrine correlates. Furthermore, we also wanted to compare natural and synthetic corticosteroids in terms of long-term adverse effects.

METHODS

Animals and treatments

Experiments were conducted in accordance with local regulations (European Union Directive 86⁄609⁄EEC) and NIH guidelines on animal care and experimentation.

Twelve adult timed pregnant Wistar Han rats (Charles-River Laboratories, Barcelona, Spain) received at day 14 of gestation were individually housed under standard laboratory conditions (12/12h light/dark cycle, with lights on at 8 a.m.; food and water ad libitum).

Subcutaneous injections of DEX (1 mg/kg, Sigma-Aldrich; n=4), corticosterone (CORT, 25 mg/kg, Sigma-Aldrich; n=4), or saline (controls, 1 mL/kg; n=4) were administered on embryonic days (ED) 18 and 19 of pregnancy (Oliveira et al., 2006). Drug dosages were chosen to achieve comparable transrepressive potencies at the glucocorticoid receptors (GR) (Schimmer BP, 2005).

Weaning was performed at postnatal day 21 and pups were pair-housed according to gender and prenatal exposure. Male offspring (2 siblings per dam; n= 4 dams/ group), were tested at 3 months for sexual behavior.

Preparation of sexually receptive females

Adult 3 months female rats were individually housed and ovariectomized, as previously described (Agmo, 1997). Sexual receptivity was induced by subcutaneous estradiol benzoate (20 µg/rat, Sigma Aldrich) and progesterone (1 mg/rat, Sigma-Aldrich) 52 and 4 hours before male exposure, respectively.

Male sexual behavior

The test arena consisted of a rectangular Plexiglas box (40x60x40cm) with a transparent top and a video camera over it. Exposure was conducted two hours after the onset of the dark phase, in a quiet room, with a dim red light. Sexually experienced males were placed in the arena ten minutes before a receptive female was presented and activity was recorded for 20 minutes; latency time and number of mounts and intromissions (vaginal penetration) were registered and intromission ratio calculated as intromissions /[intromissions+mounts].

Biometric and testosterone measurements

Animals were sacrificed one week after behavior assessment and blood collected for determination of serum total testosterone levels by electrochemiluminescense immunoassay (Elecsys Testosterone II reagent kit, Roche Diagnostics; measuring range 2.5-1500 ng/dL). Testis wet weight was assessed.

Brain cathecolamines

After brain snap freezing, the regions of interest were rapidly dissected under the scope using macrodissection of specific brain areas. The hypothalamus was isolated by placing whole brains upside down and using delicate forceps (Dumont #7 forceps, Fine Science Tools USA Inc., Foster City, CA, USA) to detach it from the rest. The hypothalamus was identified as the round shaped area in the center of the brain. NAcc was isolated using punch dissection in 2mm sections of brains (Alto^{TM} brain matrix, Stoetling Co., Wood Dale, IL, USA) and identified under a stereomicroscope (Model SZX7, Olympus America Inc., Center Valley, PA, USA). NAcc was

identified as the tissue in the vicinity of the anterior branch of the anterior commissure, according to Paxinos stereological coordinates (Paxinos and Watson, 2005). Samples were frozen in liquid nitrogen (overnight at -20ºC) after adding percloric acid 0.2M. Samples were briefly sonicated, centrifuged and 50 µl aliquots of the supernatant injected on a high performance liquid chromatography (HPLC) combined with electrochemical detection system. A mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l) and Na-EDTA (40 mg/l) was used.

Levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) were determined using a Gilson instrument (Gilson Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 M; 7.5 cm x 4.6 mm; flow rate: 1.0–1.5 ml/min; Supelco, Bellefonte, PA, USA). A standard curve was then obtained and data presented as concentration (nanogram per milligram of tissue protein).

Molecular analysis

For Real-Time PCR analysis, total RNA was isolated from frozen areas using Trizol (Invitrogen) and DNase treatment (Fermentas), according to manufacturer. Two µg of RNA were converted into cDNA using the iSCRIPT kit (Biorad). RT-PCR was performed using SyberGreen (Qiagen) and the Biorad q-PCR CFX96 apparatus. HPRT was used as a housekeeping gene. We used relative quantification to determine the fold change difference between control, CORT and DEX animals, using the ΔΔCT method as described before (Pfaffl, 2001). Primer sequences were AR_F:GGGTGACTTCTCTGCCTCTG, AR_R:CCACAGATCAGGCAGGTCTT (androgen receptor);

ESR1_F:CAGGTGCCCTACTACCTGGA, ESR1_R:GGTAGCCAGAGGCATAGTCG (estrogen receptor 1); ESR2_F:AACCGCCATGAGTATTCAGC, ESR2_R:GTAACAGGGCTGGCACAACT (estrogen receptor 2); nNOS_F:GACAACGTTCCTGTGGTCCT, nNOS_R:GAAGAGCTGGTCCTTTGTGC (neuronal nitric oxide synthase); D1R_F:TCCTTCAAGAGGGAGACGAA, D1R_R:CCACACAAACACATCGAAGG (dopamine D1 receptor); D2R_F:CATTGTCTGGGTCCTGTCCT, D2R_R:GACCAGCAGAGTGACGATGA (dopamine D2 receptor); HPRT1_F:GCAGACTTTGCTTTCCTTGG, HPRT1_R:TCCACTTTCGCTGATGACAC.

Statistical analysis

For statistical analysis, the “n” of each experiment group was considered the number of litters from which individuals were derived. Results are presented as average±SE. Data was analyzed by PASW Statistics 18.0 (SPSS Inc, Chicago, IL, USA), using ANOVA. Whenever appropriate, post hoc comparisons were performed using Tukey test; statistical significance was considered when $p<.05$.

RESULTS

Acute antenatal DEX exposure affects adult male sexual behavior

Treatment significantly affected sexual motivation and the volitive aspects of copulatory behavior ($F=20.057$, $p<.001$), as revealed by an increased latency to mount in DEX-exposed animals compared to controls ($p<.001$) and CORT subjects ($p=.029$), respectively (Figure 1). CORT animals were also different from controls ($p=.027$).

Treatment also affected the number of mounts ($F=5.233$, $p=.031$), another measure of sexual motivation (Agmo, 1997), which was significantly decreased in DEX-exposed subjects when compared to controls ($p=.027$). Interestingly, CORT rats were not different from the other groups.

In contrast to mounts, intromissions are not exclusively dependent on sexual motivation. Treatment affected this parameter ($F=23.081$, $p<.001$) as revealed by an increased latency of DEX progeny in comparison to controls ($p<.001$) and CORT subjects ($p=.016$). Again, CORT animals also took more time to intromission than controls ($p=.024$).

Moreover, the number of intromissions was affected by treatment ($F=8.083$, $p=.010$). This indicator of the easiness in the activation of ejaculatory reflexes (Agmo, 1997) was found to be decreased in DEX-exposed ($p=.008$), but not on CORT-exposed subjects ($p=.103$).

Intromission ratio (Figure 2), an indicator of efficiency of penile erection, was also affected by treatment ($F=22.972$, $p<.001$). DEX-subjects displayed significantly lower ratios compared to controls ($p<.001$) and CORT-exposed animals ($p=.003$).

Adult testosterone levels are affected by antenatal DEX

Serum testosterone levels were significantly reduced by antenatal DEX exposure ($F=15.815$, $p=.001$; vs CORT $p=.001$; vs controls $p=.004$) but not by CORT (Figure 3). Testis wet weight was similar between groups (data not shown).

Antenatal corticosteroids influence brain dopamine levels

Antenatal corticosteroids exposure influenced the levels of dopamine in NAcc ($F=39.911$, $p<.001$; Table 1). Both progeny of CORT and DEX dams displayed decreased levels of dopamine in NAcc in comparison to controls ($p<.001$ and $p<.001$, respectively). Treatment also affected dopamine turnover ($F=6.162$, $p=.021$), with an increase in DEX subjects when compared to CORT ($p=.026$) and controls ($p=.047$).

Hypothalamic levels of dopamine were also significantly affected by antenatal treatment ($F=5.817$, $p=.024$), with a decrease only in DEX-treated subjects ($p=.022$) when compared to controls. Dopamine turnover in this area was also affected ($F=10.286$, $p=.005$), with CORT and DEX animals displaying a lower ratio when compared to controls ($p=.010$ and .008, respectively).

Treatment did not affect serotonin levels ($F=3.564$, $p=.072$) nor its turnover ($F=1.076$, $p=.381$) in the NAcc. However, hypothalamic levels of serotonin were reduced ($F=14.050$, $p=.002$) while its

turnover was increased (F=7.552, p=.012) in both DEX (p=.003; p=.029) and CORT (p=.004; p=.016) groups.

Molecular correlates

In the NAcc, dopamine D1 (D1R) and D2 (D2R) receptors mRNA levels were significantly affected by prenatal exposure to corticosteroids (F=111,471, p<.001 and F=76.383, p<.001, respectively; Table 2), with an increase in DEX group when compared to controls (p<.001; p<.001, respectively) and CORT (p<.001; p<.001, respectively). A similar effect was also observed in the hypothalamus, but only for D1R (F=7.868, p=.011; vs controls p=.009); CORT animals were not different from any other group.

Levels of AR and neuronal nitric oxide synthase (nNOS) mRNA were not different between groups in the NAcc. However, in hypothalamus, there was a significant effect of exposure on AR (F=13.049, p=.002) and nNOS (F=27.056, p<.001) mRNA levels, with an increase in DEX progeny when compared to controls (p=..020; p<.001, respectively) and CORT subjects (p=.002; p=.002, respectively).

Levels of estrogen receptors 1 and 2 were not affected by treatment in neither area.

DISCUSSION

In rats, brain sexual differentiation occurs mainly during late gestation (ED 14–21) and the first 2 weeks of postnatal life (Segarra et al., 1991). The vulnerability of this time-window to several insults, including stress and corticosteroid exposure, may thus lead to long-term consequences on adult male sexual behavior (Gerardin et al., 2005, Piffer et al., 2009). In fact, previous data suggests that prenatal stress disrupts the normal maternal hormonal milieu and suppresses the fetal testosterone peak on ED18 and 19, required for later expression and maintenance of male sexual behavior (Ward and Weisz, 1984, Lalau et al., 1990). Such data sustain that interferences in the maturation of the hypothalamic-pituitary-adrenal (HPA) axis, affect the hypothalamic-pituitary-gonadal (HPG) axis, since both are regulated by common players both centrally and at the periphery (Page et al., 2001). Following the initial experimental evidence showing that late gestation whole-body restraint under bright lights results in delayed initiation of copulation (WARD 1972), more recent data correlated prolonged prenatal stress/corticosteroids with impaired adult male sexual behavior and reduced serum testosterone levels (Gerardin et al., 2005, Piffer et al., 2009). However, no previous studies focused on the potential effects of a short-term exposure which better mimics the everyday clinical practice, nor in the comparison between different types of corticosteroids.

The analysis of several behavioral parameters permitted us to distinguish between appetitive and consummatory components of male rat sexual behavior. Male rat sexual behavior is characterized by a series of mounts, either with or without vaginal penetration (intromission),

ultimately leading to ejaculation (Hull and Dominguez, 2007). While latency until the first mount reflects some of the appetitive aspects and sexual motivation, intromission and ejaculation latencies but also mount and intromission frequencies reproduce consummatory components of copulatory behavior (Pfaus et al., 1990b, Agmo, 1999). Interestingly, the impairment of male sexual behavior observed in this study was mainly characterized by alterations in sexual appetite (increase in mount and intromission latencies); moreover, the reduced number of mounts might reflect decreased sexual motivation (Agmo, 1997). Importantly, these differences are more striking in animals exposed to DEX than to CORT.

Regarding the neurobiology of sexual behavior, three major integrative systems regulate sexual motivation and genital and motor responses (Hull et al., 2004). Whereas the mesolimbic system is critical for appetitive behavior and reinforcement, the medial preoptic system contributes to genital reflexes, sexual motivation and motor patterns of copulation. Finally, the nigrostriatal system enhances the motoric readiness to respond to stimuli. Dopamine is the common key player in all three systems, easing sexual motivation, copulatory proficiency, and genital reflexes (Giuliano and Allard, 2001). The pathways for sexual excitation involve the activation of incertohypothalamic and mesolimbic dopamine transmission that targets the hypothalamic medial preoptic area (MPOA) and NAcc, respectively (Pfaus, 2009). As a result, in the male rat there is a slight increase in dopamine release in NAcc following presentation to a receptive female that is followed by a sharp increase in dopamine transmission during copulation, that gradually declines after the removal of the female (Pfaus et al., 1990a).

A previous study focusing on the effects of prolonged prenatal immobilization stress on the adult male rat correlated the absence of copulatory behaviors with unchanged NAcc extracellular levels of dopamine, DOPAC and HVA during exposure to receptive females. Such data, obtained through simultaneous sexual behavior testing and concomitant microdialysis samplig, suggested that intense environmental stressors might impair NAcc dopamine release (WANG 1995).

In our experiment, an interesting neurochemical-behavior correlate was established, as we found decreased dopamine in hypothalamus and NAcc of corticosteroid briefly exposed animals. Interestingly, we had previously reported a reduced dopaminergic innervation of the NAcc following prenatal short-term exposure to DEX, revealed by a reduced density of tyrosine hydroxylase-positive fibers in these subjects (Leao et al., 2007). In addition, the decrease in dopamine levels in NAcc herein reported is likely to be of relevance for the changes in sexual behavior if one takes into account descriptions correlating a delayed onset of copulation and ejaculation with a diminished release of this neurotransmitter in the mesolimbic tract (Hull et al., 2004). Furthermore, the increase in dopamine D1 and D2 receptors mRNA in the NAcc following DEX exposure herein shown further supports the existence of a hypodopaminergic status in these animals, and may appear as a compensatory mechanism due to the low dopamine levels observed.

Although we observed substantial differences in the dopamine levels and receptors in the NAcc, one drawback of this work is the fact that we did not discriminate between its two functionally distinct regions: the core and the shell. C-fos expression is increased in the core but not the shell during sexual behavior (Bradley and Meisel, 2001). On the contrary, administration of drugs of

abuse results in increased dopamine levels in the shell of the NAcc (Pontieri et al., 1995, Nisell et al., 1997, Di Chiara et al., 1999) (DI CHIARA 2002). This suggests that shell and core might be activated differently in response to natural reinforcers and drugs of abuse. Indeed, NAcc neurons exhibit similar neuronal activity during responding to two natural rewards - food and water, but different firing patterns during responding for a natural reward versus cocaine (Carelli et al., 2000). Therefore, considering the different functional/activational roles of core and shell, it would be interesting to analyze dopamine metabolism and receptors in each subarea in order to dissect what is the most affected area.

The hypodopaminergic status of DEX-exposed animals in the NAcc might have other behavioral consequences besides altered sexual behavior, considering the importance of correct dopamine input for feeding, reward and addiction, among others. Dopamine is released in the NAcc in response to drugs of abuse but also other consumatory behaviors such as sex and food and thus the VTA-NAcc pathway is also known as the "reward pathway"(Piazza and Le Moal, 1996). Interestingly, some studies have reported cross-sensitization between repeated exposures to pharmacological agents and natural motivated behaviors such as sex (Mitchell and Stewart, 1990a, b, Fiorino and Phillips, 1999). For example, sexual experience can cross-sensitize neuronal responses to amphetamine and this seems to depend on dopamine release in the NAcc (Bradley and Meisel, 2001).

The intricately-regulated balance between hypo- and hyper-dopaminergic states in the mesolimbic circuit, specially in the NAcc area, underlies an individual's cycles of drug-seeking behavior/abuse and response to natural rewards. While a hyperdopaminergic state seems to enhance the motivational or rewarding properties of drugs of abuse, hypodopaminergic states

appear to enhance drug-seeking behavior in parallel with reductions in the perceived motivational impact of 'natural' rewards such as food and sex (Diana et al., 1993, Diana et al., 1998, Melis et al., 2005). This theory is in agreement with our behavioural and neurochemical results, given the fact that DEX-animals have low dopamine levels in the NAcc and, concomitantly, impaired appetitive sexual behaviour. Additionally, it suggests that these animals might also display differential susceptibility to addiction, a phenomenon also observed in other models of early life stress (Kippin et al., 2008).

Dopamine in the hypothalamus, particularly in the MPOA, is essential for genital reflexes, motor patterns of copulation, and probably sexual motivation (Hull and Dominguez, 2006); several studies described the facilitative role of increased levels in the MPOA on sexual behavior, suggesting that testosterone might mediate this effect (DOMINGUEZ 2005). In the present study, conclusions on the impact of prenatal exposure to natural versus synthetic corticosteroids are limited by the fact that dissection of the whole hypothalamic area was performed instead of isolating the MPOA. Nonetheless, the decreased hypothalamic levels of dopamine herein reported in the DEX group, but not in the CORT group, is associated with a significant effect on D1 receptors mRNA. This fact is likely to be of significance to explain the differential neuroendocrine and behavioral effects of CORT from DEX.

In addition, an increase in the androgen receptor mRNA was observed in the hypothalamus of DEX progeny, possibly reflecting a reduction in the circulating androgens. Interestingly, previous

studies showed that although normal basal levels of dopamine in the MPOA are adequate to allow some copulatory behavior, efficient mating requires an androgen-dependent female-stimulated increase (Putnam et al., 2005). Also, by up-regulating nNOS in the MPOA, testosterone enhances nitric oxide production, which controls dopamine release (Sanderson et al., 2008). In the present study, we found increased levels of nNOS mRNA in the hypothalamus of DEX subjects. It would be of added value to assess if theses changes persist in MPOA samples, which would be in accordance to previous descriptions in the MPOA of gonadectomized rats (Singh et al., 2000). However, technical issues in the isolation of the MPOA and the fact that neighbor hypothalamic subareas might display different susceptibilities to circulating androgens could justify why other studies did not confirm the original findings (Sato et al., 2005).
Thus, in order to draw further conclusions on the impact of the in utero corticosteroids exposure on the adult male MPOA, it would be of interest to specifically analyze this hypothalamic area in future studies.

CONCLUSIONS

Early life exposure to short-term glucocorticoid ligands triggers lifelong programming effects in brain regions implicated in distinct aspects of male sexual behavior. The behavioral changes correlate with altered dopaminergic systems and neuroendocrine markers. These findings are of clinical relevance, as they provide support to therapeutic interventions for sexual dysfunction that modulate brain dopaminergic levels (Montorsi et al., 2003a, Montorsi et al., 2003b, Padma-Nathan et al., 2004, Miner and Seftel, 2007) and peripheral levels of testosterone (Hatzimouratidis and Hatzichristou, 2007, Traish et al., 2007, Hatzimouratidis et al., 2010). Noticeably, equipotent CORT administration triggers a less detrimental impairment than DEX, highlighting the role of the different corticosteroid receptors on the systems regulating sexual behavior.

FIGURE LEGENDS

Figure 1 – Latency times to mount and intromission were increased in dexamethasone (DEX) and corticosterone (CORT) exposed animals when compared to controls (left); the number of mounts and intromissions was significantly reduced in DEX-exposed rats (right). * $p<.05$.

Figure 2 – Intromission ratio, calculated as *intromissions/[intromissions+mounts]*, was diminished in DEX-exposed rats when compared to controls and CORT animals. * $p<.05$.

Figure 3 – Antenatal DEX administration led to diminished serum testosterone levels (ng/dL) in adult male rats, when compared to CORT and controls. * $p<.05$.

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