ORIGINAL ARTICLE

The mild stress of chronic prenatal injections may have additive effects on drugs administered during pregnancy to alter brain sexual differentiation

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Abstract

OBJECTIVE: In order to clarify the effect of the prenatal (PN) treatment of the drug 1,4,6-androstatriene-3,17-dione (ATD) which blocks the conversion of testosterone into estradiol on male sexual behavior of the rats offsprings, from the effect of the mild stress induced by the PN administration of the Propylene glycol (PG), the vehicle used to dissolve ATD.

METHODS: Pregnant Wistar rats were divided into three groups. The CON group did not receive any kind of treatment. The other two groups (PG and ATD) were injected i.p. during gestation (days 11–22) with 0 and 5 mg of ATD, dissolved in 0.1 ml of PG, respectively, doses reported by other authors. Sexual performance of the male pups was analyzed three months later in four successive tests.

RESULTS: In the first sexual test of these naive rats, the percentage of males mounting, intromitting, ejaculating and the ejaculation frequency of the ATD group decreased significantly in comparison with the CON group. Also in the first and 4th tests, mounting, intromission and ejaculation latencies, as in the post-ejaculatory refractory period, ATD group, was significantly longer in comparison with the CON group. PG males showed a male sexual behavior (MSB) similar to that observed in the ATD group, but the differences did not reach statistical significance when they were compared with the CON group.

CONCLUSION: We considered that the PN stress induced by the daily administration of PG and ATD, results in a slower execution of the MSB in both groups and avoid distinguish the effect of the ATD. Then chronic PN injections, as a route of administration, could act as mild stressor and may have additive effects on drugs affecting brain sexual differentiation.

INTRODUCTION

Sexual differentiation is a series of ordered, sequential events that begins with sex determination during the embryonic stage and continues with the action of steroid hormones during critical periods in the prenatal and neonatal stages. This process occurs at the gonadal, hormonal and brain levels, culminating in the establishment of reproductive and non-reproductive, sexually dimorphic behaviors (Arnold 1996; Arnold *et al.* 2004; Carruth *et al.* 2002; Davies & Wilkinson 2006; DeVries 2004; Morales-Otal *et al.* 2009).

The process of brain sexual differentiation is determined by two mechanisms that have been termed the organizational-activational mechanisms of sex hormone action an idea first set forth by Phoenix et al in 1959 (Phoenix *et al.* 1959). The first mechanism organizes the brain structures during embryonic development as well as the neurotransmitter systems involved in the expression of reproductive and non-reproductive sexually dimorphic behaviors. The effects involved in this mechanism are irreversible and permanent, but it is possible to permanently alter the sexual differentiation of an individual by administrating hormones or drugs during specific stages or critical periods of brain development (Arnold 2009; McCarthy 2007; Woodson & Gorski 2000).

In a later stage, steroids could act on sexually differentiated brain tissue in order to activate previously organized functions and behaviors. These effects are reversible and temporary, and they constitute the activational mechanism of sex hormones (Arnold 2009; Kruijver *et al.* 2001; McCarthy 2007; Woodson & Gorski 2000).

It has been demonstrated that two important biochemical processes occur in different areas of the brain: reduction of testosterone (TES) to dihydrotestosterone by the 5 alpha-reductase enzyme, and the aromatization process, including the transformation of testosterone into estradiol (E_2) by the cytochrome p-450 enzyme aromatase (Wilson & Davies 2007).

It has been shown that E_2 is the principal hormone in masculinization of the brain in many mammals. In humans and rodents, these biochemical processes occur during critical periods of sex differentiation, during which the brain is vulnerable to modification by sex hormones. In male rats, there are two critical periods related to plasma testosterone surges: the first occurs between days 17 and 18 of gestation (Weisz & Ward 1980) and the second during the first 1 to 3 hours after birth (Slob *et al.* 1980).

During these critical periods it is possible to manipulate the embryo and the neonate by administering or applying different drugs or stressors in order to permanently alter sex differentiation at the gonad and brain levels (Canchola *et al.* 1986; Gorski 1986).

Some researchers have argued that sexual differences in some male-female brain structures are related to the expression of sexually dimorphic behaviors, and also to gender identity and sexual orientation (Swaab 2004). This could demonstrate that there is a biological component to the regulation of sexual orientation (Byne *et al.* 2001; De Cecco & Parker 1995; Gooren 2006; James 2005; Mustanski *et al.* 2002; Swaab 2004; 2008).

In order to resolve this question, several studies have been conducted in rats, administering aromatase inhibitors during the critical periods of sexual differentiation and blocking the aromatization of testosterone into E_2 in order to modify sexual patterns during adulthood. Some studies have demonstrated that the alteration of the male sexual behavior depends on the period selected for the administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD): prenatal, neonatal or perinatal (prenatal and neonatal). For example, a significant decrease has been observed in the number of mounts, intromissions and ejaculations in adult male rats who have been treated with ATD during the neonatal (Bakker *et al.* 1993a,b; Brand *et al.* 1991) or perinatal stage (Brand *et al.* 1991; Houtsmuller *et al.* 1994; Swaab *et al.* 1995).

However, contradictory results have been found regarding the effect of prenatal ATD administration on male sexual behavior. While some researchers have not found significant differences in the number of mounts, intromissions and ejaculations between the control group and the prenatal ATD group (Brand *et al.* 1991; Houtsmuller *et al.* 1994; Swaab *et al.* 1995), as others have (Gladue & Clemens 1980; Whalen & Olsen 1981).

These differences could be partly attributed to the fact that the sexual activity obtained by the prenatal ATD males has been compared with that of control males treated prenatally with propylene glycol (PG), the vehicle used to dissolve ATD (Brand *et al.* 1991; Houtsmuller *et al.* 1994; Swaab *et al.* 1995), instead of comparing them with males from mothers who had remained intact through gestation. This is because PG administration during gestation is supposedly innocuous and incapable of altering the sexual behavior of control group males. However, some evidence suggests the contrary. For example, it has been shown that high doses of PG during short periods results in toxic effects (Glover & Reed 1996; Zar *et al.* 2007). This is the case of its use as a solvent in the preparation of synthetic multivitamins, like dihidrotaquisterol (MacDonald *et al.* 1987a,b), barbiturates like pentobarbital and phentobarbital (Lolin *et al.* 1988; Yorgin *et al.* 1997) and anxiolytics like benzodiazepine and lorazepam (Arbour 1999; Arroliga *et al.* 2004; Bedichek & Kirschbaum 1996; Chicella *et al.* 2002; Horinek *et al.* 2009; Yaucher *et al.* 2003). Also, Grimm & Frieder (1987) demonstrated that daily administration of the vehicle used to dissolve the anxiolytic diazepam, made up of 40% PG and 8% ethanol dissolved in 0.9% saline solution, from days 6 to 20 of gestation, is capable of producing noticeable changes in the behavior of these mothers' offspring. Finally, it has been demonstrated that chronic injections of saline

during late pregnancy could act as a mild stressor and induce important alterations in the Hypothalamus-Pituitary-Adrenal axis (HPA), and the behavior of the rat´s offsprings during adulthood (Dickerson *et al.* 2004; Cratty *et al.* 1995; Ward *et al.* 2000).

Thus, in order to dismiss the possible influence of PG or the mild stress produced by chronic injections during prenatal ATD treatment, it was important to add another group of control males to those who were not administered anything during gestation. This is why one of the goals of this study is to evaluate the effect of prenatal ATD administration on male sexual behavior, comparing it with prenatal PG administration and a group of untreated control males in order to determine whether or not prenatal ATD administration has an effect which alters sexual behavior in adult rats. Another of the study's goals is to more precisely analyze the effect of prenatal ATD administration on the different patterns and parameters of male sexual behavior, and not just on ejaculatory frequency and the number of mounts and intromissions preceding ejaculation, as has been done in prior studies.

MATERIAL AND METHODS

Adult female Wistar rats (200–250 g) from the Universidad Autónoma Metropolitana-Iztapalapa were kept in standard conditions under a reversed 12-hr. light-dark cycle (lights on at 01:00), with free access to commercial rat pellets and water. Female rats were always time mated. They were fertilized by way of three ejaculations from expert male rats and were then divided into three groups (n=7 rats). The first group did not receive any kind of treatment during pregnancy (control intact group, CON-I). The other two groups (PG and ATD) received an intraperitoneal (i.p.) injection of 0 and 5 mg of 1,4,6-androstatriene-3,17-dione (ATD), respectively dissolved in 0.1 ml of propilenglicol (PG) as a vehicle from days 11 to 22 of gestation. We decided the use PG as a vehicle, considering that other authors had reported the same (Brand *et al.* 1991; Houtsmuller *et al.* 1994; Swaab *et al.* 1995).

After birth the pups were counted and their sex was determined. One month later the male rats were separated according to the treatment applied to dams in three different groups: CON-I (n=7), PG (n=18) and ATD (n=48). Before the sexual tests**,** Wistar female rats (160–200 g) were ovariectomized under deep anesthesia with ether and were used after a recovery period of fifteen days. Then the ovariectomized female rats were made sexually receptive by administering a subcutaneous injection of 10 μg/100 μl oil, estradiol benzoate (Sigma Chemical Co.) and 0.5 mg/100 μl of progesterone oil (Sigma Chemical Co.), 44 and 4 hours respectively prior to testing. All behavioral tests were conducted during the early dark portion of the light/dark cycle under a dim red light. All the procedures were in strict compliance with the "Regulations of the General Health Ministry" approved by the Animal Care Commitee from the Universidad Autónoma Metropolitana.

At three months, male sexual behavior (MSB) was analyzed in all the naive males during four 30 min weekly tests. Five minutes prior to starting the test, each male rat was placed in a Plexiglas cylinder (50 cm diam., 40 cm high), with wood shavings in the floor for habituation. Upon presentation of the female, the following parameters were recorded: latencies to first mount, first intromission and first ejaculation (from the first intromission). The number of mounts, number of intromissions, ejaculation frequency (EF = number of ejaculations during 30 min. of recording), hit rate (HR = number of intromissions/number of mounts + number of intromissions), as well as the post-ejaculatory refractory period (PERP = time between ejaculation and subsequent intromission) and percentage of subjects that mounted (%SsM), intromitted (%SsI), and ejaculated (%SsE) were registered.

Statistics

All of the parameter results were analyzed statistically using non parametric Kruskall-Wallis' ANOVA, followed by Dunn analysis. A chi square was applied for the percentage cases.

RESULTS

In the pups delivered by dams treated with PG and ATD, no apparent morphological changes were observed in comparison with the control group, and the percentage of stillborns was minimal in all groups.

Figure 1 shows the percentage of rats that mounted (%SsM), intromitted (%SsI) and ejaculated (%SsE) during four consecutive sessions in all groups. With the exception of the first test, in which only one male in the CON-I group did not mount, in the other sessions all CON-I males mounted, intromitted and ejaculated. Compared with the sexual activity of the CON-I group, prenatal administration of both PG and ATD produced a reduction in the proportion of animals displaying all aspects of male sexual behavior in all the sessions. However, the first test was the only one in which these differences were statistically significant in terms of the proportion of male rats in the ATD group performing mounts (χ^2=6.0, d.f.=1, *p*<0.014), intromissions and ejaculations (χ^2=5.5, d.f.=1, *p*<0.019 in both parameters) in comparison with the CON-I group. Despite the lower values obtained by the PG males in the percentage of subjects displaying mounts, intromissions and ejaculations these results did not differ significantly from the CON-I group.

Figure 2 shows latency to mount, intromit and ejaculate for the three groups of males in the four tests during the first copulatory series, with the sample sizes per group shown in the bottom of the bars. In most tests, the CON-I group showed the shortest time to
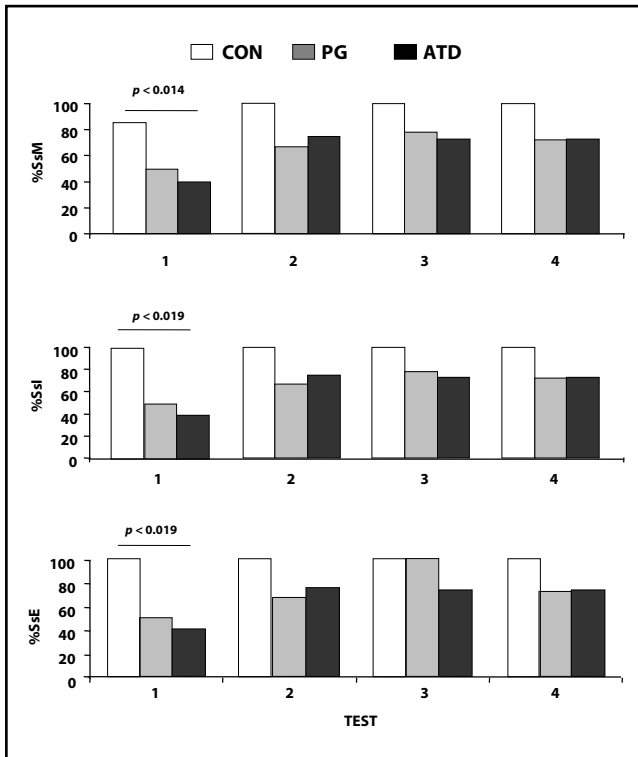
Fig. 1. Percentage of males mounting (%SsM), intromitting (%SsI) and ejaculating (%SsE) achieved by three groups of naive male rats: Control (CON, n = 7), Propilene glycol (PG, n = 18) and 1,4,6-androstatriene-3,17-dione (ATD, n = 48) during four weekly tests (Probability was obtained after CHI-Square test).

Fig. 2. Mount, intromission and ejaculatory latencies achieved by three groups of sexually naive male rats: Control (CON), Propilene glycol (PG) and ATD during four weekly tests. Numbers below bars in the bottom of the figure represents the sample sizes. (Asterisk indicates significant difference between ATD and CON groups. * $p<0.05$ after Kruskall-Wallis' ANOVA, followed by Dunn).

mount, intromit and ejaculate and the ATD needed the most time, but only in a few of the tests did the ATD group show a significant ($p<0.05$) increase in comparison with the CON-I group in terms of mounting, intromission and ejaculatory latencies (tests 1 and 4).

The ejaculatory frequency and post-ejaculatory refractory period shown in the first copulatory series by each group of male rats in the four tests are shown in Figure 3. Sample sizes per group are also shown at the bottom of the figure. The initial sample sizes of the groups were CON-I, n=7, PG, n=18 and ATD, n=48. For the first behavioral sexual parameter, the CON-I group showed a similar ejaculatory frequency in most of the four tests, with a mean of three ejaculations. In comparison with the CON-I group, both experimental groups ATD and PG showed a reduction in ejaculatory frequency in the four tests, but only in the first, second and fourth tests did the ATD males show a significant reduction ($p<0.01$, tests 1 and 4; $p<0.05$, test 2) when compared with the CON-I group. For the post-ejaculatory period, a more varied response was observed in all groups. Nevertheless, only in the third and fourth tests was the post-ejaculatory refractory period for ATD males significantly longer ($p<0.01$) in comparison with the CON-I group.
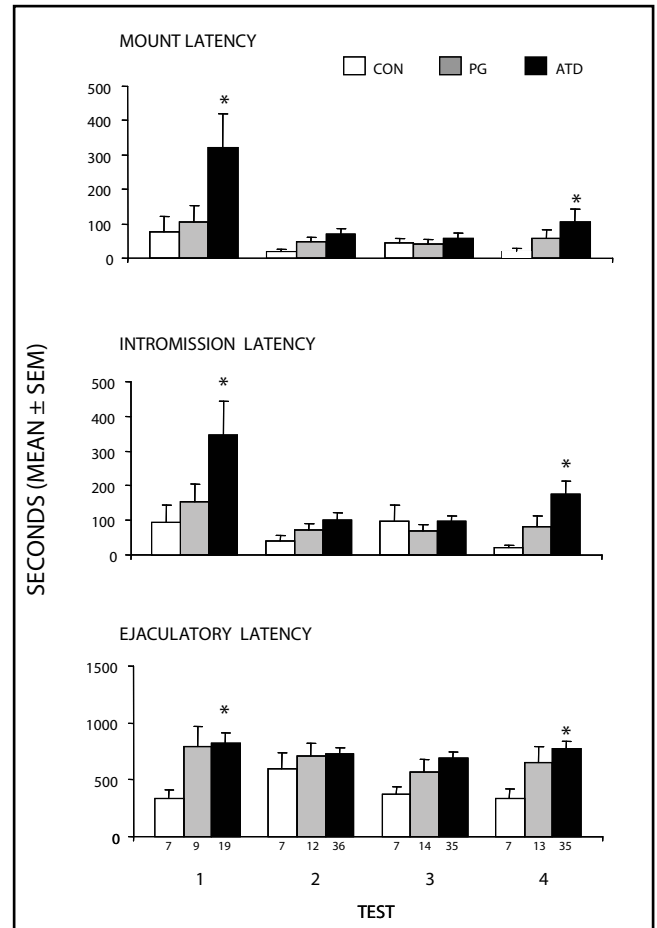
Finally, the number of mounts and intromissions preceding the first ejaculation, along with the data for hit rates for the three groups of males during the four consecutive tests (means ± SEM), are shown in Figure 4. For these three parameters, the three groups of males showed minor variations during the four tests, without any significant differences between them in each test.

DISCUSSION

Many studies have shown that adult male rats in which brain estrogen formation was inhibited through administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) show altered sexual behavior and partner preference during adulthood (Bakker *et al.* 1993a,b; 1995a,b; 1996; Brand *et al.* 1991; Dickerson *et al.* 2005; Houtsmuller *et al.* 1994; Swaab *et al.* 1995).

However, different results were obtained when ATD was administrated prenatally, neonatally or perinatally. In most cases, male rats showed bisexual behavior and
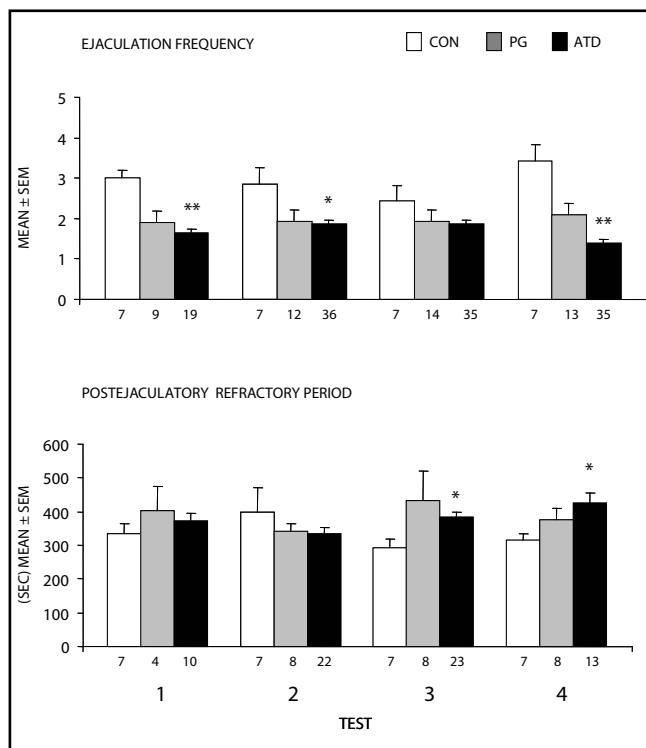
Fig. 3. The ejaculation frequency and the postejaculatory refractory period displayed during the first copulatory serie by three groups of naive male rats during four weekly tests. Sample sizes are indicated at the base of each bar. (Asterisks indicates significant difference between ATD and CON groups.** *p*<0.01; * *p*<0.05 after Kruskall-Wallis' ANOVA, followed by Dunn).

Fig. 4. Number of mounts, number of intromissions and hit rate achieved by three groups of sexually naive males: Control (CON), Propilene glycol (PG) and ATD during four weekly test. Numbers below bars in the bottom of the figure represents the sample sizes.

altered sexual behavior mainly when ATD was administered perinatally (Brand *et al.* 1991; Houtsmuller *et al.* 1994; Swaab *et al.* 1995) while contradictory or controversial results have been obtained in prenatal administration of ATD (Gladue & Clemens 1980; Whalen & Olsen 1981).

When a detailed study of the effect of prenatal ATD administration on the different patterns and parameters of male rat sexual behavior is performed and it is compared with the sexual behavior of males delivered by intact, pregnant females (CON-I group), as in this work, it may be observed that prenatal ATD also has an important effect on the development of this behavior. In our study, the ATD males demonstrated difficulty in expressing male sexual behavior, given that in the first test the percentage of ATD males who mounted, intromitted and ejaculated was significantly lower than in the CON-I group. ATD males also showed significantly greater mount, intromission and ejaculation latencies, than the CON-I group, as well as lesser ejaculatory frequency in the first test. Even though the ATD males showed improvement in these parameters in the second test, but in the fourth test they showed the same deficiency again, not just in the previously mentioned parameters, but also in terms of post-ejacu-
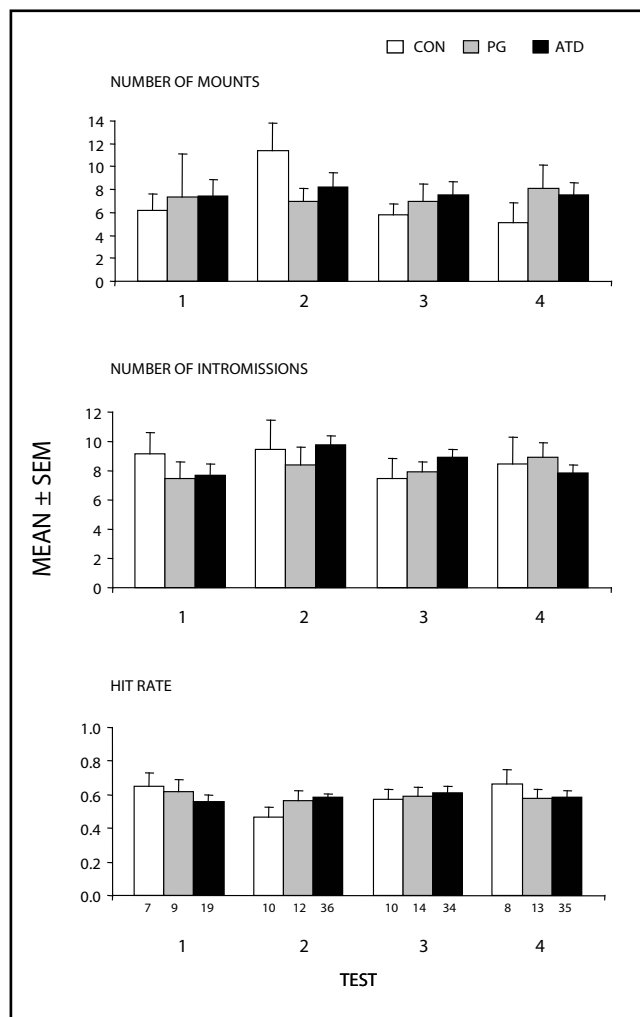
latory refractory period. All of this shows that prenatal ATD administration alters the parameters with which the appetitive/motivational component of male sexual behavior (i.e. mount latency, post-ejaculatory refractory period) as well as its consummatory components, are measured (percentage of subjects that mount, intromit and ejaculate) (Agmo 1997; Meisel & Sachs 1994).

In fact, our study is the first to demonstrate that prenatal ATD administration may cause a decrease in male ejaculation, in terms of the percentage of subjects who ejaculated and their ejaculation frequency, in a manner similar to the results obtained by other authors through perinatal or neonatal ATD administration (Brand *et al.* 1991; Gladue & Clemens 1980; Swaab *et al.* 1995; Whalen & Olsen 1981). It is important to note that our results for these sexual parameters were obtained only when we compared ATD-treated males with the CON-I group, but not with males treated prenatally with PG.

Several groups of researchers (Brand *et al.* 1991; Gladue & Clemens 1980; Houtsmuller *et al.* 1994; Swaab

et al. 1995; Whalen & Olsen 1981) have compared the effect on male sexual behavior of prenatal ATD administration (ATD prenatal group) with the effect of PG, the vehicle used to dissolve it (PG prenatal control group), in the same dosage and gestation period as our study, without finding significant differences between groups in ejaculatory frequency, percentage of subjects ejaculating, or number of mounts and intromissions preceding the first ejaculation, as was the case in our study. In this sense, one could say that our study confirms some of the findings obtained by the above mentioned researchers and that it does not furnish any substantially new information. However, the fact that we were able to find a significant decrease in the expression of various parameters of sexual behavior when comparing the prenatal ATD group with the results obtained by males delivered by females who remained intact during gestation (CON-I group) suggests that prenatal administration of PG may have, in and of itself, an effect on the expression of this behavior in the adult stage. This effect could be caused by PG or by the stress produced by the chronic injection. As we already mentioned in the introduction, it has been observed that chronic PG administration for short periods and high doses may be toxic (Glover & Reed 1996; Gooren 2006; Zar *et al.* 2007), and that it may even cause noteworthy behavioral modifications when PG is injected daily from days 6 to 20 of gestation as part of the vehicle which dissolves the anxiolytic diazepam (Grimm & Frieder 1987). These studies allow us to suggest that prenatal PG administration could have had a toxic effect in our study and may have been responsible for the observed alterations in male sexual behavior in the group, which, in most cases, resembled the prenatal ATD group more than the CON-I group. Although in our study we know that the most adequate way to proceed is to compare the effects of prenatal ATD with the results obtained by the prenatal PG group, given that this is the vehicle in which ATD was dissolved, we believe that it could be important to include an intact control group in order to determine the effect that administration of the vehicle alone could have. As we were able to demonstrate in this study, the effects obtained by the prenatal PG control group and the CON-I group were not the same, and the significance of the effect of prenatal ATD administration depended on the control group it was compared to. Other researchers have also observed different effects of prenatal ATD administration, depending on whether or not the control group used received prenatal PG or not. For example, Brand *et al.* (Brand *et al.* 1991), in a first experiment, administered ATD prenatally (days 11–22 of gestation) by way of sylastic capsules implanted subcutaneously, and the control group was made up of gestating rats who were implanted with an empty sylastic capsule during the same period. In this first study, Brand *et al.* (Brand *et al.* 1991) did not find significant differences in ejaculatory frequency between the two groups (sylastic capsule with ATD vs. empty sylastic

capsule). However, in a second study they administered the same dose of ATD we used (5 mg of ATD dissolved in 0.1 ml of PG) by way of daily subcutaneous (s.c) injections performed on female rats during the same gestation period. In this case, the control group was made up of gestating rats to whom only the vehicle was administered, that is, 0.1 ml of PG daily, the same as our prenatal PG group. In their study, Brand *et al.* (Brand *et al.* 1991) obtained significant differences in ejaculatory frequency between the prenatal ATD group and the prenatal PG control group. The results of our study, along with Brand's *et al.* (1991) results, show that the ATD administration pathway may bear an influence on the drug's effect on the sexual differentiation process in the brain. Therefore, it would be valid to speculate whether or not the administration of daily injections (that is, intraperitonal, i.p, as in our study or s.c as in Brand's work), during the period of gestation in which the organizational changes in the rat brain occur (days 11–21 of gestation), could have a stressful effect capable of altering the sexual differentiation process in the rat brain. As already mentioned, Grimm and Frieder (1987) also found behavioral alterations in the adult stage in rats whose mothers had received daily s.c. injections of the vehicle used for the anxiolytic diazepam, made up of 40% PG and 8% ethanol dissolved in saline solution.

On the other hand, other researchers have recently identified the administration of daily injections of saline solution in the last week of gestation as a mild stress factor and they have shown that these injections can modify the Hypothalamus-Pituitary-Adrenal axis (HPA), which acts modulating emotional and behavioral states, including stress and anxiety (Cratty *et al.* 1995; Dickerson *et al.* 2004; Ward *et al.* 2000). For example, it has been demonstrated that the offspring of mothers who had been exposed to this type of stress, compared to those from the control group (undisturbed dams), display, in the adult stage, adrenal hypertrophy, high levels of corticosterone in plasma, a greater content and release of the Corticotropin-Releasing Factor (CRF) (Cratty *et al.* 1995) and a greater number of CRF receptors in the amygdala (Ward *et al.* 2000). It has been observed that this imbalance on the HPA axis leads to offspring displaying, in adult life, an increase in anxiety (Cratty *et al.* 1995) which makes them fearful and show increased defensive-withdrawal behavior (Cratty *et al.* 1995; Dickerson *et al.* 2004). Based on the above, the similar decrease in our study for the groups treated prenatally with ATD and PG in the percentage of subjects who mounted (%SsM), intromitted (%SsI) and ejaculated (%SsEy) in the first test, may be due to the fear produced by the prenatal daily injections in the offspring of both groups. As mentioned in our methodology, one month after birth the offspring were weaned and separated by sex, therefore the males were no longer in contact with the females until their first sexual test. Once mating with females became familiar to males in both groups, the effect over %SsM, %SsI,

%SsE disappeared in the rest of the tests. In the same manner, the fear produced by the stress of the prenatal injection in both groups of males (prenatal ATD and PG), could explain why these males were slower to copulate since they showed similar increases in mount, intromission and ejaculation latencies as well as in the post-ejaculatory refractory period, which in turn bore an influence on the observed decrease in ejaculatory frequency. It is important to point out that both groups (prenatal ATD and PG) had very similar sexual activity in all parameters and that significant differences were only seen when the results for the prenatal ATD group were compared with the CON-I group.

On the other hand, in an as of yet unpublished study (Ferreira-Nuño *et al.* 2011) in which we analyze the female sexual behavior of the females who are the sisters of the males treated prenatally with PG in this study, we were able to demonstrate that the prenatal PG females are also slower to copulate compared with offspring females from undisturbed dams, perhaps as a consequence of the mild stress caused by chronic injection of PG during the prenatal stage, and for this reason they were more fearful.

In fact, the alterations displayed in our study by the prenatal ATD and PG males in different parameters of male sexual behavior are similar to those displayed by the Sensitive Flinders Line (FSL) of rats who shown endogenous depression (Ferreira-Nuño *et al.* 2005), given that both groups of rats show a decrease in the indicatory parameters of the appetitive component of male sexual behavior (greater mount latency and PERP and lower %SsE), without displaying any alterations in the parameters which serve to evaluate the consummatory component of this behavior (e.g. number of mounts and intromissions preceding ejaculation). Furthermore, FSL rats also exhibit alterations in the HPA axis (Malkesman *et al.* 2006; Overstreet *et al.* 2005).

In accordance with what has just been described, we believe that in our study it is difficult to separate the effect caused by ATD from the one produced by prenatal stress as a result of the injections by which this drug was administered. This is even more difficult if we consider that it has been shown that prenatal stress decreases the activity of fetal brain steroid aromatase (Weisz *et al.* 1982), a similar process to that carried out by ATD. Nonetheless, it may be pointed out that the studies on sexual differentiation in which drugs or hormones have been chronically injected during gestation must be reexamined, given the fact that, as in our study, it is possible that the effect of the aforementioned substances may be influenced by the effects of the prenatal stress produced by the chronic administration of the drugs. There are many studies which point to the importance of prenatal stress during the sexual differentiation process in rats (Braastad 1998; Rhees *et al.* 1999; Velazquez-Moctezuma *et al.* 1993; Ward & Ward 1985; Ward *et al.* 1996; 2000; 2002a,b; 2003; Weisz *et al.* 1982). These studies show that chronic stress factors, more severe than i.p. or s.c. injections, such as body immobilization, flood lights, electric foot shocks and selective REM sleep deprivation, applied during gestation can demasculinize and feminize male sexual behavior, that is, reduce the expressions of male sexual behaviors and increase those typical of females, as occurs with males treated prenatally and perinatally with ATD (Velazquez-Moctezuma *et al.* 1993; Ward & Ward 1985; Ward *et al.* 1996; Ward *et al.* 2002b). Given that in rats there is also a critical period of sexual differentiation during the first days of life, also the mild stress produced by the daily injections of drugs during this period could have a similar effect on the HPA, as occurs during the prenatal administration. In this sense, studies exist which demonstrate that neonatal injection of saline solution may alter behavior as a result of modifying the HPA axis. For example, the daily manipulation of rat offspring, as well as daily neonatal administration of saline solution, produces a decrease in the response of the HPA axis to stress in the adult stage (Grota & Ader 1969; Meaney *et al.* 1989). Also, in another rodent, the prairie vole *Microtus ochrogaster*, it has been demonstrated that the simple fact of injecting 50 µl of saline solution intraperitoneally on the day of birth is capable of reducing mating latency in females in comparison with the group of females who were only physically handled during the same period (Cushing *et al.* 2005). Furthermore, among females of the same species, daily i.p. injection of saline solution during the first seven days of life led to an increase in aggressiveness, which was not observed in females who were only physically manipulated (Stribley & Carter 1999; Ward & Ward 1985). In light of this data, is necessary to reevaluate the studies on brain sexual differentiation in which a drug or hormone is administered chronically during the first few days of life, given that part of the effects may be linked to the effect of neonatal stress produced by the injection of the substance administered. Since the groundbreaking study by Phoenix *et al.* (1959) in which the organizational and activational actions of the gonad hormones in the process of brain sexual differentiation were set forth, hormones and drugs which alter this process have been administered during the critical stages, which present themselves during the prenatal and neonatal stages, and administered either through s.c. or i.p. injections. In light of recent findings which have shown that daily, chronic injection of substances during the prenatal and neonatal stages may be a mild stress factor capable of altering the HPA axis in rat offspring, it is important to determine with clarity and precision how chronic substance injection alone is capable of altering the brain's sexual differentiation process, in order to clarify which aspects of rats' sexual behavior is modified for this reason in the adult stage and which are attributable to the action of the substance administered. For example, the fact that in our

study ATD and PG males had a similar sexual behavior, suggest that prenatal mild stress induced by the chronic injections of PG had a more noticeable effect on brain sexual differentiation than ATD.

In conclusion, our study shows that the mild stress produced by the chronic injections during the prenatal stage may have an additive effect on the action of drugs administered to alter the process of brain sexual differentiation in the rat. For this reason, if a hormone or drug is to be administered prenatally or neonatally to alter the process of brain sexual differentiation, we suggest avoid administration by way of periodic injections, in order to effectively discard the mild stress effect this procedure may produce.

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