Prenatal lipopolysaccharide exposure affects maternal behavior and male offspring sexual behavior in adulthood

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Prenatal Lipopolysaccharide Exposure Affects Maternal Behavior and Male Offspring Sexual Behavior in Adulthood

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Abstract

Objective: This study investigates the effects of prenatal lipopolysaccharide (LPS) exposure on the maternal behavior of pregnant rats and the physical development and sexual behavior of their male offspring in adulthood. Methods: For two experiments, pregnant rats were injected with LPS (250 μg/kg, i.p.) on gestation day (GD) 21. In the first experiment, the maternal behavior (postnatal day, PND, 6) and the dam's open-field general activity (PND7) were evaluated. In the second experiment, the maternal pre- and postnatal parameters, the pup's development, the offspring's sexual behavior in adulthood, and the pup's organ weights were assessed. Results: Compared to the control group, the LPS-treated dams presented reduced maternal behavior, decreased general activity, a smaller body weight difference between GD21 and PND1, a greater number of perinatal deaths, and smaller litters. For the male pups, LPS treatment resulted in a decreased body weight on PND2, whereas the anogenital distance and the day of testis descent were not modified. The male sexual behavior was impaired by prenatal LPS. Particularly the number of ejaculating animals was reduced. The testis weight was also lower in the prenatally LPS-treated rats than in the control rats. Conclusion: We propose that prenatal LPS exposure on GD21 acts as an imprinting factor that interferes with the programming of brain sexual determination in offspring.
LPS administration to pregnant rats upregulates mRNA expression of the stress-related peptide, corticotrophin-releasing hormone, in the fetal brain [11], suggesting the possibility of inducing a fetal stress response. The activation of the stress response during pregnancy was shown to have long-term consequences on the response of adult offspring to stressful situations, as demonstrated in rodents, primates, and humans [12–17].

There is growing evidence that adverse early environments can have a profound and life-long influence on the responsiveness to stress through epigenetic programming. Early life is a period of heightened susceptibility to common stressors that might permanently modify the major regulatory systems, such as the hypothalamo-pituitary-adrenocortical axis [18, 19].

For many years, the stress response induced by physical or emotional challenges has been recognized as a profound disruptive factor in reproductive function in both males and females [10, 20–23]. It has been hypothesized that prenatal stress disrupts the normal maternal hormonal milieu and suppresses the fetal testosterone peak on gestational days (GD) 18 and 19, a peak necessary for later expression and maintenance of male sexual behavior [3, 24]. In addition, the critical period for the organizational actions of the gonadal hormones on the sexual differentiation of the rat brain extends from approximately the last week of prenatal life through the first postnatal week [23, 25, 26]. Thus, sexual behavior programming is controlled by the presence or absence of the appropriate hormones and various central neurotransmitters in the prenatal and postnatal periods [16, 26, 27]. In this respect, a negative influence of maternal stress on the reproductive function of male pups has been demonstrated [3, 10, 16, 17, 19, 28]. Based on this, different types of stressful events may sometimes produce qualitatively different patterns of effects on both the behavior and physiology [29–33].

The aim of this study was to determine the effects of prenatal LPS exposure on possible changes in brain sexual determination and sexual performance of rats in adulthood. First of all, we examined the effects of prenatal LPS exposure on maternal behavior in the rat. The alterations in the maternal behavior could thus also make a strong contribution to the long-term effects of stress on the pups’ programming behaviors, including sexual brain determination [27, 33, 34]. The maternal general activity in the open field was examined to investigate the possible emotional or motor contributions on disruption of maternal behavior [26]. Finally, the prenatal LPS effects on the pups’ development and sexual behavior in adulthood were examined.

Animals and Methods

Animals

Pregnant Wistar rats were used from our own colony, weighing approximately 220–260 g each (GD0 = the presence of spermatozoa in the vaginal smear). The dams were individually housed in polypropylene cages (38 × 32 × 16 cm) at a controlled room temperature (20 ± 2°C) on a 12/12-hour light/dark cycle (lights on at 6:00 h) and had free access to food and water. The animals were distributed into control and experimental groups (n = 6/group for maternal behavior and the maternal general activity, respectively; n = 10/one pup each litter for the development and adulthood behaviors). All of the pregnant rats were allowed to give birth and nurture their offspring normally. No cross-fostering was performed, because the cross-fostering procedure per se alters the behavioral and corticosterone responses to LPS [35].

Parturition was considered to be postnatal day (PND) 1.

Two experiments were performed. In the first experiment, the maternal behavior (PND6) and dam’s general activity (PND7) were evaluated. The pups used in these experiments remained undisturbed until PND6 for the maternal behavior test. In the second experiment, other pregnant rats were employed: the maternal pre- and postnatal parameters, the pup’s development, the offspring’s sexual behavior in adulthood, and the pup’s organ weights were assessed. Thus, after delivery, on PND2, the number of pups and the litter weight were assessed. Immediately after weighing, eight offspring, four males and four females, were randomly selected (by anogenital differences, greater in males). On PND10, these offspring were weighed. On PND21, the litters were separated, weighed, housed together by sex, and grouped. In both experiments, the rats and their parents were maintained in the same laboratory conditions. Two male pups from each litter were used in the development evaluations and another two in the sexual behavior tests in adulthood, respectively. The female offspring were kept apart, because they were being used in a different experiment. The animals used in this study were maintained in accordance with the guidelines issued by the Committee on the Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil (Protocol FMVZ-USP – No. 926/2006).

Treatment

LPS (from Escherichia coli, Sigma, serotype 0127:B8, 50 μg/ml of LPS in 0.9% sodium chloride solution) was intraperitoneally administered to pregnant rats in a 250 μg/kg dose on GD21. The control group consisted of pregnant rats submitted to the same treatment schedule with saline. Each dam was administered 0.1 ml/100 g saline or LPS solution. The LPS dose chosen was based on a study by Reijo et al. [25], who showed that 200 and 250 μg/kg LPS decreased the release rates of luteinizing hormone (LH) and follicle-stimulating hormone and suggested that gonadotropin-releasing hormone (GnRH) levels in the milk also decreased. In addition, this dose was reported to induce sickness behavior and fever in rats [36]. GD21 was chosen because previous studies in our laboratory revealed that LPS (250 μg/kg) induced at least 3 consecutive days of sickness behavior in pregnant rats (data not published). This period (GD21 to PND2) is critical for GnRH release of testosterone in the milk to the pups [27].
Maternal Evaluation

To perform the maternal behavior test on PND6, the pups were removed at 07:00 h and placed in another home cage away from their mother. Sixty minutes after the maternal separation, all pups were returned to the cage of their mother, and the maternal behavior testing began. The retrieval of the first pup (in s and %), the retrieval of all pups (s and %), grouping (s and %), full maternal behavior (s and %), crouching (%), self-grooming (s), and pup grooming (s) were scored [37, 38]. The animals were scored as having full maternal behavior if they retrieved all 8 pups to the nest and displayed nursing behavior with their back arched over the pups for 3 consecutive minutes. If the animals were not scored as having full maternal behavior after 30 min of continuous observation, they were checked every 15 min until 60 min and then hourly until a full maternal behavior was observed. The events observed after the first 30 min of continuous observation were recorded at the time of the first observation (e.g. if a full maternal behavior was first observed at 60 min; the full maternal behavior latency was scored as 60 min). The same criterion was used for all of the responses.

The general activity of these dams was observed in an open-field arena for 5 min. The arena was 97 cm in diameter and 28 cm in height. This arena was washed with a 5% alcohol solution before the behavioral testing to eliminate a possible bias due to odors left by previous subjects. On PND7, the pups were removed at 08:00 h and placed in another home cage away from their mother. Sixty minutes later, the dam rats were placed in the open-field arena and evaluated for locomotion frequency (the number of floor units entered), rearing frequency (the number of times the animal stood on its hind legs), the immobility duration (the total number of seconds without movement), and the total grooming duration (measured in seconds) [39].

Pre- and Postnatal Evaluations

The maternal body weight was measured on GD1, GD20, GD21, and on PND1, after parturition. The maternal body weight difference (BWD) between these days was calculated. The time between the treatments and delivery was measured. On PND2, the number of pups/litter was assessed.

Offspring Evaluations

Each pup’s body weight was measured on PND2, PND10, and PND21. The male pups’ anogenital distance was measured on PND2 and PND21 by a pachimeter. The day of the male pups’ testis descent was also assessed between PND15 and PND25.

The male offspring sexual behavior was measured in adulthood (PND100). All of the tests to evaluate the sexual behavior in the male rats were performed in a 12-hour inverted light-dark cycle (lights on at 22:00 h) between 14:00 and 17:00 h, i.e. during the dark phase of the light-dark cycle. The sexual behavior was observed in a wooden cage (56 × 35 × 31 cm) with a moveable cover and frontal glass. A sawdust layer served as the bedding for the animals. During the observation, two 25-watt infrared lamps provided room illumination. The rats were accustomed for 15 days to the reversed light/dark cycle before beginning the sexual behavior studies [40, 41].

To investigate the sexual behavior, male rats were allowed to mount ovariectomized females, sexually activated with exogenous estradiol (50 μg/kg s.c., 54 h before the tests) and progesterone (2.0 mg/kg s.c., 6 h before tests). These female lure rats were tested for receptivity before being placed with the males. The females that presented lordosis after a male mount were selected for the study. Each naive male rat was individually allowed to acclimate to a behavioral box for 5 min. A receptive female was then introduced, and the sexual behavior was measured during a 40-min time period. Each lure female was used in only two tests. The following parameters were recorded: the first mount (a mount without intromission); the first intromission (a mount with vaginal insertion), and the first ejaculation latencies; the number of mounts and intromissions until the first ejaculation; the post-ejaculatory mount latency (ML); the number of ejaculations, and the number of animals ejaculating in each group. Additionally, other derived parameters were calculated: the total mounts until ejaculation; the total mounts in 40 min; the mount frequency by minute (the number of mounts divided by the time from the first mount to ejaculation); the intromission frequency by minute (the number of intromissions divided by the ejaculation latency, EL), and the copulatory efficiency or hit rate (the number of mounts, NM, plus the number of intromissions, NI, divided by the time from the first mount until ejaculation × 100). The sexual activity index (SAI) was calculated as proposed by Agmo et al. [42]:

\[
SAI = \log\left(\frac{1}{ML} \times t\right) + \log\left(\frac{1}{IL} \times t\right) + \log\left(\frac{1}{EL} \times t\right) \cdot \left(\frac{NM}{NI} + Y\right)
\]

where \(t\) is the time of observation, IL equals intromission latency and Y means four when an animal’s ejaculation occurred and zero when it did not.

All latencies were calculated in minutes and only the animals that ejaculated were included in statistical analysis, except for SAI.

After the sexual behavior observations (PND100), the animals were anesthetized and the testis, seminal vesicle, and ventral prostate gland were removed for the wet-weight determination.

Statistical Analysis

Homooscedasticity was verified through a Kolmogorov-Smirnov test. Thus, Student’s t test and two-way ANOVA were used, since the observed data were parametric. For the nonparametric data, the Mann-Whitney U test was employed. To analyze the frequency data, Fisher’s test was employed. In all cases, the results were considered significant if \(p < 0.05\). The statistical computer programs GraphPad Instat and GraphPad Prism were used throughout.

Results

Maternal Evaluation

Compared to the data of the control group, the LPS-treated dams presented a significantly decreased percentage of crouching and displayed full maternal behavior (table 1). Moreover, the animals from the LPS group presented increased latencies in retrieving the 3rd to 8th pup with respect to the control group (fig. 1). The animals in the LPS group continued digging in the wood chips and building nests instead of retrieving pups, resulting in longer latencies to retrieving the pups. Such disruptive ef-
Effects were not observed in the control group. Finally, maternal crouching, pup grooming, as well as maternal grooming were similar between the control and experimental groups (Table 1).

LPS treatment reduced the dams’ general activity, indicated by a significant difference between the open-field parameters in the control and experimental groups: the locomotion frequency (control group = 106.20 ± 7.8; LPS group = 84.00 ± 6.00, p = 0.048), rearing frequency (control group = 24.10 ± 1.70; LPS group = 15.11 ± 1.10, p = 0.002), immobility duration (control group = 45.10 ± 6.10; LPS group 108.00 ± 9.70, p = 0.0006), and grooming duration (control group = 10.20 ± 3.30; LPS group = 1.8 ± 0.7, p = 0.032).

**Pre- and Postnatal Evaluation**

A statistically significant difference between the control group and the LPS-treated dams regarding weight between GD21 and PND1 was observed. The experimental dams had significantly less BWD than the controls (control group: 96.27 ± 8.46; experimental group: 78.06 ± 4.05, p < 0.0050). There was no difference in the BWD between GD1 and GD20 and the time between the LPS administration and delivery in the control and experimental groups (data not shown). A further analysis showed that the animals treated with LPS presented 5 perinatal deaths related to different litters, whereas those of the control group presented just one. The number of offspring per litter on PND2, before the pups were culled, was significantly different: it was 12.43 ± 1.99 in the control group and 9.50 ± 2.13 in the experimental group (p < 0.01).

**Offspring Evaluation**

Figure 2 illustrates the offspring body weights of the prenatally LPS-treated and saline-treated rats on PND2, PND10, and PND21. Two-way ANOVA showed the existing differences between the days [F (2/54) = 8.967, p = 0.0041] and the performed treatments [F (1/54) = 3.163, p = 0.0001]. No interaction was observed between the factors. The post hoc test indicated a decreased body weight in the prenatal LPS-treated pups on PND2.

### Table 1. The effects of LPS administration (250 µg/kg on GD21) on the maternal behavior observed on PND6

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>LPS group</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pup retrieval</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st pup, s</td>
<td>10.53 ± 4.52</td>
<td>12.83 ± 4.98</td>
<td>0.739</td>
</tr>
<tr>
<td>1st pup, %</td>
<td>100</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>All pups, s</td>
<td>116.17 ± 22.58</td>
<td>413.60 ± 132.13</td>
<td>0.037</td>
</tr>
<tr>
<td>All pups, %</td>
<td>100</td>
<td>66.66</td>
<td>0.455</td>
</tr>
<tr>
<td>Grouping, s</td>
<td>221.40 ± 28.80</td>
<td>717.00 ± 189.00</td>
<td>0.027</td>
</tr>
<tr>
<td>Grouping, %</td>
<td>100</td>
<td>66.66</td>
<td>0.455</td>
</tr>
<tr>
<td>Full maternal behavior, s</td>
<td>1,008.00 ± 169.20</td>
<td>1,608.00 ± 37.20</td>
<td>0.029</td>
</tr>
<tr>
<td>Full maternal behavior, %</td>
<td>100</td>
<td>66.66</td>
<td>0.455</td>
</tr>
<tr>
<td>Crouching, s</td>
<td>13.67 ± 3.84</td>
<td>16.57 ± 3.21</td>
<td>0.562</td>
</tr>
<tr>
<td>Crouching, %</td>
<td>100</td>
<td>66.66</td>
<td>0.002</td>
</tr>
<tr>
<td>Self-grooming, s</td>
<td>10.20 ± 3.63</td>
<td>17.00 ± 3.23</td>
<td>0.562</td>
</tr>
<tr>
<td>Pup grooming, s</td>
<td>42.50 ± 6.23</td>
<td>33.67 ± 4.86</td>
<td>0.289</td>
</tr>
</tbody>
</table>

Data are means ± SEM or percentages; n = 6/group. Statistically significant differences are italicized. The data given in seconds were analyzed by Student’s t test, and those given as percentages were analyzed using Fisher’s exact test.
The anogenital index and the day of testis descent were not different between the groups (data not shown).

Table 2 shows the results of the sexual behavior obtained from the animals prenatally treated or not treated with LPS on GD21. The percentage of animals with ejaculation was significantly smaller in the experimental group than in the control rats (control group = 12/12 rats, 100%; experimental group 9/12 rats, 60%; p = 0.02). In relation to the rats in the control group, the latency to the first ejaculation was higher in the experimental animals (p < 0.05). The number of mounts in 40 min (p < 0.01), the mount frequency (p < 0.05), and the sexual index (p < 0.01) were also reduced in the prenatally LPS-treated animals compared to those in the control group.

No differences between the control and experimental groups were observed for the remaining parameters of sexual behavior analyzed, i.e. the number of mounts and intromissions until the first ejaculation, the number of intromissions in 40 min, the latencies to the first mount, the intromission after the first mount ejaculation, the frequency of intromissions/min, and the copulatory efficiency.

Means ± SEM. n = 10/group. p < 0.05 vs. the control group (Student’s t test).
The absolute testis weight was significantly decreased (p < 0.05) in the experimental group compared to the control rats (table 3). Furthermore, body weight (p = 0.219), seminal vesicle (p = 0.082), and prostate (p = 0.728) absolute weights did not differ.

**Discussion**

There is considerable evidence that perinatal LPS administration induces long-lasting effects in offspring development and behavior [12–17]. The present study reveals the novel finding that exposure to the bacterial endotoxin in neonatal life modifies a dam's maternal behavior and the offspring's sexual behavior in adulthood.

The LPS administration on the last day of pregnancy impaired the ongoing maternal behavior. In fact, this endotoxin decreased the interactions between the dams and their pups, reflected by longer latencies in retrieving the pups and pup grouping. Moreover, LPS treatment significantly decreased the dam's full maternal behavior performance, since the latency to perform this parameter was higher in LPS-treated dams than in controls.

The retrieving and nest building behaviors may be more indicative of maternal motivation, whereas the nursing behavior may be indicative of a more reflexive maternal response [43]. In other words, retrieving may represent an active voluntary response, which reflects interest in and an attraction toward pup-related stimuli, and nursing may be transiently activated as a reflex when the female wanders near pups and they crawl under her. Our data show that LPS treatment makes the lactating animal less motivated to interact with their pups as well as reduced the nursing behavior. Besides, the dams had decreased exploratory and grooming behaviors, suggesting a reduced motivational state [44–46].

The reduced locomotion and rearing frequencies, as well as the increased immobility duration in the open-field test, may mean a decrease in the exploration caused by a low motivational state. In fact, the LPS-induced sickness behavior reduces the general activity of the rats [36]. In addition, this decrease in the open-field behaviors could be attributed to the maternal motor disturbance. However, the increased latency to retrieve pups was only apparent after the third pup, thus suggesting that motivational factors also have a part in the maternal behavior and the open-field disruption because the two distinct behavioral patterns displayed in both situations might be compromised by a more general phenomenon: an LPS-induced change in the motivational state. The reduced grooming duration observed here is in agreement with the decreased motivational state induced by LPS [47].

At the maternal level, a smaller BWD between GD21 and PND1, a greater number of perinatal deaths, and smaller litters were observed. Since LPS did not cross the body cellular barriers such as the placenta in normal conditions [48, 49], it is possible that LPS-released inflammatory mediators, such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6, reached the fetus within the uterus causing the perinatal deaths [50–52]. However, it is also possible that the maternal exposure to infection alters proinflammatory cytokine levels in the fetal environment, which may have a significant impact on fetus survival [12–17]. In addition, increased proinflammatory cytokine levels could affect the brain of the developing fetus and may be responsible for the inhibition of the reproductive axis and its normal function in the fetuses and newborn male rats [18].

Prenatal LPS (250 μg/kg) exposure produced only slight effects on the pup's development. In fact, a reduced body weight was detected just after delivery. No interferences were seen in the anogenital index and the day of testis descent. Thus, it is feasible to suggest that the maternal LPS inflammation at the end of pregnancy induced no effects on the hormonal processes involved in male sexual maturation in the early periods of the pup's life, particularly the GnRH [27]. However, the possibility may not be discarded that the different levels in the fetal and newborn reproductive axis may be effected by maternal cytokine exposure resulting in fetal and newborn levels of lower circulating hormones.

LPS exposure was liable to induce long-lasting effects in male offspring sexual behavior in adulthood. Indeed, the percentage of rats presenting ejaculation in the initial 10 min of the experiment was significantly reduced in rats exposed to prenatal LPS. Moreover, the latency to the first mount was increased, whereas the number of mounts in 40 mounts, the hit hate (frequency of mounts/min), and the SAI were decreased. Altogether, these behavioral changes suggested that prenatal LPS disrupted male offspring sexual behavior in adulthood.

The method to analyze the male offspring sexual behavior here employed allows us to distinguish between sexual motivation (the ease by which sexual behavior is activated or 'libido') and the execution of the copulatory acts (performance or 'potency') [53].

The mount latency is a measure of sexual motivation. The same is valid for the intromission latency, but in this case, it requires penile erection and coordinated activity of the striated penile muscles; therefore, it is not entirely
determined by sexual motivation [54]. Thus, it seems that the prenatal LPS exposure did not modify the offspring sexual motivation and penile erection. The number of mounts also reflects sexual motivation, but it may be confounded by other intervening factors and should be interpreted with caution [55]. In this respect, the rats showed a decreased number of mounts, i.e., a smaller mount frequency, suggesting that the motor aspects of sexual behavior might have been affected by prenatal LPS treatment. In addition, the latency to the first ejaculation and the smaller number of ejaculations seemed to reflect a decrease in the sexual behavior potency. As a result, the sexual performance (SAI) was also impaired.

Actually, we showed that prenatal LPS treatment impaired several aspects of male sexual behavior in adulthood. LPS treatment induces high levels of stress and releases corticosterone in rats [56]. It is now established that maternal stress has a demasculinizing effect on male sexual behavior in rats [31]. However, prenatal stress disrupts the normal maternal hormone threshold, resulting in the suppression of a surge in fetal testosterone on GD18–GD19, which is necessary for the later expression and maintenance of male sexual behavior [30]. In our experiment, LPS was administered on the last day of pregnancy. Thus, maternal sickness behavior remained at least 3 days after the treatment, thus reaching the postnatal period. Since it is known that newborn rats pass through a stress-nonresponsive period for the first 10 days after birth [57], a stress demasculinizing effect was not the cause of the sexual disruption observed here.

Immediately after birth and for at least 3 days after birth, while the females were still under the LPS effects, the cytokines released might have changed the GnRH concentrations in the mothers’ milk. Cytokines are responsible for the pattern of gonadotropin release that occurs during the infection process: IL-1 inhibits hormone secretions such as GnRH, LH, corticotropin-releasing factor, and growth hormone, and stimulates prolactin secretion [58–62]. Thus, despite the fact that we did not measure these hormones, the prenatal LPS exposure could have indirectly reduced the GnRH levels in the milk in the present set of experiments. Since milk GnRH is critical to the brain sexual determination in rats during the early lactation period [27], inhibition of this hormone, which is released into the milk, might have been responsible for the disruption in the male sexual behavior observed here. Thus, the significant reduction in maternal care could be responsible for the reduction in milk GnRH. The decrease found in the prenatally LPS-treated pups’ body weight on PND2 and on the testis weights is also in accordance with this proposition. In fact, the testis weight is determined by the number of Sertoli cells present in the testis, for which proliferation occurs during fetal life and continues into neonatal life and is dependent on gonadotropins and androgens [63].

Moreover, the reduction in GnRH milk transferred from the mother to the pups might be related to the maternal care. In our study, LPS disrupted maternal behavior [64]. Thus, it is possible that the pups received smaller quantities of milk as a consequence and therefore also a smaller amount of milk GnRH. A decrease in maternal milking might also explain the decreased body weight on PND2 in the pups.

Thus, we propose that prenatal LPS exposure on GD21 interferes with the pup’s brain sexual masculinization as a result of the decrease in the dam’s milk supply, thus resulting in a decreased GnRH release at birth by maternal sickness behavior. This is the first study demonstrating the effect of prenatal LPS exposure on male sexual behavior in adulthood. The study on the long-term neurobehavioral impact of the postnatal environment in rats is of primordial importance. This study suggests a remarkable correlation between pre- and postnatal maternal sickness behavior and offspring sexual behavior disruption later in life.

The cytokine and hormone levels may provide clearer data and point out the mechanism leading to the suggested change in the masculine behavior.

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