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The reduction in sexual behavior induced by neonatal immune stress is not related to androgen levels in male rats

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ABSTRACT

Purpose: It is known that various types of stress in early life increase the incidence of diabetes, myocardial infarctions, and psychiatric disorders in adulthood. We examined the mechanism by which neonatal immune stress reduces sexual behavior in adult male rats.

Methods: Male rats were randomly divided into 3 groups: the control (n = 17), postnatal day 10 lipopolysaccharide (PND10LPS) (n = 31), and PND25LPS (n = 16) groups, which received intraperitoneal injections of LPS (100 μg/kg) or saline (injection volume: ≤0.1 ml/g) on postnatal days 10 and 25. In experiment 1, male rats (age: 11 to 12 weeks) were put together with female rats in a one-to-one setting for mating, and sexual behavior (mounting, intromission, and ejaculation) was monitored for 30 minutes. The serum levels of luteinizing hormone (LH) and testosterone (T) and the hypothalamic mRNA expression levels of factors related to sexual behavior were examined. After experiment 1 finished, the remaining 37 male rats were used for experiment 2: the control group (n = 8), PND10 LPS group (n = 21) and PND25LPS group (n = 8) these rats had been given an i.p. injection of the saline during the expriment1. All of the rats were orchidectomized at 14 weeks of age. After a 3-week recovery period, a silastic tube containing crystalline T was subcutaneously implanted into the back of each rat. The rats' sexual behavior, serum hormone concentrations, and hypothalamic mRNA expression levels were assessed.

Results: In experiment 1, preputial separation occurred significantly later in the PND10LPS group than in the control group. The frequency of sexual behavior was significantly lower in the PND10LPS group than in the control group. The serum T concentrations of the PND10LPS and PND25LPS groups were significantly lower than that of the control group, but the serum LH concentrations of the 3 groups did not differ significantly. The hypothalamic mRNA expression levels of progesterone receptor B (*PRB*) and gonadotropin-releasing hormone (*GnRH*) were significantly lower in the PND10LPS and PND25LPS groups than in the control group, whereas the hypothalamic *PRA + B* mRNA expression levels of the 3 groups did not differ significantly. In experiment 2, after T supplementation the frequency of sexual behavior was significantly lower in the PND10LPS and PND25LPS groups than in the control group, although there were no significant differences in the serum T or LH concentrations or the hypothalamic *PRB*, *PRA + B*, or *GnRH* mRNA expression levels of the 3 groups.

Conclusion: In male rats, immune stress in the early neonatal period delayed sexual maturation, reduced sexual behavior, suppressed the serum T concentration, and downregulated the hypothalamic mRNA expression levels of *GnRH* and the *PR* in adulthood. The delayed sexual maturation was presumed to have been caused by the reduction in the serum T concentration. However, the rats that experienced neonatal stress exhibited reduced sexual behavior irrespective of their serum T concentrations.

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1. Introduction

It has been reported that experiencing immune, psychological, or metabolic stress in the early neonatal period has various long-lasting effects on physiological functions in adulthood, and it is also related to various diseases. For example, in humans undernutrition in the prenatal period increased the incidence of coronary heart disease in late adulthood, which is known as developmental origins of health and disease (DOHaD) or metabolic programming (Barker, 1995; Godfrey and Barker, 2000). In addition, maternal psychological stress in the prenatal period was found to be associated with lower cognitive and linguistic abilities in children aged five and a half years (Laplante et al., 2008). In rats, undernutrition in the prenatal period was demonstrated to be associated with an increased risk of type 2 diabetes (Park et al., 2008) and enhanced hypothalamic mRNA expression of the orexigenic peptide neuropeptide Y (Tungalagsuvd et al., 2016) in late adulthood. Furthermore, immune stress in the early neonatal period enhanced the activity of the hypothalamic-pituitary-adrenal (HPA) axis, increased the incidence of tumor metastasis, and caused body weight to rise in adulthood (Shanks et al., 1995, 2000; Hodgson et al., 2001; Boisse et al., 2004; Ellis et al., 2005).

In addition to metabolic functions, experiencing stress early in life can also have an impact on reproductive functions. We and others have reported that prenatal undernutrition delayed sexual maturation in male and female rats (Engelbregt et al., 2000; Iwasa et al., 2010a, b; Castellano et al., 2011; Matsuzaki et al., 2017), and immune stress in the early neonatal period delayed sexual maturation in male and female rats (Knox et al., 2009; Wu et al., 2011; Walker et al., 2011) and strongly suppressed the pulsatile secretion of gonadotropin-releasing hormone (GnRH) after the administration of lipopolysaccharide (LPS) in adult male rats (Li et al., 2007). Furthermore, maternal separation in the early neonatal period, which is a form of psychological stress, reduced sexual behavior in male rats in adulthood (Rhees et al., 2001), and immune stress in the neonatal period downregulated sexual behavior in male and female rats in adulthood (Walker et al., 2011).

Hypothalamic sex hormones, progesterone receptors (PR), and kisspeptin (Kiss1) play important roles in sexual behavior. It is well known that testosterone (T) stimulates sexual behavior in male rats (Stone, 1939; Beach and Holz-Tucker, 1949; Malmnäs, 1977) and most mammalian species (Meisel and Sachs, 1994). The androgen receptor is expressed in particular hypothalamic areas; i.e., the medial preoptic area (MPOA) and ventromedial nucleus (VMH) (Simerly et al., 1990), which have been demonstrated to contribute to male sexual behavior (Davidson, 1966; Hrat and Leedy, 1985; Harding and McGinnis, 2003). T stimulates neuronal activity in the MPOA in castrated male rats (Pfaff and Pfaffmann, 1969; Kendrick, 1983; Jansen et al., 1993). PR-expressing neurons located in the ventrolateral part of the VMH (VMHvl), are required for mating and aggression in male mice, according to studies involving the ablation of these neurons (Yang et al., 2013). In addition, the central administration of Kiss1 evoked erections in male rats, and kisspeptin receptor knockout mice did not exhibit any sexual activity (Kauffman et al., 2007; Gresham et al., 2016). Thus, these factors are involved in the control of sexual behavior.

In this study, we tried to identify the mechanism by which neonatal immune stress reduces sexual behavior in adult male rats, focusing on T and hypothalamic factors.

2. Materials and methods

2.1. Animals

Fifteen pregnant female Sprague-Dawley rats were purchased (Charles River, Japan Inc., Tokyo Japan) and housed individually in a room with controlled lighting (a 14 h light, 10 h dark cycle) and temperature 24 °C conditions. After all dams had delivered their pups, the male pups were used in this study. The pups were cross-fostered and weaned on postnatal day (PND) 21. After weaning, three pups were maintained in each cage. All animal experiments were conducted in accordance with the ethical standards of the animal care and use committee of Tokushima University.

2.2. Experiment 1. Influence of immune stress in the early neonatal period on sexual maturation and sexual behavior

2.2.1. Study design

The male pups were randomly divided into 3 groups. The rats in the control group (n = 17) were given intraperitoneal (i.p.) injections of saline (volume: 0.1 ml/g body weight) on PND10 and PND25. The rats in the PND10 group (n = 32) were given an i.p. injection of LPS (100 μg/kg dissolved in saline) derived from *Escherichia coli* (0111:B4; Sigma, St. Louis, MO, USA) on PND10 and an i.p. injection of saline on PND25. The rats in the PND25LPS group (n = 16) were given an i.p. injection of saline on PND10 and an i.p. injection of LPS on PND25. PND10 and PND25 were the models of neonatal period and juvenile period, respectively.

2.2.2. Body weight

The body weights of all rats were recorded every week throughout experiment 1.

2.2.3. Sexual maturation

From PND35, the pups were inspected daily for preputial separation (PS), which is an indicator of male puberty. The penis was evaluated by examining and attempting to retract the prepuce to determine if it had separated from the glans penis (Stoker et al., 2000; Matsuzaki et al., 2017).

2.2.4. Sexual behavior

Sixty-four sexually mature adult female rats were prepared as partners for a sexual behavior test. They were bilaterally ovariectomized at 9 weeks of age under sodium pentobarbital (40–50 mg/kg, i.p.) or sevoflurane-induced anesthesia. Two weeks later, estradiol benzoate (10 μg/body) dissolved in 0.1 mL sesame oil was injected subcutaneously (s.c.) into the backs of the female rats at 48 h and 24 h before the test session. Progesterone (1 mg/body) dissolved in 0.2 mL sesame oil was injected s.c. into the female rats 4 h before the test session (Govic et al., 2008; matsuzaki et al., 2017). At 11 and 12 weeks of age, sexual behavior tests were conducted under the following conditions. Male rats and receptive female rats were placed into plastic transparent boxes (41 × 21 × 21 cm; one male and one female rat were placed into each box). Five min after male rat were habituated in the cage, female rats were introduced there. Video of the rats' activity was recorded for 30 min to study their sexual behavior. The rats' sexual behavior was evaluated by assessing the frequency of mounting (the male rat assumes a copulatory position behind the female rat, but does not insert its penis into the female's vagina), intromission (the male rat assumes a copulatory position behind the female rat and inserts its penis deep into the female's vagina. Then, the male rat licks its penis immediately after pulling out. In addition, the male rat does not exhibit any mounting behavior soon after the intromission), and ejaculation (the male rat assumes a copulatory position behind the female rat and inserts its penis deep into the female's vagina for 1–3 seconds. After that, the male rat separates from the female rat and licks its penis) (Ågmo, 1997).

2.2.5. Sampling

Two weeks after the sexual behavior test, 24 male rats were killed by decapitation (8 rats from the control group, 8 rats from the PND10LPS group, and 8 rats from the PND25LPS group were randomly selected). Blood was collected for hormone assays of luteinizing hormone (LH) and T, and brain tissue was immediately excised and used to analyze mRNA expression. The brain tissue samples were then frozen

rapidly in liquid nitrogen and stored at −80 °C until use. Serum was separated by centrifugation and stored at −20 °C until use. Before the RNA analysis, hypothalamic explants were dissected out from the brain tissue samples using the following method. Each explant was dissected out by making coronal cuts 1 mm anterior from the anterior border of the optic chiasm and the posterior border of the mammillary bodies. The explant was cut 2.5 mm from the bottom of the hypothalamus and then trimmed 2.5 mm lateral from the midline of each side.

2.3. Experiment 2. Influence of T supplementation on sexual behavior in castrated rats that experienced immune stress in the neonatal period

2.3.1. Study design

After experiment 1 and the sampling had been performed, the remaining 37 male rats 14 weeks of age (8 rats from the control group, 21 rats from the PND10LPS group, and 8 rats from the PND25LPS group) were used for experiment 2. As described in the study design of the experiment 1, the rats in the control group had been given i.p injections of saline on PND10 and PND25. The rats in the PND10 group had been given an i.p. injection of LPS (100 μg/kg) on PND10 and an i.p. injection of saline on PND25. The rats in the PND25LPS group had been given an i.p. injection of saline on PND10 and an i.p. injection of LPS on PND25. All rats were orchidectomized at 14 weeks of age under sodium pentobarbital (40–50 mg/kg, i.p.) or sevoflurane-induced anesthesia. After a 3 week recovery period, a silastic tube (inner diameter: 3 mm, outer diameter: 5 mm, length of the filled part: 30 mm) containing crystalline T was s.c. implanted into each rat's back (Urban et al., 1993; De Vries et al., 1994; Harding and Velotta, 2011; Iwasa et al., 2016). On day 18 after the implantation procedure (at the age of 19 weeks), 30-min sexual behavior test videos were recorded, as explained above. At the end of the day on which the sexual behavior test was performed, all of the rats were killed by decapitation. Their blood and brain tissue were collected and stored until use.

2.4. Hormone assays

The serum LH concentration was measured using the I-125 radioimmunoassay kit (rat LH [I-125] RIA kit; Institute of Isotopes Co., Ltd., Tokyo, Japan). The analytical sensitivity of the LH assay was 0.1 mUI/ml, and the intra-assay co-efficient of variation was 6.5%. The serum T concentration was measured using an electrochemiluminescence immunoassay kit (ECLusys TESTO II; Roche Diagnostics, K.K., Tokyo, Japan). The analytical sensitivity of the T assay was 0.45 ng/mL, and tests of a specimen whose T concentration was close to the values seen in our experiment produced inter- and intra-assay coefficients of variation of 2.5% and 2.1%, respectively.

2.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from the hypothalamic tissue using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® mini kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with oligo (deoxythymidine) primers at 50 °C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (RT-PCR; Invitrogen Co.). RT-PCR analysis was performed using the StepOnePlus™ RT-PCR system (PE Applied Biosystems, Foster City, CA, USA) and SYBR® green fast (Applied Biosystems). We chose GAPDH as an internal control because it is the most stable housekeeping gene in the brain (Vandesompele et al., 2002; Munkhzaya et al., 2015). The following forward and reverse primers were used: *GAPDH*: F: 5'-ATG GCA CAG TCA AGG CTG AGA-3', R: 5'-CGC TCC TGG AAG ATG GTG AT-3'; *GnRH*: F: 5'-GCA GAA CCC CAG AAC TTC GA-3', R: 5'-TGC CCA GCT TCC TCT TCA AT-3'; *PRA + B*: F: 5'-GGT CTA AGT CTC TGC CAG GTT TCC-3', R: 5'- CAA CTC CTT CAT CCT CTG CTC ATT C-3'; *PRB: F*: 5'-GCA TCG TCT GTA GTC TCG CCA ATAC-3', R: 5'-GCT CTG GGA TTT CTG CTT CTT CG-3'; *Kiss1*: F: 5'- ATG ATC TCG CTG GCT TCT TGG-3', R: 5'-GGT TCA CCA CAG GTG CCA TTT-3'; The PCR cycling conditions were as follows: initial denaturation and enzyme activation were performed at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C (*PRA + B*) for 30 s, 60 °C (*PRB*) for 30 s, 65 °C (*kiss1*) for 30 s, 64 °C (*GnRH*) for 30 s, or (*GAPDH*) 64 °C for 30 s; and an extension step of 72 °C for 10 min. The copy numbers of the *GnRH*, *PRA + B*, *PRB* and *Kiss1* transcripts were normalized against that of the *GAPDH* transcript.

2.6. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test. Values are expressed as the mean ± SE, and statistical significance was defined as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Experiment 1. Influence of immune stress in the early neonatal period on sexual maturation and sexual behavior

3.1.1. Effects of neonatal LPS injection on body weight and sexual maturation

Body weights did not show significant difference among the groups throughout experiment 1 (data not shown). PS occurred significantly later in the PND10LPS group (40.53 ± 0.33 days, $p < 0.05$, mean ± SE) than in the control group (38.4 ± 0.58, $p < 0.05$). No significant difference in the timing of PS was detected between the PND25LPS group (40.12 ± 0.66 days, $p < 0.05$) and the other groups (Fig. 1A). Body weight on the day of PS did not differ significantly among the groups (Fig. 1B). The cumulative frequency of PS was significantly lower in the PND10LPS group than in the control group from PND36 to PND40 (Fig. 1C).

3.1.2. Effects of neonatal LPS injection on sexual behavior in adulthood

The frequency of mounting was significantly lower in the PND10LPS group than in the control group ($p < 0.01$ vs. control group) (Fig. 2A). The frequency of intromission was significantly lower in the PND10LPS group than in the control group ($p < 0.05$ vs. control group) (Fig. 2B). Furthermore, the frequency of ejaculation was significantly lower in the PND10LPS group than in the control group ($p < 0.01$ vs. control group) (Fig. 2C). No significant differences were detected between the sexual behavior of the PND25LPS group and that of the other groups.

3.1.3. Effects of neonatal LPS injection on serum hormone concentrations in adulthood

The serum LH concentration did not differ significantly among the groups (Fig. 3A). On the other hand, the serum T concentration was significantly lower in the PND10LPS and PND25LPS groups than in the control group ($p < 0.01$) (Fig. 3B).

3.1.4. Effects of LPS injection on the hypothalamic mRNA expression levels of PRA + B, PRB, GnRH, and Kiss1

Hypothalamic *PRB* mRNA expression was significantly lower in the PND10LPS group and PND25LPS group than in the control group ($p < 0.01$) (Fig. 4A). Hypothalamic *PRA + B* mRNA expression did not differ significantly among the groups (Fig. 4B). Hypothalamic *GnRH* mRNA expression was significantly lower in the PND10LPS and PND25LPS groups than in the control group ($p < 0.01$) (Fig. 4C). *Kiss1* mRNA expression did not differ among the groups (data not shown).

3.2. Experiment 2. Influence of T supplementation on sexual behavior in castrated rats that experienced immune stress in the neonatal period

3.2.1. Effects of T supplementation on sexual behavior

The frequency of mounting was significantly lower in the PND10LPS

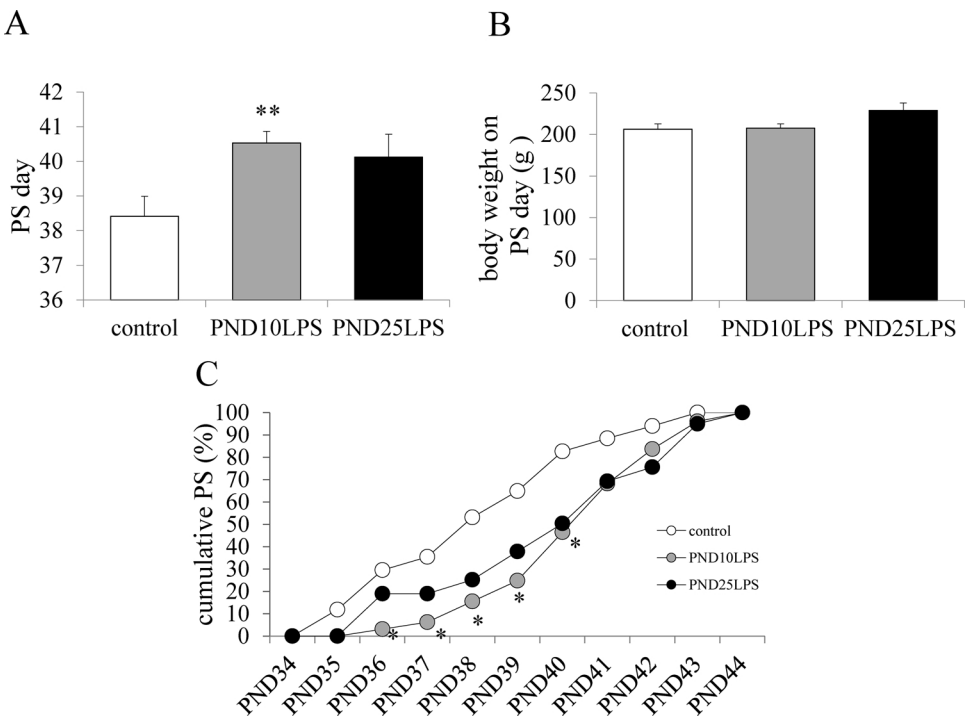

Fig. 1. Preputial separation (PS) occurred later in the postnatal day 10 lipopolysaccharide (PND10LPS) group than in the control group (A). No significant difference in body weight on the day of PS was detected among the groups (B). The cumulative frequency of PS was significantly lower in the PND10LPS group than in the control group during the period from postnatal day (PND) 36 to 40 (C). Data are presented as mean ± SE values. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

and PND25LPS groups than in the control group, in which the testes were removed and T was administered ($p < 0.05$) (Fig. 5A). The frequency of intromissions was significantly lower in the PND10LPS and PND25LPS groups than in the control group ($p < 0.05$) (Fig. 5B). Furthermore, the frequency of ejaculation was significantly lower in the PND10LPS and PND25LPS groups than in the control group ($p < 0.05$) (Fig. 5C).

3.2.2. *Effects of T supplementation on serum hormone concentrations*

The serum T and LH concentrations of the 3 groups did not differ significantly (Fig. 6A and B).

3.2.3. *Effects of T supplementation on the hypothalamic mRNA expression levels of PRA + B, PRB, and GnRH in castrated rats*

The hypothalamic mRNA expression levels of *PRB*, *PRA + B*, and *GnRH* did not differ significantly among the 3 groups (Fig. 7A–C).

4. Discussion

It has been reported that experiencing immune, psychological, or metabolic stress in the early neonatal period can have various long-lasting effects on physiological functions in adulthood, e.g., it is associated with metabolic, psychological, and reproductive dysfunction. Regarding reproductive functions, immune stress in the early neonatal period suppressed hypothalamic *Kiss1* mRNA expression in pre-pubertal female rats (Knox et al., 2009), delayed sexual maturation in male and

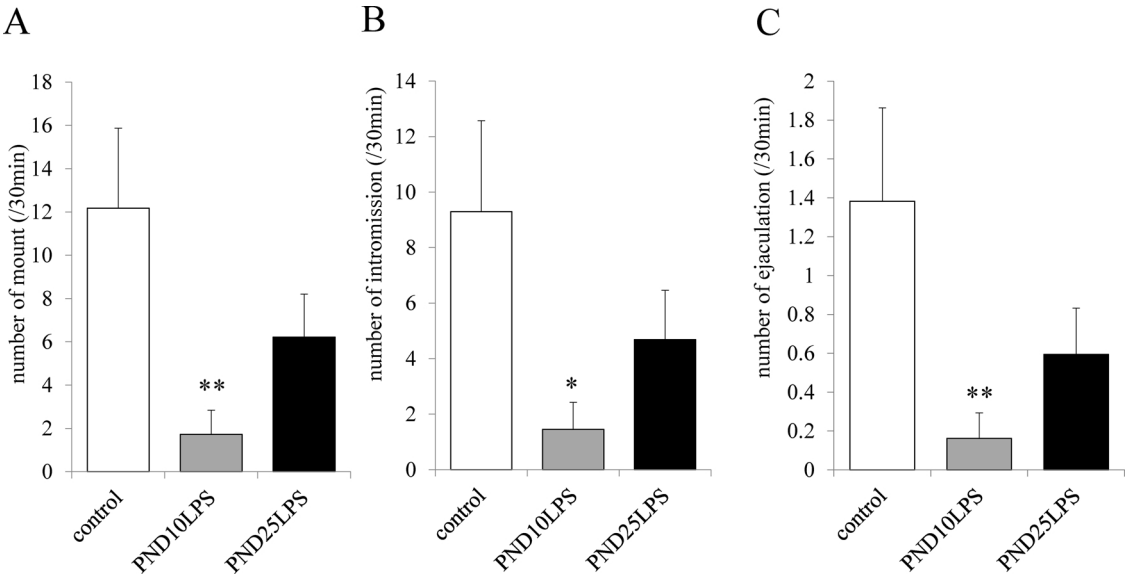

Fig. 2. The total numbers of mounting, intermission, and ejaculation events in the 30-min observation period were significantly lower in the PND10LPS group than in the control group (A, B, and C). Data are presented as mean ± SE values. $^{**}p < 0.01$ vs. control.

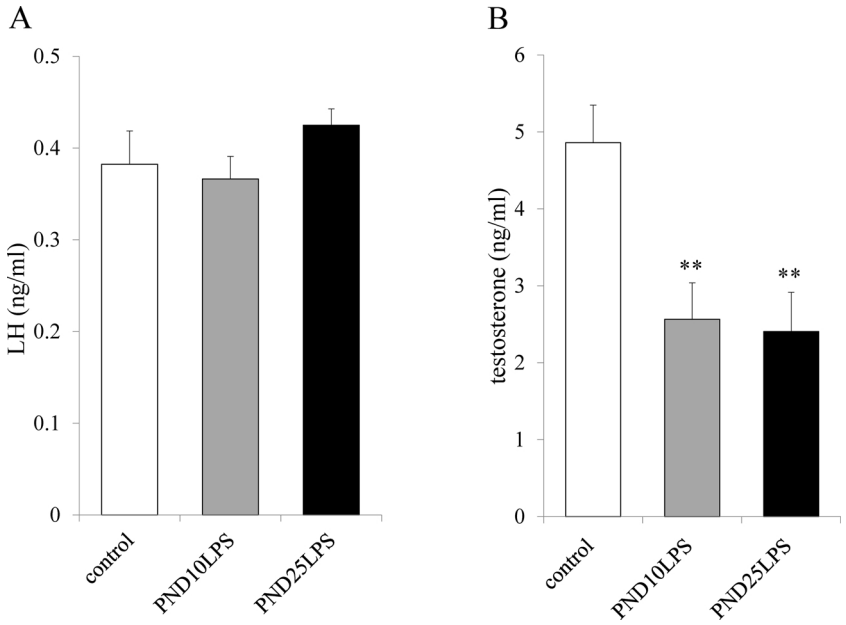

Fig. 3. The serum luteinizing hormone (LH) concentration did not differ significantly among the groups (A). However, the serum testosterone (T) concentration was significantly lower in the PND10LPS and PND25LPS groups than in the control group (B). Data are presented as mean ± SE values. $^{**}p < 0.01$ vs. control.

female rats (Knox et al., 2009; Walker et al., 2011; Wu et al., 2011), disrupted the estrous cycle (Wu et al., 2011), reduced sexual behavior in adulthood in male rats (Walker et al., 2011), and suppressed the serum T concentrations of male rats (Iwasa et al., 2009).

In this study, we examined the relationship between sexual behavior and serum T levels in adult male rats that experienced immune stress during the neonatal period and found that the frequency of sexual behavior reduced, irrespective of the serum T concentration. This is the first study to demonstrate that there is no relationship between the serum T concentration and sexual behavior in male rats that are exposed to immune stress in the neonatal period.

In the current study, inducing immune stress with LPS in the neonatal period (on PND10 or 25) delayed sexual maturation in male rats. This agrees with the study by Walker A et al., in which LPS injections were administered on PND3 and 5 (Walker et al., 2011). In addition, experiencing other kinds of stress, such as undernutrition, in the prenatal or early neonatal period can also delay sexual maturation in male rats (Chernoff et al., 2009; Hernández-Arteaga et al., 2016). Female rats that were subjected to prenatal undernutrition also exhibited delayed sexual maturation (Iwasa et al., 2010a, b; Castellano et al., 2011). We previously found that hypothalamic *Kiss1* mRNA expression in the pubertal period was decreased in prenatally undernourished rats, which

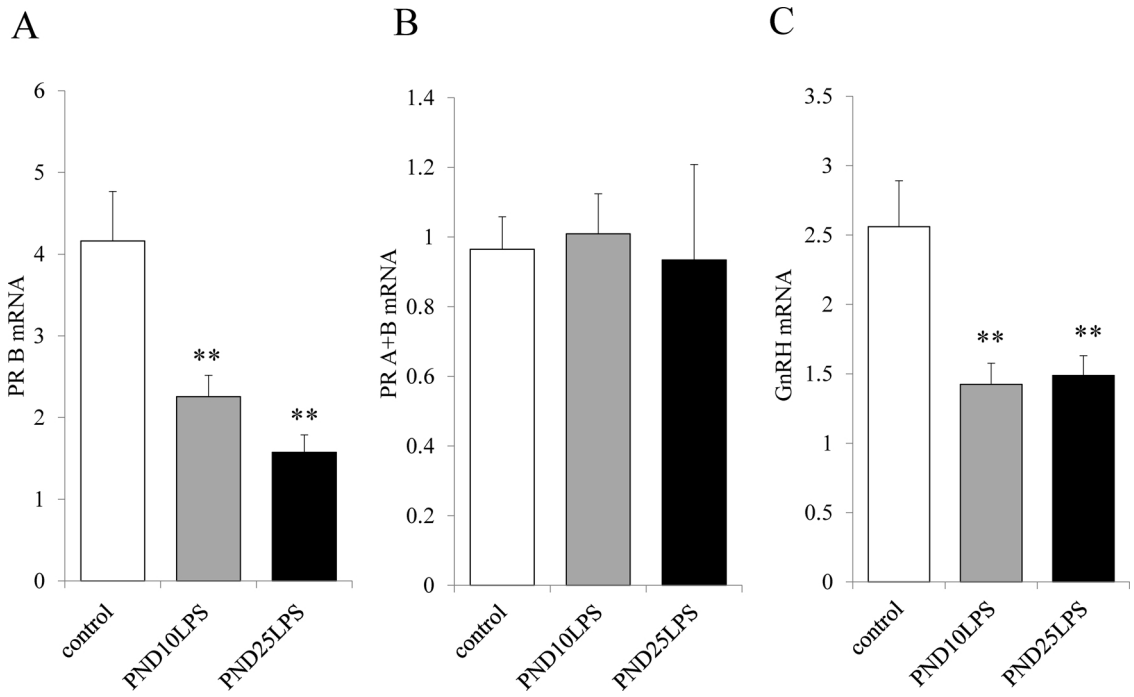

Fig. 4. The hypothalamic mRNA expression levels of progesterone receptor B (*PRB*) and gonadotropin-releasing hormone (*GnRH*) were significantly lower in the PND10LPS and PND25LPS groups than in the control group (A and C). However, the hypothalamic *PRA + B* mRNA expression level did not differ significantly among the groups (B). Data are presented as mean ± SE values. $^{**}p < 0.01$ vs. control.

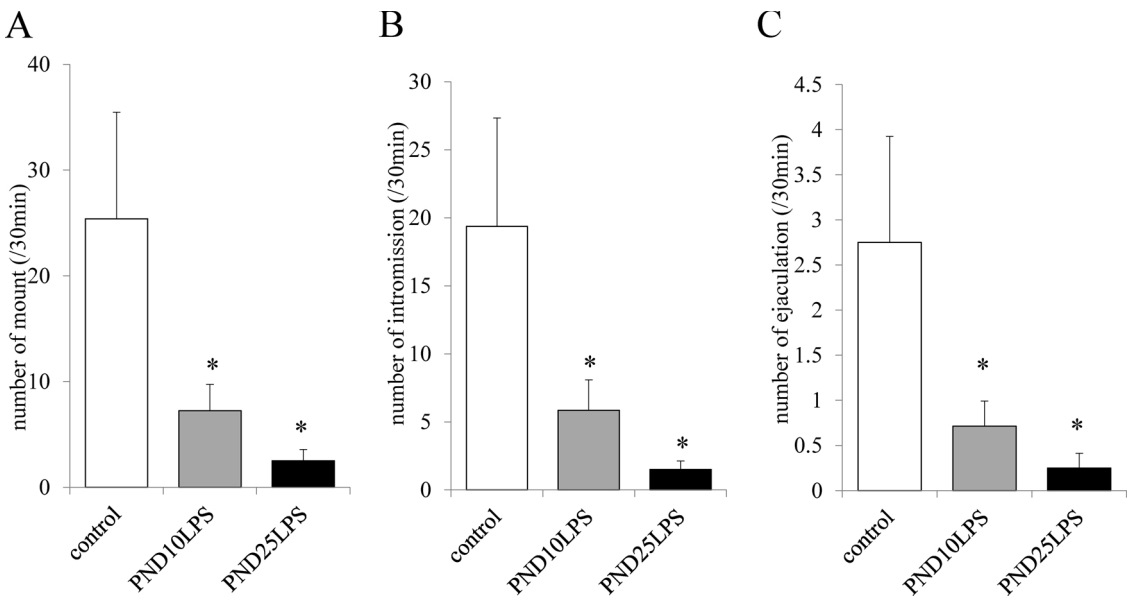

Fig. 5. The numbers of mounting, intermission, and ejaculation events in the 30-min observation period were significantly lower in the PND10LPS and PND25LPS groups than in the control group (A, B, and C). Data are presented as mean ± SE values. *$p < 0.05$ vs. control.

would have caused delayed sexual maturation (Iwasa et al., 2010a, b). Furthermore, the injection of LPS strongly suppressed the serum T concentrations of adult male rats that had previously been injected with LPS in the neonatal period (Iwasa et al., 2009). In the present study, the rats subjected to neonatal immune stress exhibited lower serum T concentrations in adulthood than the control group. Therefore, the hypothalamic-pituitary-gonadal (HPG) axis would have been suppressed in the pubertal period, resulting in delayed sexual maturation, in the male rats that experienced immune stress in the neonatal period.

As for sexual behavior, in our study the frequencies of mounting, intromission, and ejaculation were lower in the PND10LPS group than in the control group, which is similar to Walker's finding that the frequency of mounting was reduced by the injection of LPS during the early neonatal period (Walker et al., 2011). Maternal separation during the period from PND2-10, which is a model of psychological stress, also prolonged the time to the first mount and intromission events, and reduced the number of ejaculation events in male rats (Rhees et al., 2001; Gerardin et al., 2005). Similarly, in another rat study maternal restraint stress during gestation prolonged the time to the first mounting/intromission events, reduced the number of ejaculation events, and suppressed the serum T level in male offspring (Gerardin et al., 2005; Pereira et al., 2006; Hernández-Arteaga et al., 2016). These studies indicated that psychological or immune stress in the prenatal or early neonatal period can suppress sexual behavior in adulthood in male rats.

It is well known that immune stress in adulthood suppresses the HPG axis by downregulating GnRH synthesis and the serum levels of LH and T in male rats (Battaglia et al., 1997; Li et al., 2007; Walker et al., 2011; Iwasa et al., 2012). However, there have not been any studies about why experiencing immune stress in the neonatal period suppresses sexual behavior in adulthood. Exposure to stress in a specific neonatal period called the stress hyporesponsive period (SHRP) has

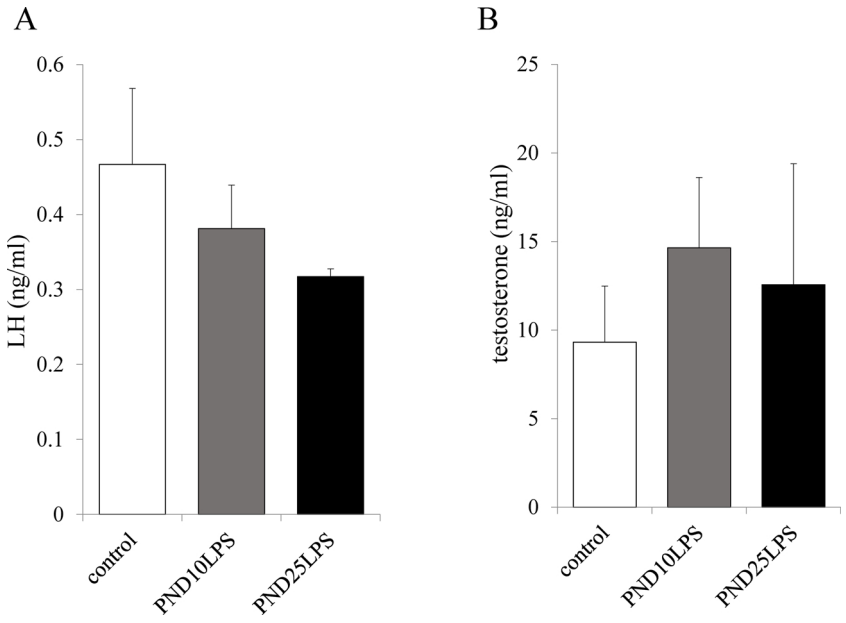

Fig. 6. No significant differences in the serum T or LH concentration were seen among the 3 groups (A and B). Data are presented as mean ± SE values.

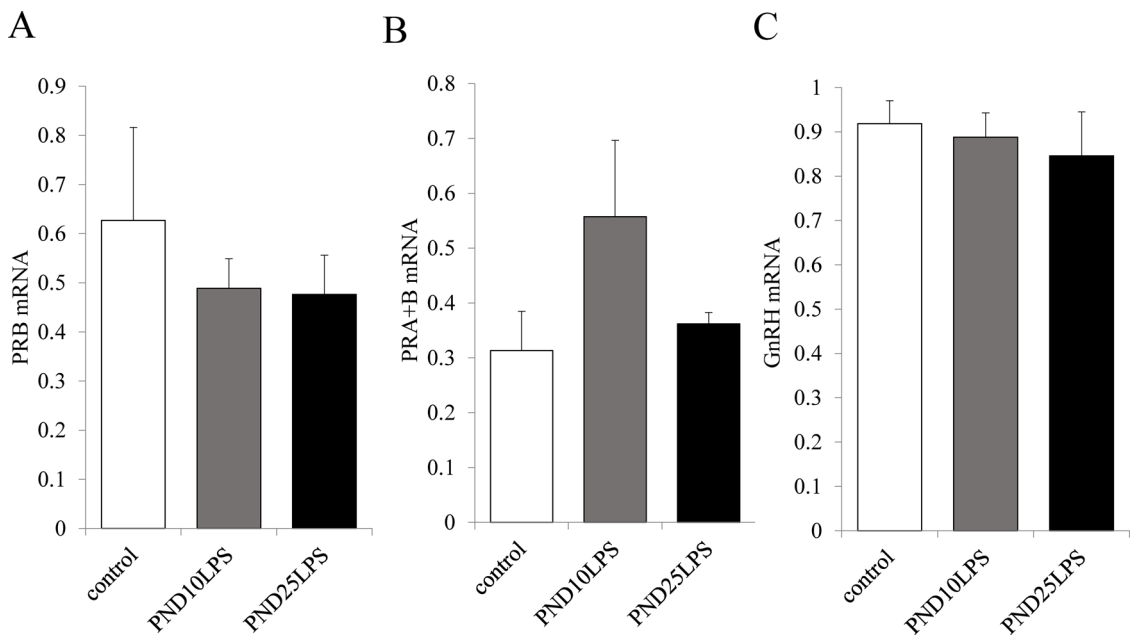

Fig. 7. No significant differences in the hypothalamic mRNA expression levels of *PRA + B*, *PRB*, or *GnRH* were seen among the 3 groups (A, B, and C). Data are presented as mean ± SE values.

long-lasting effects on physical functions (Walker et al., 1986). The original SHRP was related to the HPA axis, but we have demonstrated that an HPG axis-related SHRP also exists (Munkhzaya et al., 2015). For example, the HPG axis and hypothalamic inflammatory cytokine expression were found to be hyporesponsive to immune stress in immature male and female rats (Munkhzaya et al., 2015). In fact, exposure to immune stress in the early neonatal period; i.e., within the SHRP, enhanced HPA axis activity (Shanks et al., 1995, 2000) and suppressed HPG axis activity (Battaglia et al., 1997; Li et al., 2007; Walker et al., 2011; Iwasa et al., 2012) in adulthood. Our previous studies and the present study have shown that male rats that were given LPS in the early neonatal period had lower serum T concentrations in adulthood (Iwasa et al., 2009). It is also known that the HPG axis plays a critical role in reproductive function and sexual behavior. Castration in adult rats reduced sexual activity and serum T levels, and post-castration T supplementation restored sexual activity, clearly indicating that T is a stimulator of sexual behavior (Harding and Velotta, 2011). A previous study examined the role of T in the mechanism by which stress in early life suppresses sexual behavior in adulthood. As a result, it was found that maternal restraint stress suppressed the serum T concentration and sexual behavior in adulthood (Gerardin et al., 2005), and continuous serum T replacement from PND22 in such animals restored sexual behavior in adulthood (Pereira et al., 2006), indicating that the suppression of the serum T concentration was responsible for the reduction in sexual behavior observed in adulthood in this setting. On the other hand, although exposure to immune stress in the neonatal period also led to reductions in the serum T concentration and sexual behavior in adulthood in male rats (Walker et al., 2011), continuous T supplementation from week 17 did not restore sexual behavior in the present study. Differences in the types of stress and the timing of T supplementation could explain this discrepancy. However, in the current study exposure to immune stress in the neonatal period seemed to suppress sexual behavior in adulthood, irrespective of the serum T level.

Hypothalamic factors, such as GnRH, corticotropin-releasing hormone (CRH), and Kiss1, are also important for regulating sexual behavior (Schiml and Rissman, 2000; Sakuma, 2002; Nakamura et al., 2016). We previously reported that the injection of a high dose of LPS into adult female rats suppressed hypothalamic *Kiss1* and *GnRH* mRNA expression (Iwasa et al., 2007, 2014). However, in the present study, steady-state *Kiss1* mRNA expression levels in adulthood were not influenced by neonatal immune stress, indicating that Kiss1 was not involved in the observed suppression of sexual behavior. Lim WL et al. reported that maternal dexamethasone exposure decreased the number and dendritic development of early postnatal GnRH-expressing neurons in the organum vasculosum of the lamina terminalis/preoptic area (Lim et al., 2014). Some of our present results might be explained by suppressed dendritic development in GnRH-expressing neurons, assuming that this phenomenon occurs in rats exposed to LPS in the neonatal period. In addition, in our study neonatal immune stress reduced hypothalamic *GnRH* mRNA expression and suppressed sexual behavior. Therefore, suppressed dendritic development and/or lower hypothalamic expression of GnRH could help to explain the low sexual activity levels seen in the male rats that were exposed to immune stress in the neonatal period. Because LH is secreted in a pulsatile manner and serum LH level varies over time (Dierschke et al., 1970; Turgeon et al., 1974), one point measurement in our study might not be enough to evaluate serum levels of LH. Therefore, we speculate that pulsatile secretion of LH would be suppressed in the LPS treated groups although basal LH level was not suppressed. Moreover, we found that neonatal immune stress decreased hypothalamic *PRB* mRNA expression in adulthood. PR-expressing neurons in the VMHvl have been reported to maintain mating behavior and aggression in male and female mice (Yang et al., 2013). Furthermore, neonatal exposure to LPS suppressed the serum progesterone level in adulthood in female rats (Nilsson et al., 2002). Thus, lower hypothalamic PR expression could be another reason for the low sexual activity seen in the male rats that were exposed to immune stress in the neonatal period.

Researchers have also focused on epigenetic mechanisms as a reason for the long-term effects of stress in early life on physical functions in later life. It has been reported that prenatal and neonatal stress induced epigenetic modulations in the genome, such as DNA methylation and histone acetylation (Henry et al., 1996; Tenk et al., 2008). For example, the separation of neonatal male rats from their mothers, which is a form of psychological stress, enhanced the hypothalamic *CRH* mRNA expression induced in response to restraint stress in adulthood, which was found to be associated with decreased methylation of the *CRH* promoter (Chen et al., 2012). The separation of neonatal male mice from their

mothers also resulted in decreased methylation of the pro-opiomelanocortin (*POMC*) promoter in the pituitary gland (Wu et al., 2014), and increased *POMC* mRNA expression in the pituitary gland combined with elevated serum levels of corticosterone under stressful conditions or at the peak of the circadian rhythm in adulthood (Murgatroyd et al., 2009). It has also been reported that the DNA methylation of the arginine vasopressin (*AVP*) enhancer region was decreased in the hypothalamus, the *AVP* mRNA expression level was increased, and the frequency of depression-like behavior was increased in such mice. On the other hand, the DNA methylation level in the hippocampus was markedly increased in adulthood in such mice (McCoy et al., 2016). Numerous epigenetic modulations are seen in the brains of adult animals that experience stress in early life. Therefore, an epigenetic mechanism, such as the DNA hypermethylation of the promoter of the *PR* gene or hypoacetylation of *PR*-associated histone molecules, which could have resulted in a reduction in sexual behavior, might have been involved in the lower hypothalamic expression of *PR* mRNA detected in the male rats exposed to neonatal immune stress in the present study. Although the administration of T after castration meant that the reduction in hypothalamic *PR* mRNA expression observed in adulthood was not significant, lower *PR* expression might contribute to the suppression of sexual behavior in neonatally stressed male rats. Prenatal and neonatal stress can have long-term suppressive effects on sexual behavior in male rats; however, these effects differed among previous studies and are mediated by various factors. The mechanism responsible for the reduction in sexual behavior seen in male rats exposed to immune stress in the neonatal period remains to be elucidated.

In conclusion, in male rats immune stress in the neonatal period delayed sexual maturation, reduced sexual behavior, and suppressed the serum T concentration and the hypothalamic mRNA levels of *GnRH* and *PR* in adulthood. The delayed sexual maturation was presumed to have been caused by the reduction in the serum T concentration. However, the reduction in the frequency of sexual behavior occurred irrespective of the serum T level and might have been caused by the epigenetic modulation of hypothalamic factors.

Conflicts of interest

The authors declare that no conflicts of interest exist.

Human and animal rights

This article does not contain any experiments involving human subjects. All of the institutional and national guidelines for the care and use of laboratory animals were followed. The protocol for the research project was approved by a suitably constituted ethics committee.

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