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The suppressive effect of immune stress on LH secretion is absent in the early neonatal period in rats

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ABSTRACT

Some physiological functions display weak responses to stress in the early neonatal period; i.e., they exhibit stress hyporesponse periods. In this study, we evaluated whether gonadotropin regulatory factors exhibit stress hyporesponsive periods in male and female rats. Rats were intraperitoneally injected with lipopolysaccharide (100 μg/kg) (LPS group) or saline (control group) on postnatal day (PND) 5, 10, 15, or 25. Then, their serum luteinizing hormone (LH) concentrations and hypothalamic mRNA levels of gonadotropin regulatory factors; i.e., kisspeptin (*Kiss1*), the kisspeptin receptor (*Kiss1r*), and gonadotropin-releasing hormone (*GnRH*), were measured at 2 h after the injection. The hypothalamic mRNA levels of pro-inflammatory cytokines were also measured because they suppress gonadotropin secretion. The serum LH concentration of the LPS group was lower than that of the control group at PND25 in both sexes, but no such difference was seen at PND5, 10, or 15 in either sex. In both sexes, the hypothalamic tumor necrosis factor (*TNF*)α and interleukin (*IL*)*-6* mRNA expression levels of the LPS group were higher than those of the control group at PND25, but not at PND5 or 10. The hypothalamic *IL-1β* mRNA expression level of the LPS group was higher than that of the control group at all time points. The hypothalamic *Kiss1, Kiss1r*, and *GnRH* mRNA expression levels of the LPS and control groups did not differ at any time point in either sex. These findings suggest that gonadotropin regulatory factors exhibit stress hyporesponse periods. The hypothalamic–pituitary–gonadal axis (HPG) might become responsive to immune stress between PND15 and 25, which could be related to enhanced hypothalamic cytokine expression. The avoidance of infectious stress during the early neonatal period might be important for normal development of the HPG axis.

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1. Introduction

In rodents, the brain undergoes significant development during the first few weeks of life. As the central stress regulatory systems are not fully established in this period, they display weak physiological responses to stress; i.e., they exhibit stress hyporesponse periods (SHRP) (Witek-Janusek, 1988; Spencer et al., 2006). For example, the hypothalamic-pituitary-adrenal (HPA) axis is not responsive to immune stress in the early neonatal period, but is responsive to it in the pre-pubertal period (Schmidt et al., 2003; Dinel et al., 2014). It is assumed that exposure to stress in the SHRP alters the developmental pattern of the brain to enable it to cope with stressful environments (Shanks et al., 1995; Boisse et al., 2004; Ellis et al., 2005), and similar alterations seem to continue throughout life and can cause physiological dysfunctions in adulthood (Hodgson et al., 2001; Nilsson et al., 2002).

On the other hand, the developmental changes in the stress responses of the hypothalamic-pituitary-gonadal (HPG) axis, which is an essential hormonal system for reproductive function, have not been fully elucidated. In a previous study, we showed that in male rats the serum gonadotropin level was not affected by immune stress in the early neonatal period, whereas it was suppressed in later periods (Iwasa et al., 2012). Similarly, we also showed that the serum gonadotropin level was not influenced by fasting in the early neonatal period in female rats (Iwasa et al., 2010). Although these findings indicate that, like the HPA axis, the HPG axis exhibits SHRP, the mechanism responsible for this period has not been clarified. In addition, the response of the HPG axis to immune stress during the developmental period has not been examined in female rats. As it has been shown that immune stress in the early neonatal period

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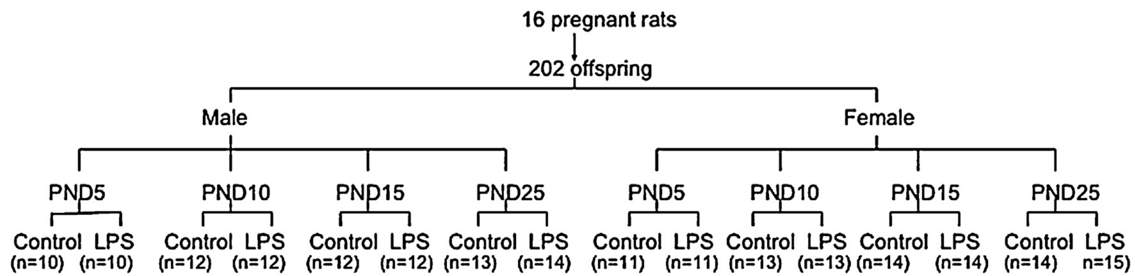

Fig. 1. Experimental design.
Sixteen pregnant rats and their 202 offspring were used in this study. The male and female offspring were randomly divided into 4 groups according to the postnatal day on which they were injected with LPS or saline (the PND5, 10, 15, and 25 groups). Each group was further divided into 2 subgroups (the control and LPS groups; n = 10–15 per group).

causes long-lasting alterations in the HPG axis and disturbs future reproductive function (Wu et al., 2011; Knox et al., 2009; Li et al., 2007), it is important to clarify the mechanism responsible for the HPG axis' SHRP.

It has been determined that pro-inflammatory cytokines such as tumor necrosis factor-α (*TNFα*) and interleukin-1β (*IL-1β*) play critical roles in the immune stress-induced suppression of the HPG axis. For example, the administration of these cytokines suppressed hypothalamic gonadotropin-releasing hormone (*GnRH*) secretion and decreased serum gonadotropin levels (Rivier and Vale, 1990; Ebisui et al., 1992; Yoo et al., 1997; Kalra et al., 1998; Kang et al., 2000; Watanobe and Hayakawa, 2003; Matsuwaki et al., 2004; Herman et al., 2013). In addition, immune stress attenuates the expression of kisspeptin (*Kiss1*), which is a potent positive regulator of GnRH, resulting in a reduction in the serum gonadotropin level (Iwasa et al., 2008; Iwasa et al., 2014). Therefore, we hypothesized that the responses of these GnRH/gonadotropin regulatory factors to immune stress are not fully established in the early neonatal period, and that such immaturity is involved in the SHRP exhibited by the HPG axis.

In this study, developmental changes in the responses of the serum level of LH, a representative gonadotropin, and the hypothalamic expression levels of *GnRH* and gonadotropin regulatory factors; i.e., pro-inflammatory cytokines and *Kiss1*/the kisspeptin receptor (*Kiss1r*), to immune stress were evaluated in both male and female rats.

2. Materials and methods

2.1. Animals

Sixteen pregnant female Sprague–Dawley rats were purchased (Charles River, Japan Inc., Tokyo Japan) and housed individually under controlled lighting (14 h light, 10 h dark cycle) and temperature (24 °C) conditions. All rats delivered their offspring after 7 days. The offspring were weaned on PND21 and maintained at 3 offspring per cage. All animal experiments were conducted in accordance with ethical standards of the institutional Animal Care and Use Committee of the University of Tokushima.

2.2. LPS injection and sampling of serum and hypothalamic tissue

The male and female offspring were randomly divided into 4 groups according to the postnatal day on which they were scheduled to receive an injection of LPS or saline; i.e., into the PND5, 10, 15, and 25 groups (Fig. 1). Each group was subdivided into 2 groups: the control and LPS groups (*n* = 10–15 per group). The rats' body weight was measured just before each injection (Table 1). Then, the rats were intraperitoneally injected with LPS (100 μl/kg, Sigma, St. Louis, MO, USA) or saline. The mean body weight of the

Table 1
Body weight of experimental group just before injections.

	Male		Female	
	Control	LPS	Control	LPS
PND5	9.60 ± 0.27	8.80 ± 0.36	9.20 ± 0.28	9.25 ± 0.24
PND10	19.96 ± 0.21	20.88 ± 0.54	16.97 ± 0.19	18.75 ± 0.18
PND15	33.64 ± 0.87	29.26 ± 0.36	30.25±0.78	33.54 ± 0.58
PND25	67.72 ± 0.91	65.42 ± 0.84	77.29 ± 1.23	74.26 ± 0.89

(Data are presented as mean ± SE)

control and LPS subgroups did not differ significantly in any PND group in either sex. The rats were killed by decapitation 2 h after the injection. The time course suppression of serum LH was tested previously at 60 min intervals for 180 min after LPS injection and serum LH was suppressed 120 and 180 min after injection (Iwasa et al., 2008). Therefor, we chose 2 h as sampling time for LH. Their blood and brains were collected immediately as described previously. Before RNA analysis, brain section was dissected out using coronal cuts that were 1 mm anterior from the border of the optic chiasm and the posterior border of the mammillary bodies. The section was cut 2.5 mm from the bottom of the hypothalamus and then trimmed to 2.5 mm lateral from the midline of each side (Matsuzaki et al., 2011). Blood and hypothalamic tissue were used to measure their serum LH concentration and hypothalamic mRNA expression levels of *Kiss1; Kiss1r; GnRH*; and pro-inflammatory cytokines; i.e., *TNFα, IL-1β,* and *IL-6* (Iwasa et al., 2012; Iwasa et al., 2015). The serum was isolated by centrifugation and stored at -20 °C, and the brain tissues were snap frozen and stored −80 °C.

2.3. Hormone assay

The rats' serum LH concentrations were determined using an I-125 radioimmunoassay kit (rat LH [I-125] RIA kit, Institute of Isotopes Co., Ltd., Tokyo, Japan).

2.4. Quantitative real-time polymerase chain reaction

Total RNA was extracted from the hypothalamus using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy Mini kit (Qiagen, Hilden, Germany). Complementary DNA 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.) according to the manufacturer's instructions. Eight micrograms of total RNA were mixed with oligo(dt) primers and dNTP in a total volume of 10 μL and then incubated at 65 °C for 5 min. Next, RT buffer, $MgCl_2$, dithiothreitol, RNaseOUT, and SuperScript III RT were added, and then the mixture was incubated at 50 °C for 50 min. The reverse transcriptase reaction was terminated by heating the reaction mixture at 85 °C for 5 min. One microliter of RNase H (2 U) was added to the reaction

mixture, which was then incubated at 37 °C for 20 min. We measured the mRNA expression levels of *GAPDH, Kiss1, Kiss1r, GnRH, TNFα*, *IL-1β*, and *IL-6* using fast SYBR green master mix. Sequence-specific primers were generated on the basis of the published rat sequences. 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′; *Kiss1*: F: 5′- ATG ATC TCG CTG GCT TCT TGG-3′, R: 5′- GGT TCA CCA CAG GTG CCA TTT T -3′; *Kiss1r*: F: 5′-TGT GCA AAT TCG TCA ACT ACA TCC-3′, R: 5′-AGC ACC GGG GCG GAA ACA GCT GC-3′; *GnRH*: F: 5′-GCA GAA CCC CAG AAC TTC GA-3′, R: 5′-TGC CCA GCT TCC TCT TCA AT-3′; *TNFα*: F:5′- AGC CCT GGT ATG AGC CCA TGT A-3′, R: 5′- CCG GAC TCC GTG ATG TCTA AGT-3′; IL-1β: F5′-GCT GTG GCA GCT ACC TAT GTC TTG-3′, R: 3′-AGG TCG TCA TCC CAC GAG-5′; and IL-6: F: 5′- TCC TAC CCC AAC TTC CAA TGC TC-3′, R: 5′-TTG GAT GGT CTT GGT CCT TAG CC-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 15s; annealing at 64 °C for 30s (*GAPDH*), 65 °C for 30 s (*Kiss1*), 65 °C for 30 s (*Kiss1r*), 64 °C for 30s (*GnRH*), 65.5 °C for 30 s (*TNFα*), 66 °C for 30 s (*IL-1β*), or 67 °C for 30 s (*IL-6*); and extension at 72 °C for 10 min. The copy numbers of the transcripts were normalized against that of the *GAPDH* transcript for each sample. We chose GAPDH as internal control because it is the most stable gene in brain (Vandesompele et al., 2002). Mean mRNA expression level of each control group was adjusted as 1 in each PND or sex.

2.5. Statistical analysis

The differences between the serum LH concentrations and hypothalamic mRNA expression levels were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Tukey–Kramer test and Student's *t* test. All data are presented as mean + SE values. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of LPS injection on the serum LH concentration

At PND25, the serum LH concentration of the LPS group was significantly lower than that of the control group in both males (control: 0.46 ± 0.05 ng/ml, LPS: 0.32 ± 0.03 ng/ml, $p < 0.05$; mean + SE) and females (control: 0.71 ± 0.03, LPS: 0.61 ± 0.02, $p < 0.01$; mean + SE). On the other hand, the serum LH concentrations of the LPS and control groups did not differ at PND5, 10, or 15 in either sex (Fig. 2).

3.2. Effects of LPS injection on the hypothalamic mRNA expression levels of Kiss1, Kiss1r, GnRH, and pro-inflammatory cytokines (TNFα, IL-1β, and IL-6)

The hypothalamic mRNA expression levels of *Kiss1, Kiss1r,* and *GnRH* did not differ significantly between the control and LPS groups at any PND in either sex (Fig. 3).

The hypothalamic *TNFα* mRNA expression level of the LPS group was significantly higher than that of the control group at PND25 in males ($p < 0.001$) and at PND15 ($p < 0.001$) and 25 ($p < 0.01$) in females. However, at PND5 and 10 no significant difference in hypothalamic *TNFα* mRNA expression was detected between the control and LPS groups in either sex. The *TNFα* mRNA expression level of LPS group was significantly higher in PND25 than in other PNDs in male rats (one-way ANOVA; $F\ (3,\ 39) = 12.7$, $p < 0.001$). On the other hand, this expression level in female was significantly higher in PND15 than in PND5 and 10 (one-way ANOVA; $F\ (3{,}48) = 7.84$, $p < 0.001$). As for sex difference, that *TNFα* mRNA expression level was significantly higher in PND10 (two-way ANOVA; $F(1, 45) = 4.74$, $p < 0.05$) and 25 in male rats (two-way ANOVA; $F\ (1,\ 55) = 19.3$, $p < 0.001$), and significantly higher in PND15 in female (two-way ANOVA; $F\ (1,\ 46) = 5.18$, $p < 0.05$). The hypothalamic *IL-1β* mRNA expression level of the LPS group was significantly higher than that of the control group at all PND in both sexes ($p < 0.01$). The *IL-1β* mRNA expression level of LPS group was significantly higher in PND25 than in other PNDs in male (one-way ANOVA; $F\ (3.39) = 8.86$, $p < 0.001$). On the other hand, this expression level in female was significantly higher in PND15 than in PND5 and 10 (one-way ANOVA; $F\ (3,\ 49) = 5.3$, $p < 0.01$). As for sex difference, that *IL-1β* mRNA expression level was significantly higher in PND25 in male (two-way ANOVA; $F\ (1{,}55) = 4.38$, $p < 0.05$), and in PND15 in female (two-way ANOVA; $F\ (1{,}46) = 8.5$, $p < 0.001$). The hypothalamic *IL-6* mRNA expression level of the LPS group was significantly higher than that of the control group at PND5 ($p < 0.01$), 15 ($p < 0.05$), and 25 ($p < 0.001$) in males and at PND15 ($p < 0.01$) in females. The hypothalamic *IL-6* mRNA expression levels of the LPS and control groups did not differ significantly at PND10 in males and at PND5, 10, and 25 in females. The *IL-6* mRNA expression level of LPS group was significantly higher in PND25 than in PND10 in male (one-way ANOVA; $F(3, 39) = 3.54$, $p < 0.05$). On the other hand, this expression level in female was significantly higher in PND15 than in PND10 and 25 (one-way ANOVA; $F\ (3{,}49) = 3.7$, $p < 0.05$). As for sex difference that *IL-6* mRNA expression level was significantly higher in PND25 in male (two-way ANOVA; $F(1, 55) = 14.15$, $p < 0.001$) (Fig. 4).

4. Discussion

In this study, we found that the HPG axis exhibits SHRP, as has previously been demonstrated for the HPA axis. Specifically, the serum LH concentrations of male and female rats were not affected by the injection of LPS until postnatal day 15, whereas they fell after the injection of LPS on postnatal day 25. Our previous study showed that in male rats LH secretion was not affected by the injection of LPS at PND10, whereas it was reduced by LPS injection at PND15 and 25 (Iwasa et al., 2012). These results indicate that the HPG axis displays SHRP.

The HPA axis has also been reported to exhibit SHRP. Specifically, the response of the HPA axis to immune and metabolic stress was demonstrated to be weaker in the first few weeks of life than in adulthood or other stages of the life in male rats and mice (Dent et al., 1999; Enthoven et al., 2009). Witek–Janusek reported that the serum corticosterone levels of rats were increased by the injection of endotoxin on PND5, 10, or 14; however, the increases observed in these rats were smaller than those seen in the rats injected with endotoxin on PND1, 2, or 21 or adulthood (Witek-Janusek, 1988). Our study focused on whether the HPG axis also exhibits SHRP. In male rats, no reductions in LH secretion were seen in response to immune stress on PND5, 10, or 15; however, LH secretion was reduced by the injection of LPS on PND25 (Iwasa et al., 2012). This indicates that the HPG axis acquires sensitivity to immune stress around PND25 in rats, which is similar to the age at which the HPA axis becomes sensitive to immune stress (Witek-Janusek, 1988; Spencer et al., 2006). In adulthood, immune stress activates HPA axis (Shanks et al., 1995; Ellis et al., 2006) and induces hypothalamic CRH mRNA expression, which suppresses LH secretion mediated by CRH-R2 on GnRH neuron (Li et al., 2005, 2006; Iwasa et al., 2007, 2009). Suppressed LH secretion at PND 25 might be related to HPA axis that acquire sensitivity to LPS at PND 21 (Witek-Janusek, 1988).

The hypothalamic expression of pro-inflammatory cytokines is increased under immune stress conditions in adulthood. As pro-inflammatory cytokines suppress GnRH neuron activity, immune stress results in a reduction in the serum gonadotropin level in adulthood. The central and peripheral administration of pro-

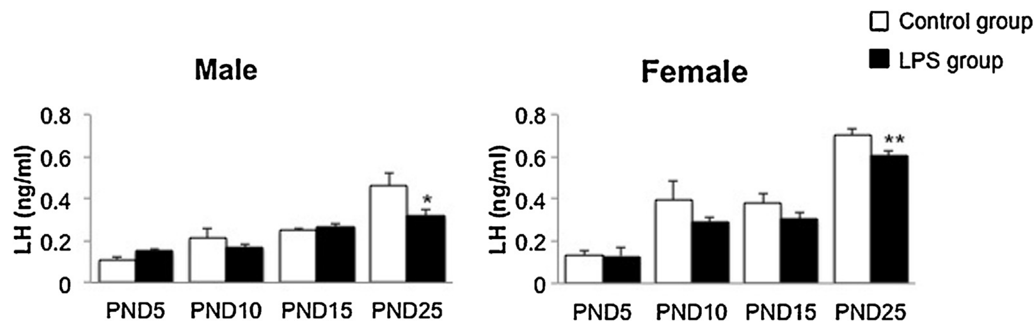

Fig. 2. Effects of the i.p. injection of LPS (100 mg/kg) or saline (equal volume) on the serum LH concentration at PND5, 10, 15, and 25 in male and female rats.
Blood samples were collected 2 h after each injection ($n = 10–15$ per group). In both the male and female rats, the serum LH concentration of the LPS group was significantly lower than that of the control group at PND25. Data are presented as mean + SE values. $^{**}p < 0.01$ $^{*}p < 0.05$ vs. control.

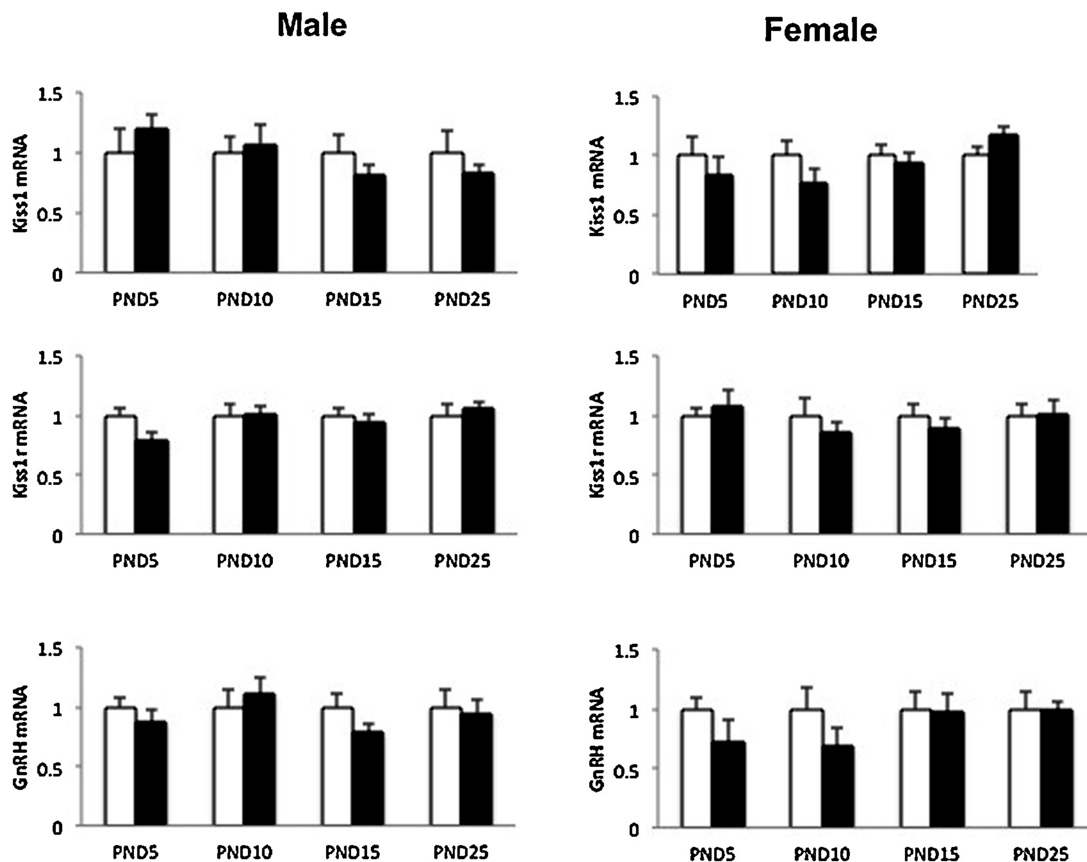

Fig. 3. Hypothalamic KiSS1, KiSS1r, and GnRH mRNA expression levels at PND5, 10, 15, and 25 in male and female rats.
Brain samples were collected 2 h after each injection (n = 10–15 per group). Relative KiSS1, KiSS1r, and GnRH mRNA expression levels were calculated by dividing the expression level of each molecule by the mRNA expression level of GAPDH. No significant differences in the expression levels of the examined molecules were detected between the LPS and control groups in the male or female rats. Data are presented as mean + SE values.

inflammatory cytokines such as TNFα and IL-1β were found to suppress GnRH release and LH secretion in mature rats and sheep (Yoo et al., 1997; Williams et al., 2001; Watanobe and Hayakawa, 2003; Matsuwaki et al., 2004; Sirivelu et al., 2009). The present study indicated that the mRNA expression levels of *TNFα* and *IL-6,* which would have suppressive effects on GnRH release in adulthood, were increased by LPS injection on PND15 and 25, but not on PND5 or 10, in both sexes. Although *IL-1β* mRNA expression was significantly increased by LPS injection on PND5 and 10 in male and female rats, these increases were smaller than those seen on PND15 and 25. Induction of hypothalamic pro-inflammatory cytokines mRNA expressions by LPS were the highest on PND25 in male and on PND15 in female rats. It seems that hypothalamic cytokine expression would also have SHRP to LPS. Pro-inflammatory cytokines seem to acquire sensitivity to LPS at PND15 in female or PND25 in male rats, and these ages are earlier than the age at which the serum LH level becomes sensitive to LPS (PND25). Therefore, we assume that the hyporesponsiveness of these cytokines might be one of the causes of the HPG axis SHRP.

We reported that acute food deprivation suppressed hypothalamic *Kiss1* and *Kiss1r* mRNA expression at PND25, but not at PND5 or 15 in female rats (Iwasa et al., 2010). Therefore, it is assumed that the HPG axis exhibits a period of hyporesponsiveness to metabolic stress and that this is caused by the hyporesponsiveness of the Kiss1-Kiss1r-GnRH system. Our group and others have found that the expression levels of kisspeptin and its receptor, which are

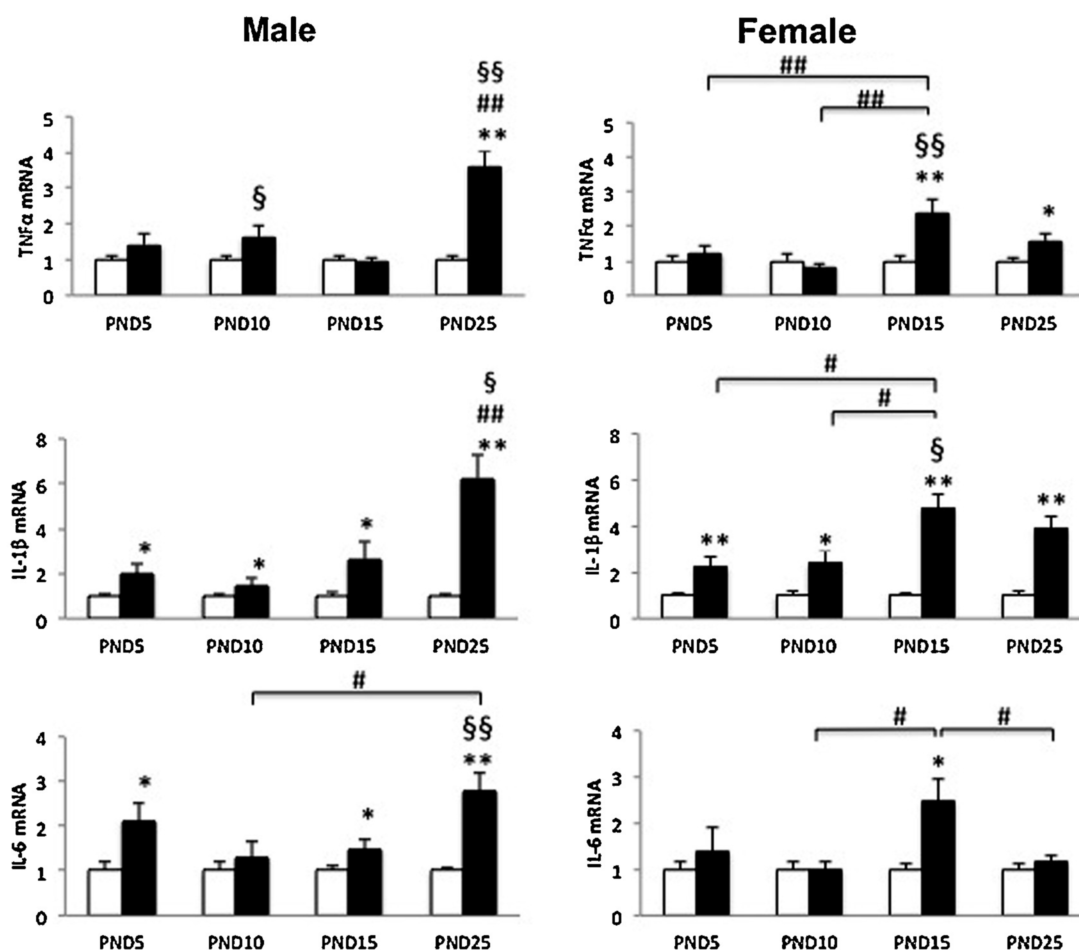

Fig. 4. Hypothalamic TNFα, IL-1β, and IL-6 mRNA expression levels at PND5, 10, 15, and 25 in male and female rats.
Brain samples were collected 2 h after each injection ($n = 10–15$ per group). Relative TNFα, IL-1β, and IL-6 mRNA mRNA expression levels were calculated by dividing the expression level of each molecule by the mRNA expression level of GAPDH. Data are presented as mean + SE values. $^{**}p < 0.01$ $^{*}p < 0.05$ vs. control; ##$p < 0.01$ #$p < 0.05$ vs. within LPS groups; §§$p < 0.01$ §$p < 0.05$ vs. between sexes.

positive regulators of GnRH, are also sensitive to immune stress (Watanobe and Hayakawa, 2003; Kisney-Jones et al., 2009). We reported that high dose LPS injection suppressed hypothalamic *Kiss1, Kiss1r*, and *GnRH* mRNA expression in female rats (Iwasa et al., 2007; Iwasa et al., 2014). However, the expression levels of these molecules were not affected by the injection of a lower dose of LPS at any examined age in the present study. Therefore, we assume that these factors do not play significant roles in the HPG axis SHRP. The mechanisms responsible for SHRP might differ according to the type of stress involved; i.e., immune or metabolic stress.

LH concentration was suppressed, as expected, at PND 25 after LPS injection. However, GnRH mRNA expression level remained stable. Suppression of LH secretion in acute phase such as 2 h would be induced by suppression of pulsatile release of GnRH. Our previous studies showed that orexinergic peptides, orexin A and ghrelin injection acutely suppressed the frequency of pulsatile LH secretion and mean level of LH in female rats (Irahara et al., 2000; Iwasa et al., 2007). On other hand, it is reported that high dose LPS injection (2 mg/kg, i.p.) suppressed GnRH and GnRH-R mRNA expressions acutely in mature female rats (Nappi and Rivest., 1997). Although our LPS dose (0.1 mg/kg, i.p.) was much lower than their dose, GnRH-R mRNA might be suppressed and involved in our data. Lack of estimation of GnRH-R mRNA is a limitation of our study.

The GnRH neuron appeared at 19 day of gestation, and detectable number of GnRH neuron increased until 2 weeks after birth when it showed adult level (Watanabe, 1980). During developing period until at least 5 days after birth, the HPG axis seemed to have a principal role on the development of sexual behavior in the rat (Lim et al., 2014; Holson et al., 1995). The neuronal, metabolic, and immune systems develop rapidly in the SHRP, and increased stress in this period might affect the development of some physiological functions. Some groups have reported that early (PND3) neonatal exposure to stress such as LPS increased the serum corticosterone and adrenocorticotropic hormone levels and decreased the serum progesterone levels of adult male rats (Nilsson et al., 2002), resulting in impaired tumor immunity (Hodgson et al., 2001). Therefore, it is possible that high stress levels during the SHRP induce long-lasting alterations in the HPG axis and cause reproductive dysfunction. Walker et al. reported that early neonatal exposure to LPS reduced the serum LH level in adult rats of both sexes; disrupted the weight-to-age ratio until the onset of puberty; and impaired sexual performance such as the number of kicks, hops, mounts, and interaction time (Walker et al., 2011). Further studies are needed to clarify the mechanisms responsible for these changes. We are planning to test long-term effect of LPS injection in SHRP in rat, such as onset of puberty and sexual behavior.

In conclusion, the HPG axis exhibited a SHRP against immune stress in immature male and female rats. Hypothalamic inflammatory cytokines expression also exhibited hyporesponse against immune stress, which might be responsible for these phenomena. As exposure to significant stress during the SHRP induces long-lasting changes in physiological functions and increases the future

risk of some disorders related to the HPA axis, immune stress during the neonatal period might also induce the risk of reproductive disorders related to the HPG axis. Avoiding infectious stress in the early neonatal period might be important for normal development of the HPG axis.

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