論文の内容の要旨

応用動物科学専攻

平成 28 年度博士課程入学

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論文題目　Study on the role of kisspeptin in neonatal testosterone surge in male rats

（新生子期オスラットのサージ状テストステロン分泌におけるキスペプチンの役割に関する研究）

Mammalian species have different sexual behaviors between males and females at adult. In rodents, females in estrous display female-specific sexual behavior called lordosis behavior responding to mounting behavior of males. The development of sexual behaviors is bipotential during the early life and male rats gain the ability to show male specific sexual behaviors and lose the ability to show female specific sexual behaviors soon after birth, which is defined as masculinization and defeminization, respectively. Sexual differentiation of sexual behavior is caused by a dramatic elevation in plasma testosterone (testosterone surge) during perinatal period in male rats by the following processes: testosterone secreted from testes rapidly arrives brain and is locally converted to estradiol by aromatase in brain, especially in the hypothalamus, then the estradiol organizes and establishes numerous neuron circuits which defeminize and masculinize the male brain and consequently causes the development of mounting behavior and the disability to display lordosis behaviors at adult. Yet the mechanism underlying the generation of perinatal testosterone is unclear.

Kisspeptin, a neuropeptide encoded by *Kiss1* gene, has been recognized as a crucial regulator that is at the top of the hierarchy of the hypothalamus-pituitary-gonadal axis (HPG axis) controlling puberty onset and reproductive functions in mammals. It is well established that kisspeptin induces testosterone production by directly stimulating the release of gonadotropin-releasing hormone (GnRH) and the following secretion of gonadotropins (follicle-stimulating hormone and luteinizing hormone) in adulthood, but we know less about the functions of kisspeptin before puberty including perinatal period. Our previous paper using *Kiss1* knockout (*Kiss1*$^{-/-}$) rats revealed that *Kiss1*$^{-/-}$ males showed a high level of lordosis behaviors as found in females and impaired mounting behaviors, indicating *Kiss1*$^{-/-}$ male rats does not undergo complete defeminization and masculinization during development. However, our previous paper also suggested that both plasma testosterone level and the expression level of aromatase mRNA in hypothalamus in *Kiss1*$^{-/-}$ neonatal male rats were comparable to that of *Kiss1*$^{+/+}$ males.

Administration of either kisspeptin or estradiol within 2 hours after delivery could rescue the defeminization and masculinization of sexual behaviors in *Kiss1*$^{-/-}$ male rats in our previous paper. This means that kisspeptin should play a role in brain sexual differentiation in the upstream of estrogen. In other words, kisspeptin affects either the generation of neonatal testosterone surge or the conversion of testosterone to estradiol in the hypothalamus. One previous report using *Gpr54* (kisspeptin receptor) knockout (*Gpr54*$^{-/-}$) mice demonstrated a significant difference in plasma testosterone concentrations between wildtype and *Gpr54*$^{-/-}$ male mice within 2 hours after birth, which is contradictory to our previous result with *Kiss1*$^{-/-}$ rats. Thus, the possibility that kisspeptin has a role to induce neonatal testosterone surge in male rats still remains. Therefore, the present study aimed to reinvestigate the role of kisspeptin in differentiation of sexual behavior in male rats.

Since blood samples of our previous paper were only collected from one time point, I conducted more frequent blood collection at several different time points during perinatal periods (embryonic day 21, 0 hour after birth, 0–1.5 hours after birth, 1.5–3 hours after birth and 3–4.5 hours after birth) from *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ male rats and measured the concentration of plasma testosterone by enzyme immunoassay (EIA). There was a significant elevation in plasma testosterone concentrations in *Kiss1*$^{+/+}$ males at 0–1.5 hours and 1.5–3 hours

after birth ($p < 0.05$) while the plasma testosterone concentrations were kept at low levels throughout the perinatal period in *Kiss1*$^{-/-}$ male rats. The plasma testosterone concentrations in *Kiss1*$^{+/+}$ males were significantly higher than that in *Kiss1*$^{-/-}$ males at 0–1.5 hours and 1.5–3 hours ($p < 0.01$). In addition, subcutaneous injection of 1 nmol kisspeptin within 2 hours after birth significantly increased the plasma testosterone levels in *Kiss1*$^{-/-}$ neonatal male rats ($p < 0.05$). These results indicate that kisspeptin stimulates the generation of testosterone surge in male rats during perinatal periods.

Next, to determine the involvement of HPG axis for the induction of neonatal testosterone surge by kisspeptin, the elevation of plasma luteinizing hormone (LH) and the activation of GnRH neurons during perinatal period in *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ male rats were examined. Plasma LH concentration was measured by radioimmunoassay (RIA) and the activity of GnRH neurons was evaluated by dual-immunohistochemistry (IHC) for cFos and GnRH. Plasma LH concentration significantly increased in *Kiss1*$^{+/+}$ males at 0 hour after birth compared with that of embryonic day 21 (E21, one day before expected delivery day), whereas plasma LH was kept at undetectable levels in *Kiss1*$^{-/-}$ males. Plasma LH concentration at 0 hour after birth was significantly higher in *Kiss1*$^{+/+}$ males than that in *Kiss1*$^{-/-}$ males ($p < 0.05$), suggesting that an LH surge occurs during the perinatal periods in male rats and is induced by kisspeptin. On the other hand, cFos protein was rarely detected in GnRH neurons either in *Kiss1*$^{+/+}$ ($0.29 \pm 0.29\%$) or in *Kiss1*$^{-/-}$ ($0.26 \pm 0.26\%$) neonatal male brains at 0 hour after birth. When rats were subcutaneously treated with 1 nmol kisspeptin within 2 hours after birth, plasma LH concentration and the number of activated GnRH neurons were significantly increased both in *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ males ($p < 0.05$). Taken these results together, it is likely that kisspeptin induces neonatal testosterone surge by stimulating HPG axis at the time of birth in male rats.

As kisspeptin and its receptor GPR54 are also expressed in testes, I next aimed to investigate if kisspeptin from testes affects the neonatal testosterone surge in male rats. With testes during perinatal period (E15.5, E17.5, E19.5, E21.5, 0 h after birth and within 2 h after birth), the expression of *Kiss1* and *Gpr54* (gene for GPR54) mRNAs in *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ rats were confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Both *Kiss1* and *Gpr54* mRNAs were detected as early as E15.5. Plasma testosterone concentrations in

the late embryonic periods (E17.5, E18.5 and E19.5), which is essential for the masculinization of genitals, had no significant difference between *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ males ($p > 0.05$). IHC for 3βHSD, a key enzyme for testosterone synthesis, revealed that the number of Leydig cells that generate androgens did not differ between *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ neonatal testes within 2 hours after birth ($p > 0.05$). Moreover, *Kiss1*$^{-/-}$ testes from rats within 2 hours after delivery could increase testosterone secretion by the treatment of 100 ng/ml LH for 4 hours *in vitro* culture. These results suggest that the lack of kisspeptin does not affect the development and functions of fetal Leydig cells. To examine if kisspeptin directly stimulates the neonatal testosterone surge secreted from testes, testes of *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ rats obtained within 2 hours after birth were cultured with rat kisspeptin (0.2 μM or 2 μM) or vehicle for 4 hours and testosterone concentrations in the medium were measured. The treatment with 0.2 μM or 2 μM kisspeptin did not significantly increase testosterone levels comparing with vehicle treatment both in *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ testes ($p > 0.05$), indicating that kisspeptin in testes is not involved in the generation of neonatal testosterone surge in male rats.

In summary, the present dissertation demonstrated that 1) the incomplete defeminization and masculinization of sexual behavior in *Kiss1*$^{-/-}$ male rats is due to the absence of neonatal testosterone surge; 2) an LH surge occurring before neonatal testosterone surge is kisspeptin dependent; 3) HPG axis but not sole testis can respond to kisspeptin in newborn male rats; 4) the development and functions of fetal Leydig cells are not affected by kisspeptin. Taken together, these findings suggest that the initiation of testosterone surge in perinatal male rats, which is essential for differentiation of sexual behaviors, is mediated by kisspeptin neuron-induced activation of HPG axis.