# 博士論文（要約）

# 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)**

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## List of publications concerning this dissertation

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# Abstract

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.

As the contents of the chapter 2 to chapter 4 and part of chapter 5 are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

# Table of Contents

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# List of abbreviations

| | |
|---|---|
| ***Kiss1*$^{-/-}$** | *Kiss1* knockout |
| ***Kiss1*$^{+/+}$** | wildtype |
| **HPG axis** | hypothalamus-pituitary-gonads axis |
| **ARC** | arcuate nucleus |
| **AVPV** | anteroventral periventricular nucleus |
| **VMH** | ventromedial nucleus |
| **AMH** | anti-Mullerian hormone |
| **SDN-POA** | sexually dimorphic nucleus of the preotic area |
| **SNB** | spinal nucleus of the bulbocavernosus |
| **ER** | estrogen receptor |
| **AR** | androgen receptor |
| **PGE2** | prostaglandin E2 |
| **GnRH** | gonadotropin-releasing hormone |
| **GPR54** | G protein-coupled receptor 54 |
| ***hpg* mice** | hypogonadal mice |
| **LH** | luteinizing hormone |
| **FSH** | follicle-stimulating hormone |
| **hCG** | human chorionic gonadotropin |
| **AMH** | anti-Mullerian |
| **Kp** | kisspeptin |
| **Veh** | vehicle |
| **PND0** | postnatal day 0 |
| **EIA** | enzyme immunoassay |

| | |
|---|---|
| **RIA** | radioimmunoassay |
| **IHC** | immunohistochemistry |
| **DHT** | dihydrotestosterone |
| **NMDA** | *N*-methyl-D-aspartate |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **IHH** | isolated hypogonadotropic hypogonadism |
| **OVX** | ovariectomized |
| **GDX** | gonadectomized |
| **HRP** | horseradish peroxidase |

# CHAPTER 1: General introduction

## Sexual differentiation

In sexually reproducing species, two sexes, male and female, are required to produce offspring. There are huge differences in various organs or tissues between two sexes including gonads, genitalia and some parts of brain. These sex differences result from sexual differentiation, a common process that promotes two sexes to develop different structures and functions of any part of body, which consists of masculinization, defeminization and feminization. Masculinization and defeminization lead an individual to be more like male and less like female, respectively, while feminization makes female different from male.

Sexual differentiation is not a simple and uniform process, instead it is induced and maintained by numerous factors, such as sex specific genes, sex hormones, epigenetic modifications and environmental factors that especially plays a key role in human. Among these factors, sex specific genes and sex hormones have been well investigated. Masculinization and defeminization are primarily driven by sex hormones and male-specific genes; feminization is emerging as a factor-induced process which had been considered as a default process for a long time: female typical structures and functions were developed spontaneously without any sex-specific factors.

### Sexual differentiation on reproductive system

***Gonads*** In all mammals, genetic sex is determined by sex chromosomes: X and Y chromosome. *Sry*, a dominant sex-determining gene locating on Y chromosome, initiates primordia gonads differentiate to testes[1]. The number of X chromosome has no effect on the determination of gonadal sex. If *Sry* is absent, ovaries develop whatever in XX female or XY male[2,3].

***Genitalia*** Once the differentiation of gonads is settled, hormones become the main factors to affect the following sexual differentiation. Sertoli cells and Leydig cells in testes generate anti-Mullerian hormone (AMH) and androgens, respectively, which act together to defeminize and masculinize both internal and external genitalia during fetal life[4]. AMH targets Mullerian ducts, the precursors of female

internal genitalia (e.g., oviducts and uterus), inducing the regression of Mullerian ducts. Androgen not only stabilizes the differentiation of Wolffian ducts to male internal genitalia (i.e., vas deferens, epididymis and seminal vesicles), but also causes the differentiation of primordia external genitalia to male external genitalia (i.e., penis and scrotum).

On the other hand, granulosa cells and thecal cells instead of Sertoli cells and Leydig cells are formed in female ovaries that do not secrete AMH and androgen. Hence, in the female, Mullerian ducts develop to female internal genitalia with the spontaneous regression of Wolffian ducts, and the female external genitalia are formed from the primordia of the external genitalia spontaneously, too.

Estrogen appears to be unnecessary during the sex differentiation of genitalia in fetuses. Fetuses castrated before sexual differentiation of genitalia developed female genitalia regardless of genetic sex[5]. Mice lacking *Ftz-F1* (Steroidogenic factor1, SF-1 gene), a key regulator of steroidogenic enzymes, had no gonads but normal oviducts, uterus and vagina in both neonatal males and females[6]. Moreover, in estrogen receptor (ER) α or ERβ null mice, males and females show normal genital development during perinatal periods[7,8]. Together, these studies support the idea that androgen make the male different from the female, but estrogen is not required to make the female different from the male in terms of the sex differentiation of genitalia.

**Sexual differentiation on sexual behaviors**

Males and females show different sexual behaviors. For instance, male rodents mount a female in estrus who will display lordosis behaviors exposing vagina to male during copulation. Like gonads, the development of sexual behaviors is bipotential and sex hormones-dependent during perinatal period and then is sexually differentiated in adult. There are two actions of gonadal hormones that firstly introduced by Phoenix *et al.* in 1959[9]: organizational effects and activational effects. The former one makes the sexual behaviors modified permanently and irreversibly; the latter one is reversible and dependents on the concentration of circulating gonadal hormones.

The organizational effects are induced by the perinatal testosterone surge in male rodents, which cause masculinization and defeminization of sexual behavior. Phoenix et al. injected testosterone to pregnant guinea pigs and found that those prenatally androgenized genetic females showed less lordosis behaviors and more mount behaviors compared to the females that were not androgenized before birth [9]. Another experiment performed by Corbier et al. suggested that male rats castrated at 0 hour after birth displayed high frequency of lordosis behavior at the same level as females, which frequency was returned to low level by the immediate administration of testosterone after castration[10]. However, when castration was conducted at 6 hours or afterward, it became more difficult to inhibit defeminization[10,11]. These results suggest that the masculinization and defeminization are induced by testosterone secreted from testes within a very narrow period in male rodents.

It is now well established that estrogen converted from testicular testosterone surge by aromatase in local brain is the primary factor that induces differentiation of sexual behaviors. The first study about this phenomenon was done by Feder. Feder injected testosterone or estrogen to neonatal female rats and found that these two sex hormones had same effects on the development of sexual behavior, this is, lordosis behavior in adulthood was inhibited both in testosterone- and estrogen-treated females[12]. Thereafter, researchers showed that prenatal administration of dihydrotestosterone (DHT), a non-aromatizable androgen, failed to affect lordosis behaviors in adult female guinea pigs[13]. Moreover, injection of aromatase inhibitor during the perinatal period strongly increased lordosis frequency in male rats[14]. Taken together, these evidences suggest that conversion of testosterone to estradiol is indispensable in sexual differentiation of sexual behaviors.

**The mechanisms underlying the sexually dimorphic behaviors**

Many brain regions undergo sexual differentiation, among which the most pronounced differentiation occurs in the neural nuclei where are tightly relevant to sexual behaviors, including the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the ventromedial nucleus of the

hypothalamus (VMH).

The POA is known to be the center of male sexual behaviors. In rodents, lesions of the POA inhibits the display of male sexual behaviors and electrical activation of the POA stimulates males to mate with females[15,16]. Male type of the POA, containing more cell numbers and dendritic synapses, is formed by perinatal testosterone. Estrogen converted from testosterone in local brain protects the neurons in the POA from apoptosis and stimulates the generation of dendritic synapse's connection[17,18]. The density of dendritic synapses rather than the number of neurons closely correlates with mounting behaviors[19].

Estrogen masculinizes the neural circuits in the POA via prostaglandin E2 (PGE2). In the POA, estrogen upregulates the activity of cyclooxygenases-2 (COX-2) to enhance the generation and release of PGE2 in neurons[19]. PGE2 then stimulates neighboring astrocytes and microglia: PGE2 acts on astrocytes to induce the release of glutamate that in turn activates adjacent neurons, ultimately causing the formation of dendritic spine synapses[20,21] (Figure 1-1). Additionally, PGE2 acts on surrounding microglia to stimulate the generation of PGE2 in a feedforward way[22], which can sustainably induce the formation of dendritic spine synapses. Administration of PGE2 to neonatal females masculinizes the number of dendritic spine synapses and leads them to behave male sexual behaviors in adult[19]. Conversely, inhibition of COX-2 or microglia activity in neonatal males decreases the concentration of PGE2 and permanently downregulates the number of dendritic spines, which profoundly impairs male sexual behaviors[19,22].

VMH is to the center of female sexual behavior what POA is to the center of male sexual behavior. Electrical stimulation or estrogen implant to the VMH facilitate the display of lordosis behavior[23,24], while the impairment of the VMH reduces lordosis behavior[25]. Likewise, the number of dendritic spines in the VMH is larger in males than in females. However, the defeminization of the VMH is not caused by estrogen-induced up-regulation of PGE2 and astrocytes are not needed for this signal pathways. In case of the VMH, estrogens directly induce the glutamate release from presynaptic terminals via PI3K

activation, a non-genomic signal transduction. Glutamate then acts on its receptors, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), located on the postsynaptic terminals to activate MAPK and consequently promote the spine formation[26] (Figure 1-1). Blocking of NMDA in neonatal males decreases dendritic spines in the VMH and increases female sexual behaviors in adults, whereas giving NMDA to neonatal females increases dendritic spines in the VMH and inhibits female sexual behaviors in adulthood[27,28].

Different from the sexual differentiation of gonads and genitalia, estrogen plays an essential role in sexual differentiation of the brain and sexual behaviors. The mechanisms underlying estrogen-induced masculinization and defeminization are multiple but specific to each brain regions. PGE2 upregulated by estrogen increases the dendritic spines in the POA but not in the hippocampus and the VMH[19,29]; inhibition of NMDA leads dendritic spines in the VMH to be feminized but has no effects on the POA[21]. Hence, masculinization and defeminization of sexual behaviors seem to be regulated separately. Treatment of female neonates with PGE2 dramatically increases the male sexual behaviors and has no effects on female sexual behaviors, suggesting that PGE2 can induces masculinization without inducing defeminization[29]. Similarly, inhibition of NMDA receptors in neonatally androgenized females rescues lordosis behaviors but does not impair mount behaviors, indicating that activation of NMDA receptors is necessary for defeminization but not for masculinization[27]. These results indicate that induction of masculinization of sexual behaviors may occur independent of defeminization. On the other hand, activation of NMDA defeminizes the sexual behaviors while masculinizes the copulatory behaviors, suggesting that the initiation of defeminization is linked to masculinization, which is likely to make sure an adult to develop at least one kind of sexual behavior[26,27] (Table 1-1). Since glutamate is involved in both masculinization and defeminization, these actions are not surprising and seem to be relevant (Figure 1-1).

## Kisspeptin is essential for mammalian reproduction

### HPG axis is the basic system regulating reproduction in mammals

Gonadal steroid hormones are required for developing and maintaining sex characters and reproductive functions during puberty and adulthood. It is well known that hypothalamus modulates gonadal activities and functions through controlling the anterior pituitary gland in mammals, and this classic network is called hypothalamic-pituitary-gonadal (HPG) axis. The main components of HPG axis are: 1) gonadotrophin-releasing hormone (GnRH), a decapeptide, secreted from GnRH neurons in the hypothalamus; 2) gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), synthesized and released by gonadotrophs in anterior pituitary gland; 3) androgen and estrogen which are generated by Leydig cells in testis and granulosa cells in ovary, respectively. These components interact with each other via a positive feedback or a negative feedback control. In addition to steroidogenesis, the development and maturation of gametes (i.e., spermatogenesis, folliculogenesis and ovulation) are the primary activity of gonads, which are also regulated by HPG axis.

Responding to different activities in gonads, especially in ovaries, there are two modes of GnRH secretions: one is pulsatile GnRH secretion and the other is surge-mode of GnRH secretion[30]. Pulsatile GnRH secretion modulates the tonic secretion of gonadotropins, which is responsible for spermatogenesis in testes, folliculogenesis in ovaries and steroidogenesis in both gonads. This mode of secretion is under the control of negative feedback action of sex steroids. On the other hand, the surge-mode of GnRH secretion only exists in females, which is induced by the positive feedback of dramatically elevated estrogen during preovulatory stage and evokes ovulation by inducing LH surge.

### Kisspeptin is the upstream of GnRH neurons

Kisspeptin, encoded by *Kiss1* gene, was originally identified as a metastasis suppressor[31,32]. In 2003, two studies independently found that mutations of kisspeptin receptor gene (*Gpr54*) can be a

cause of isolated hypogonadotropic hypogonadism (IHH) in human[33,34]. Patients with *Gpr54* mutation show the absence of puberty due to the deficiency of circulating gonadotropins and sex steroids, indicating that kisspeptin-GPR54 system is essential for the onset of puberty and the regulation of HPG axis. Seminara et al. reported that *Gpr54*-defecient mice also show IHH[33]. After this breakthrough, researchers have discovered that kisspeptin and GPR54 are expressed in a variety of mammals, such as rats, pigs, sheep and monkies[35–38].

It is well established that kisspeptin induces the release of GnRH and LH/FSH by direct stimulation of GnRH neurons as GnRH neurons express GPR54[39]. Intracerebroventricular infusion of kisspeptin in sheep induces a dramatic release of GnRH into the cerebrospinal fluid with a parallel rise in plasma LH and FSH. Conversely, the stimulatory effect of kisspeptin is blocked by pretreatment of GnRH antagonist. Moreover, kisspeptin has no effects on *Gpr54*-knockout (*Gpr54*$^{-/-}$) mice. Accumulated researches have shown that the functions of kisspeptin and GPR54 are highly conserved in mammals[40].

In rodents, there are two hypothalamic populations of kisspeptin neurons: one is the arcuate nucleus (ARC) and the other is the anteroventral periventricular nucleus (AVPV)[41]. These two populations are considered to have different roles in reproduction.

Kisspeptin neurons in the ARC are thought to regulate the pulsatile GnRH/gonadotropin secretion by receiving negative feedback signal of sex steroids. As ARC kisspeptin neurons coexpress ER and/or androgen receptor (AR)[42], a lot of studies have been performed to reveal the effects of gonadal hormones on kisspeptin neurons. Castration of male mice results in a significant increase of *Kiss1* mRNA in the ARC, which can be completely reversed by testosterone or estrogen replacement[42]. Similarly, ovariectomized (OVX) female mice show higher expression level of *Kiss1* mRNA in the ARC, and estrogen implantation decreases the *Kiss1* mRNA level[43]. Thus, it is suggested that estrogen and/or androgen exert negative effect directly on kisspeptin neurons in the ARC, which raises the possibility that ARC kisspeptin neurons are responsible for the generation of GnRH pulses.

Recently, three elegant studies using optogenetic strategies performed by Herbison's group further supported the hypothesis that ARC kisspeptin neurons are the GnRH pulse generator[44–46]. In one of the studies, they introduced channelrhodopsin (ChR2), a protein that functions as a light-gated ion channel, into kisspeptin neurons in the ARC, and showed that the kisspeptin neurons expressing ChR2 can be activated by 473-nm blue light[44]. High amplitude, pulse-like increments in LH secretion were observed in both anesthetized male and diestrous female mice at 20 Hz of optogenetic activation. In OVX female mice, 5 Hz was enough to trigger the LH pulses. In another study, using GCaMP6 fiber photometry technology, monitered the population activity of the ARC kisspeptin neurons in conscious-behaving mice[46]. They showed that ARC kisspeptin neurons in intact male mice exhibited episodes of synchronized activity with a very wide range of intervals. Gonadectomy resulted in dramatic changes in the dynamics of the ARC kisspeptin neurons with much higher frequency of synchronized activity. Furthemore, continuous blood sampling revealed a perfect correlation between the activity of the ARC kisspeptin neurons and LH pulses in intact and short-term gonadectomized (GDX) mice. The results provide insights into the ARC kisspeptin neurons as a GnRH/LH pulse generator and the target of negative feedback control of HPG axis.

On the other hand, kisspeptin neurons in the AVPV are thought to generate the preovulational GnRH/LH surge in females. AVPV kisspeptin neurons are sexually differentiated, and there are much more kisspeptin neurons in the AVPV in adult females than adult males, which is organized by perinatal testosterone as with the SDN-POA[47]. Most of kisspeptin neurons in the AVPV express ERs (mainly ERα)[43,48]. Opposite to kisspeptin neurons in the ARC, *Kiss1* mRNA in the AVPV is positively regulated by estrogen: the expression level of *Kiss1* mRNA is reduced after OVX and increased with estrogen treatment; the level of *Kiss1* mRNA during estrous cycle peaks in the evening of proestrus[43,48]. Most kisspeptin neurons in the AVPV coexpress cFos, an immediate early gene that indicates the activity of neurons, coincidently with the LH surge but little coexpress cFos on diestrus[48,49]. Infusion of kisspeptin

antibody to the POA blocks the estrogen-induced LH surge in rats[49]. These evidences suggest that kisspeptin neurons in the AVPV may induce the GnRH/LH surge responding to positive feedback of estrogen. Further direct experiments are required to uncover this problem.

**The funciton of kisspepitn in testes**

The expression of kisspeptin as well as GPR54 has been detected in testes in various species including humans, rodents and goats[50–53]. Several investigations have evidenced that kisspeptin might have direct effects on testes. In the adult male rhesus monkey, kisspeptin administration significantly elevated human chorionic gonadotropin (hCG)-stimulated testosterone levels in acyline, a GnRH antagonist, pre-treated monkeys when compared with controls[54]. Samir et al. observed that the production of testosterone in Leydig cells isolated from testes of adult goats were suppressed by kisspeptin antagonist compared with controls[55]. These results are indicative of a direct effect of testicular kisspeptin on steroidogenesis in testes. On the other hand, studies on mice showed that kisspeptin can directly increase neither basal testosterone release nor hCG- or LH-stimulated testosterone release in adult mouse testes[52,56]. These different results are likely to be caused by the difference of animals used.

## Kisspeptin plays a role in sexual differentiation

Kisspeptin and GPR54 have been found to be involved in the sexual differentiation of brain and sexual behavior. Male mice with *Gpr54* gene mutations show female-like tyrosine hydroxylase neurons and consequently their partner preference is altered: they do not prefer estrous females[57,58]. Kisspeptin neurons in the AVPV and the volume of SNB are also feminized both in *Gpr54*$^{-/-}$ male mice and *Kiss1*-knockout (*Kiss1*$^{-/-}$) male rats[57,59]. Moreover, the size of the SDN-POA is larger in wild type male rats than in *Kiss1*$^{-/-}$ male rats which is as small as females[59] .

*Gpr54*$^{-/-}$ male and female mice do not display sexual behaviors, however, sex hormone-replaced

GDX *Gpr54*$^{-/-}$ males and females exhibit appropriate gender-specific sexual behaviors[57]. *Kiss1*$^{-/-}$ male rats exhibit no male-type sexual behaviors, and the ability to show male sexual behaviors is recovered by long-term testosterone replacement from the peripuberty to adulthood. Notably, *Kiss1*$^{-/-}$ male rats can display the lordosis behaviors as shown in females, which is rescued by kisspeptin administration in male rats within 2 hours after birth[59]. Taken together, these evidence indicate that the organizational action on brain nuclei and neural circuits for sexual behaviors during perinatal period, especially defeminization, is influenced by kisspeptin.

## Objective

The present dissertation aims to investigate the mechanism that kisspeptin induces sexual differentiation in male rats. In chapter 2, to determine the effect of kisspeptin in plasma testosterone profiles during perinatal periods in wild type and *Kiss1*$^{-/-}$ male rats, blood samples at several time points during perinatal period were collected and plasma testosterone concentrations were measured. It is well established that kisspeptin stimulates steroidogenesis by regulating HPG axis in adult rats; however, little is known about the relation between kisspeptin and HPG axis in neonatal rats. In chapter 3, to reveal the involvement of HPG axis in generation of testosterone surge in neonatal male rats, plasma LH concentration and GnRH neuron activity in perinatal male rats with or without kisspeptin treatment were examined. The reports on the direct effect of kisspeptin on testes are limited, therefore in chapter 4, to investigate if testicular kisspeptin affects the neonatal testosterone surge directly, the expression profiles of the mRNA of *Kiss1* and *Gpr54* during embryogenesis were examined by RT-PCR firstly, then the fetal plasma testosterone concentrations and the number of Leydig cells in neonatal male rats within 2 hours after birth were evaluated by EIA and IHC, respectively. Lastly, the effect of kisspeptin on the generation of testosterone in neonatal testes were examined *in vitro*.

## Figure and table

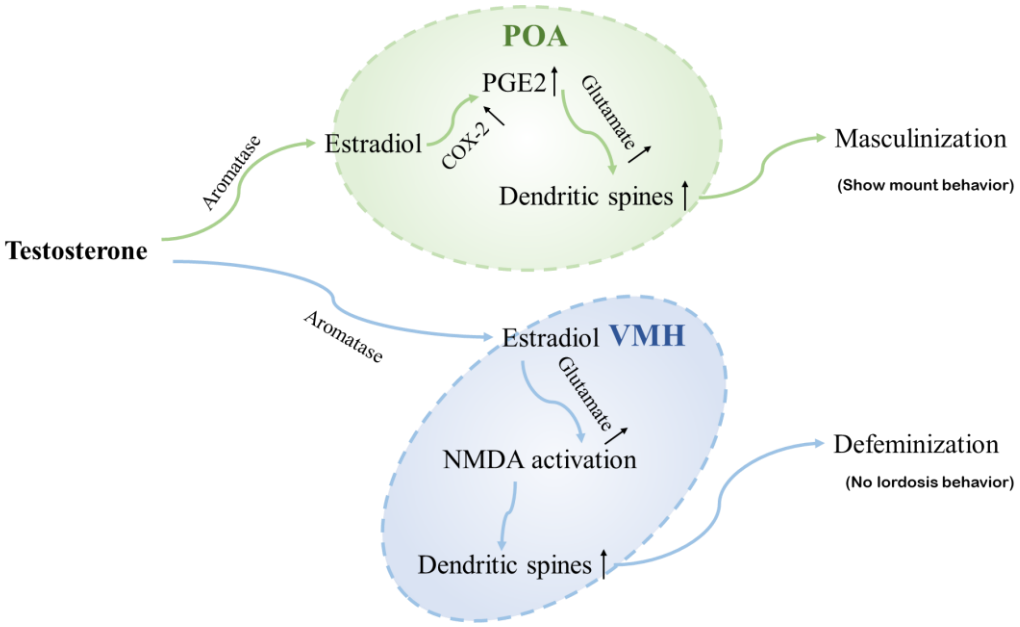


**Figure 1-1. Main signaling pathways in masculinization and defeminization of the brain in neonatal males.** Testosterone secreted by testes is converted to estradiol by aromatase in the brain. Estradiol in the POA increases the synthesis of COX-2 in neurons, which in turn enhances the generation of PGE2 by neurons. PGE2 synthesized by neurons stimulates surrounding microglia to generated PGE2 and activates astrocytes to release glutamate, inducing the synthesis of new dendritic spines and further masculinization of sexual behavior. Meanwhile, estradiol in the VMH enhances the release of glutamate from presynaptic terminals, which in turn activates NMDA, one of the glutamate receptors, on postsynaptic neurons. Activation of NMDA increases the synthesis of new dendritic spines and further defeminization of sexual behavior. COX-2, cyclooxygenase-2; NMDA, *N*-methyl-D-aspartate receptor; PGE2, prostaglandin-E2.

| Sex | Treatment | Mount behavior | Lordosis behavior | Differentiation |
|---|---|---|---|---|
| ♂ | No treatment | + | - | Ma & De |
| | COX inhibitor | - | No data | |
| ♀ | No treatment | - | + | No Ma & No De ① |
| | E | + | - | Ma & De ② |
| | E + NMDA antagonist | + | + | Ma & No De ③ |
| | PGE2 | + | + | Ma & No De ④ |
| | NMDA agonist | + | - | Ma & De ⑤ |

**Table 1-1. Summary of the studies for the differentiation of sexual behavior in mice with a variety of treatments on PND0**. Despite whether the masculinization occurs or not, the defeminization can be suppressed (①, ③ and ④). If defeminization occurs, masculinization will occur (② and ⑤) Differentiation indicates masculinization or defeminization of sexual behaviors. Ma, masculinization; De, defeminization.

# CHAPTER 2: The effects of kisspeptin on perinatal testosterone generation

As the contents of this chapter is anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

# CHAPTER 3: The effects of kisspeptin on HPG axis

As the contents of this chapter is anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

# CHAPTER 4: The effects of kisspeptin on perinatal testes

As the contents of this chapter is anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

# Chapter 5: General discussion

## Perinatal testosterone surge is a universal phenomenon in mammals

In addition to rodents, perinatal testosterone surge has been found in a variety of other species, such as humans, non-human primates and ruminants. As I introduced in chapter 1, in rats and mice, the testosterone surge required for sexual differentiation of brain and sexual behavior occurs within hours after birth. Estradiol, aromatized from testosterone in local brain, plays the dominant role in masculinization and defeminization of brain and sexual behavior. The time of occurrence and their functions, however, are species dependent.

For domestic animals, a large amount investigation about the effects of perinatal testosterone surge have been performed by sheep. In sheep, the sexual differentiation of brain and sexual behavior are occurred during embryogenesis. There is a testosterone elevation in fetuses from day 30 to 70 with a peak in day 70 of gestation (term 145 days)[116], and differentiation of brain occurs at around day 50 to 80 of gestation[117]. Fetal ewes exposed to exogenous testosterone or estrogen between day 50 and 80 of gestation display increased mounting behavior and most of them fail to show receptivity at adult[118,119]. Dissociated to sexual behavior, LH surge can be induced in androgenized ewes, whose peak value is much lower than that of control ewes[118,120]. Unlike rats that perinatal testosterone administration completely differentiates the sexual behavior and LH secretion pattern at the same time[121,122], it seems that broader period and additional factors are required for sheep. For cattle and goats, it is assumed that the testosterone surge occurring in mid-gestation is involved in the sexual differentiation of brain and sexual behavior, but the effects of mid-gestation testosterone or estrogen exposure are ambiguous[119,123], so more studies are needed to confirm it.

There are two surges of testosterone, mid-gestation and the first few months after birth, in humans and rhesus monkeys[62,124,125]. A lot of investigations have shown that mid-gestation testosterone surge rather than postnatal testosterone surge is more important for sexual differentiation of brain and behavior. Androgen but not estrogen is suggested to be involved in the sexual differentiation in primates, since

DHT, a non-aromatizable testosterone, shows equal effects as testosterone in rhesus monkeys[124] and genetic XY human males with androgen insensitivity syndrome that is caused by mutations in the androgen receptor show female type preference[126].

As the rest of the contents of this chapter is anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years.

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