Functional Analyses of Kisspeptin in Controlling Gonadal Functions

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Abstract

Kisspeptin (also termed as metastin) is a peptide that has been isolated from the human placenta as the cognate ligand of the G-protein-coupled receptor KISS1 receptor (KISS1R, also termed as GPR54). However, its physiological functions were not fully investigated when kisspeptin was identified. In this thesis, through the functional analysis in rats, I first show that the subcutaneous administration of kisspeptin or its agonist analogues activates the hypothalamic gonadotropin releasing hormone (GnRH) neurons, associated with robust elevations in plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels. These results suggest that kisspeptin is regarded as a newly characterized regulator of GnRH and gonadotropin release in the hypothalamic-pituitary-gonadal (HPG) axis. I then study the effect of continuous subcutaneous administration of kisspeptin agonist analogues on the HPG-function in male rats, and demonstrate that the continuous administration results in abrupt reductions of sex hormone levels including testosterone. Mechanistic studies suggest that continuous administration of kisspeptin analogues disrupts endogenous kisspeptin signals to suppress intrinsic GnRH pulses by desensitization of KISS1R on GnRH neurons. I further investigate the potential application of kisspeptin agonist analogues, and demonstrate continuous administration of kisspeptin agonist analogues leads to rapid and profound reductions in testosterone levels and also in testosterone-dependent organs' weight including prostate, seminal vesicles, and testes. In addition, in a pre-clinical rat model of androgen dependent prostate cancer, the JDCaP rat model, continuous administration of kisspeptin agonist rapidly decreased plasma prostate specific antigen (PSA) levels, which is clinically relevant prostate cancer marker. Thus, my thesis demonstrates that kisspeptin is a novel and perhaps physiologically meaningful GnRH secretagogue and kisspeptin agonist analogues may hold promise as a novel therapeutic approach for suppressing reproductive functions and hormone-dependent diseases such as prostate cancer.

Abbreviations

ADT	androgen deprivation therapy
AR	androgen receptor
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
CHO	Chinese hamster ovary
CI	confidence interval
DMSO	dimethylsulfoxide
DW	distilled water
E2	estradiol
EC50	half maximal effective concentration
eCG	equine chorionic gonadotropin
EIA	enzymeimmuno assay
ER	estrogen receptor
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GPCR	G-protein coupled receptor
hCG	human chorionic gonadotropin
IC50	half maximal inhibitory concentration
IHH	idiopathic hypogonadotropic hypogonadism
KISS1R	KISS1 receptor
Kp-10	kisspeptin-10
Kp-54	kisspeptin-54
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LH	luteinizing hormone
ME	median eminence

MUA	multiunit electrical activity
MW	molecular weight
NMDA	N-methyl-D-aspartate
ORX	orchiectomy
OVLT	organum vasculosum of the lamina terminalis
P4	progesterone
POA	preoptic area
PVN	paraventricular nucleus
RIA	radioimmuno assay
SON	supraoptic nucleus
T	testosterone

Introduction

Reproductive function in mammals is regulated by the three critical components, hypothalamus, pituitary, and gonad. In 1940's Harris *et al.* proposed the fundamental concept of the neuroendocrinology (Charlton, 2008). Historically it had been known that sexual functions including estrus cyclicity and ovulations were regulated by the hypothalamus and pituitary but little understandings about its precise mechanism had not been elucidated. The simplest plausible mechanism was the direct neural innervation from the hypothalamus to the pituitary and/or gonads but such kind of anatomical evidences only existed in oxytocin and vasopressin, both of these are secreted from the nerve terminals in the posterior pituitary innervated from the hypothalamus. Harris *et al.* addressed this question with surgical approach and demonstrated that a portal vein between the hypothalamus and the pituitary efficiently conveyed the substances released from the hypothalamic nerve terminals to the pituitary (Charlton, 2008). The actual substance released from the hypothalamus had not been identified until early 1970's by Schally *et al.* and Guillemin *et al.* (Schally, 1999). They independently identified a peptide sequence of gonadotropin releasing hormone (GnRH, also termed as luteinizing hormone releasing hormone or LHRH) from pigs and sheep as a critical peptide in stimulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. Subsequently the peptide structure of GnRH in other species including human were identified with highly conserved structure.

In rats and mice, the majority of GnRH neurons exist in preoptic area (POA) within the posterior hypothalamus, and the neurons project to median eminence (ME) close to the arcuate nucleus (ARC) region (Fig. 1). GnRH is released from the ME to the hypothalamic-pituitary portal vein, which eventually conveys GnRH to the pituitary. There are two modes of GnRH release; GnRH pulses and GnRH surge (Maeda & Tsukamura, 2011). The physiological importance of pulsatile GnRH release was first demonstrated by Belchetx *et al.* in 1978 (Belchetz *et al.*, 1978). They tested intermittent and continuous GnRH administration in rhesus monkeys with hypothalamic lesions, and demonstrated that intermittent GnRH administration, once per hour, elevated serum gonadotropin levels while the continuous infusion suppressed the gonadotropin release, which is known as

"desensitization" of the pituitary to the continuous exposure to GnRH. Following this observation, pulsatile GnRH release was finally demonstrated in domestic animals (eg. ewes and goats) in which the hypothalamic-pituitary portal blood was collected by excellent surgical technique (Tanaka *et al.*, 1997). Importantly, the pulsatile GnRH release into the portal blood was completely paralleled to the pulsatile LH release from the pituitary, suggesting GnRH pulses are the critical driver of LH pulses. Furthermore, pulsatile neural activity, called multiple unit activity (MUA) volley, was observed by means of electrodes implanted into the basal hypothalamus, and this MUA volley was also completely paralleled to pulsatile LH release (Mori *et al.*, 1991; Cardenas *et al.*, 1993). These evidences demonstrated that GnRH and LH pulses were regulated by pulsatile neural activity around the area of basal hypothalamus. On the other hand, the other mode of GnRH release, GnRH surge, is only observed in female animals, and this leads to pre-ovulatory LH surge and subsequently ovulation. The LH surge is associated with the strong activation of the GnRH neurons, as evidenced by c-Fos protein expressions within the GnRH neurons (Lee *et al.*, 1990; Attardi *et al.*, 1997).

Both GnRH/LH pulses and surge are under control of gonadal steroid hormones, termed steroid hormonal feedback mechanism (Maeda & Tsukamura, 2011). GnRH/LH pulses are observed both in males and females, where elevations in circulating testosterone or estradiol levels lead to suppression of the GnRH/LH release (negative feedback). On the other hand, preovulatory GnRH/LH surge is triggered when ovarian follicles are maturated enough associated with apparent elevations in circulating estradiol levels (positive feedback). These steroidal positive and negative feedback systems have been widely acknowledged while its precise mechanism, especially the responsible neurons were remained to be elucidated for long time. Given that GnRH neurons do not express steroid hormone receptors (estrogen receptor alpha or ERa etc.), the existence of GnRH afferents which critically control GnRH neural activity and release have been suggested (Nishihara *et al.*, 1999) (Fig. 1). As shown in Fig. 1, the GnRH neuronal body exists within the hypothalamic region of the preoptic area (POA), and the GnRH neurons project their nerve terminals to the median eminence (ME). Several experimental approaches, such as surgical denervation or local estrogen implantation, had revealed that the neurons responsible for GnRH/LH surge generation exist within the region of anteroventral periventricular nucleus (AVPV), while the neurons responsible for GnRH/LH pulses

exist within the region of ARC. Several research activities had tried to identify these GnRH/LH surge and pulse generators, but still remained to be elucidated (Nishihara *et al.*, 1999).

Kisspeptin is the cognate ligand of a G-protein coupled receptor (GPCR) KISS1R (also termed as GPR54) (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Since kisspeptin is a gene product of the human metastasis suppressor gene, *KISS1*(Lee *et al.*, 1996; Lee & Welch, 1997a; b), earlier studies on kisspeptin were focused on its activity as a tumor metastasis suppressor (Hori *et al.*, 2001; Ohtaki *et al.*, 2001; Stafford *et al.*, 2002). Actually kisspeptin significantly suppressed cellular motility of KISS1R-expressing CHO cells *in vitro* and tumor metastasis of KISS1R-expressing melanoma *in vivo*, while its physiological role was not well understood (Hori *et al.*, 2001; Ohtaki *et al.*, 2001). *KISS1* gene encodes a 145-amino acid precursor polypeptides, which is further enzymatically cleaved into a mature 54-amino acid peptides, Kp-54, with C-terminally amidated modification (Ohtaki *et al.*, 2001). Within the 54-amino acid sequence, its C-terminal 10 amino acid sequence, Kp-10, is critical in associating and activating KISS1R. This Kp-10 sequence is well conserved in vertebrates including fish, frogs, rodents, monkeys, and human (Table 1).

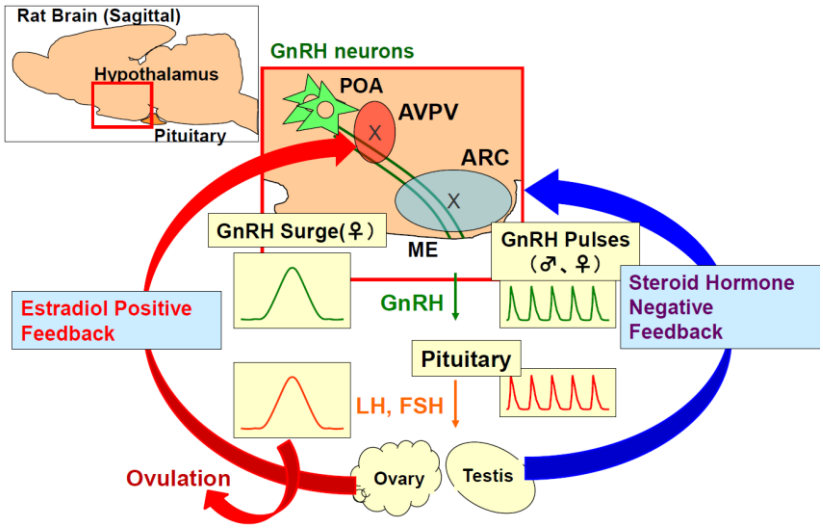

Fig. 1 Schematic Illustration of the Hypothalamic-Pituitary-Gonadal Axis (Rodent Model)
The majorities of GnRH neural perikarya exist in the POA region, and project their neural terminals to the ME, where GnRH is released into the portal blood. There are two modes of GnRH release, GnRH pulses and GnRH surges. The GnRH pulses are common both in males and females, and controlling steroidogenesis, spermatogenesis, and follicular maturations. GnRH pulses are negatively controlled by gonadal steroid hormones (negative feedback), and the historical evidences have suggested some neurons within the ARC region receive this negative feedback signals. On the other hand, GnRH surge is observed exclusively in females and triggers ovulation in response to estradiol positive feedback. Historical evidences have suggested some neurons within the AVPV region receive this positive feedback signals. See text in detail.

Table 1 Putative Kisspeptin-10 Sequences
Bold and shaded letters indicate conserved sequences across species.

	Species Name	C-terminal 10 Amino Acid Residues										GenBank No.
KISS1 Gene Product	Homo sapiens (human)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	F	AAC79512.1
	Macaca mulatta (Rhesus monkey)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	F	XP_001098284.2
	Pan troglodytes (chimpanzee)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	F	AA05977.1
	Gorilla gorilla gorilla (western lowland gorilla)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	F	XP_004028270.1
	Sus scrofa (pig)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAH47553.1
	Bos taurus (cattle)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAM28696.1
	Capra hircus (goat)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAH89066.1
	Ovis aries (sheep)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	AFW03832.1
	Felis catus (domestic cat)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	XP_003999526.1
	Mus musculus (house mouse)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAM11250
	Rattus norvegicus (Norway rat)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	EDM09773.1
	Suncus murinus (house shrew)	**Y**	**N**	R	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAL02985.1
	Xenopus laevis (African clawed frog)	**Y**	**N**	W	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAI50705.1
	Danio rerio (zebrafish)	**Y**	**N**	L	**N**	**S**	**F**	**G**	**L**	**R**	Y	ABV03802.1
	Carassius auratus (goldfish)	**Y**	**N**	L	**N**	**S**	**F**	**G**	**L**	**R**	Y	ACI96030.1
	Oryzias latipes (Japanese medaka)	**Y**	**N**	L	**N**	**S**	**F**	**G**	**L**	**R**	Y	BAF98208
	Cyprinus carpio (common carp)	**Y**	**N**	L	**N**	**S**	**F**	**G**	**L**	**R**	Y	AFM08413.1
	Dicentrarchus labrax (European seabass)	**Y**	**N**	L	**N**	**S**	**F**	**G**	**L**	**R**	Y	ACM07422.1
	Species Name	**C-terminal 10 Amino Acid Residues**										**GenBank No.**
KISS2 Gene Product	Danio rerio (zebrafish)	**F**	**N**	Y	**N**	**P**	**F**	**G**	**L**	**R**	**F**	ACJ50541.1
	Carassius auratus (goldfish)	**F**	**N**	Y	**N**	**P**	**F**	**G**	**L**	**R**	**F**	ACS34769.1
	Oryzias latipes (Japanese medaka)	**F**	**N**	Y	**N**	**P**	**F**	**G**	**L**	**R**	**F**	BAG86623.1
	Cyprinus carpio (common carp)	**F**	**N**	Y	**N**	**P**	**F**	**G**	**L**	**R**	**F**	AFM08414.1
	Dicentrarchus labrax (European seabass)	**F**	**N**	F	**N**	**P**	**F**	**G**	**L**	**R**	**F**	ACM07423.1

In this thesis, I first focus on investigating potential physiological functions of kisspeptin in reproductive axis in rats, and demonstrate peripherally administered kisspeptin induces ovulation associated with the robust elevations in plasma gonadotropins (FSH and LH). Furthermore, I focus the mechanism underlying the induction of gonadotropin-release by kisspeptin with the aid of a gonadotropin-releasing hormone (GnRH) antagonist Cetrorelix, c-Fos and GnRH double-labeling immunohistochemistry, and the primary culture of rat anterior pituitary cells.

Following my first study, several evidences have shown that kisspeptin/KISS1R system is a key regulator of GnRH release from the hypothalamic GnRH neurons. In 2003, inactivating mutations in the *KISS1R* gene from idiopathic hypogonadotropic hypogonadism (IHH) patients were reported, as well as the murine phenotype of defective onset of puberty in mice lacking the *Kiss1r* gene (de Roux *et al.*, 2003; Funes *et al.*, 2003; Seminara *et al.*, 2003). Several groups have demonstrated that, in accordance with my study in Chapter 1, central or peripheral bolus administration of kisspeptin resulted in marked GnRH and gonadotropin release in several species including human (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Thompson *et al.*, 2004; Dhillo *et al.*, 2005). It is currently well accepted that kisspeptin directly activates GnRH neurons as evidenced by: (a) kisspeptin administration induces c-Fos immunoreactivity, a marker of neuronal activity (Kovacs, 1998), in GnRH neurons as shown in

this Thesis Chapter 1 and by Irwig *et al.* (Irwig *et al.*, 2004); (b) GnRH neurons express *Kiss1r* promoter activity (Irwig *et al.*, 2004; Messager *et al.*, 2005); and (c) *ex-vivo* cultured GnRH neurons are depolarized following exposure to kisspeptin (Han *et al.*, 2005; Liu *et al.*, 2008; Pielecka-Fortuna *et al.*, 2008). Finally, while the exact site of action of kisspeptin within the hypothalamus has not been fully elucidated, both synaptic and nonsynaptic associations of kisspeptin neurons with GnRH neurons, specifically in the region of the preoptic area and median eminence, respectively, have been identified by immunohistochemistry (IHC) as potential sites of action (Kinoshita *et al.*, 2005; Clarkson & Herbison, 2006; Ramaswamy *et al.*, 2008).

As described above, acute administration of kisspeptin increases GnRH and gonadotropin levels and activates the reproductive system. In contrast, several groups have reported that chronic administration of kisspeptin results in attenuation of pituitary or gonadal function in rats (Thompson *et al.*, 2006), monkeys (Seminara *et al.*, 2006; Ramaswamy *et al.*, 2007), and women (Jayasena *et al.*, 2010). Thus, the kisspeptin/KISS1R system can be considered a rational target for therapeutic regulation of reproductive functions. Although the N-terminally truncated human Kp-10 polypeptide is responsible for receptor binding and agonistic activity for KISS1R *in vitro* (Ohtaki *et al.*, 2001), high susceptibility of Kp-10 to cleavage enzymes *in vivo* results in a short half-life which limits its potential use as a clinical therapeutic agent (Asami *et al.*, 2012a; Asami *et al.*, 2012b; Asami *et al.*, 2013; Asami *et al.*, 2014). Asami *et al.* have developed several analogue peptides of Kp-10 including KISS1-305, TAK-448, and TAK-683 (Asami *et al.*, 2012a; Asami *et al.*, 2012b; Asami *et al.*, 2013; Asami *et al.*, 2014). KISS1-305 is a prototype peptide that resists plasma protease degradation but has sub-optimal KISS1R agonistic activity *in vitro*; TAK-448 and TAK-683 has good protease resistance and retains potent agonist activity comparable to Kp-10.

In the second part of this Thesis, I study the potential clinical application of kisspeptin agonist analogues. Prostate cancer is the second most common cancer in the world (Jemal *et al.*, 2011). Since Huggins and Hodges first proposed hormonal therapy to treat prostate cancer in 1941 (Huggins & Hodges, 1941), testosterone-lowering therapy has been the mainstay for prostate cancer treatment. Though the majority of prostate cancer is diagnosed as localized disease and curative treatments such as radical prostatectomy are standard, patients with metastatic prostate cancer are encouraged to

receive androgen deprivation therapy such as bilateral orchiectomy (ORX) or gonadotropin-releasing hormone (GnRH) analogue-based therapy. Currently, continuous administration of GnRH analogues, such as leuprolide, is the mainstay of treatment for prostate cancer. Therapy with GnRH analogues is initially associated with substantial testosterone elevation especially during the first week of therapy (Bubley, 2001). This is followed by desensitization of the pituitary, which eventually leads to a decrease in testosterone levels. To explore the pharmacodynamic effects of kisspeptin analogues, I compared the effect of repetitive (once daily) or continuous administration of TAK-448 and TAK-683 with that of leuprolide on plasma testosterone levels as well as the weights of the reproductive organs in adult male Sprague-Dawley rats. I also evaluated the therapeutic potential of TAK-683, as a representative of kisspeptin agonist analogue, in a preclinical rat model of androgen-dependent human prostate cancer JDCaP (Kimura *et al.*, 2009) by measuring serum prostate specific antigen (PSA) levels as a biomarker of antitumor activity.

Furthermore, I investigated the mechanism of action of testosterone-lowering effect with these agents by (a) evaluating their effects on pituitary and hypothalamic responsiveness; (b) assessing the impact of chronic administration on the expression of *Kiss1*, *Kiss1r,* and *Gnrh* mRNA; and (c) determining GnRH peptide content in the hypothalamus as well as FSH and LH expression in the pituitary. My findings reveal that chronic administration of these kisspeptin analogues desensitizes GnRH neurons to endogenous kisspeptin input and reduces hypothalamic GnRH content. Together, these effects combine to suppress HPG function and consequently lower circulating concentrations of T. These results are discussed in relation to their impact on the elucidation of the biological and cellular processes that influence the control of the HPG axis as well as the pharmacological effects of these analogues on the control of T concentrations in plasma.

Materials and Methods

Materials

Human Kp-54, KISS1-305 ([D-Tyr^{46},D-Pya(4)^{47},azaGly^{51},Arg(Me)^{53}] metastin(46-54), molecular weight [MW]: 1165.3), TAK-448 (Ac-[D-Tyr^{46},Hyp^{47},Thr^{49}, azaGly^{51},Arg(Me)^{53},Trp^{54}]metastin(46-54), MW: 1225.4), TAK-683 (Ac-[D-Tyr^{46}, D-Trp^{47},Thr^{49},azaGly^{51},Arg(Me)^{53}, Trp^{54}]metastin(46-54), MW: 1298.5) (Asami *et al.*, 2013), and leuprolide acetate (MW: 1209.40 as acetate-free form) were synthesized Takeda Pharmaceutical Company Limited (Osaka, Japan). eCG and hCG (Teikoku Hormone MFG, Tokyo, Japan), synthetic GnRH peptide (Peptide Institute, Osaka, Japan), GnRH antagonist Cetrorelix (ANASPEC, San José, USA), DNase I, pancreatin, fetal calf serum (FCS), and 17-β estradiol (Sigma-Aldrich, St. Louis, MO), Collagenase A (Roche Diagnostics, IN, USA), Dulbecco's modified Eagle's medium (DMEM, with or without phenol red, Invitrogen, CA, USA), and charcoal/dextran treated FCS (HyClone, UT, USA) were used *in vivo* and *in vitro* experiments. Osmotic ALZET mini-pumps (Model 2001 or 2004) were from Durect Co. (Cupertino, CA). Other general reagents were dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO), aprotinin (Trasylol™, Bayer HealthCare Pharmaceuticals, Leverkusen, Germany), and EDTA-2Na (Dojindo Laboratories, Kumamoto, Japan). Rat FSH (rFSH-RP-2) and LH (rLH-RP-3) as reference peptides, and radioimmunoassay (RIA) sets were obtained from the National Hormone & Peptide Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Los Angeles, CA). A commercially available rat LH kit (rat LH [^{125}I] assay system, GE Healthcare UK Ltd, Buckinghamshire, UK) was also used when specified. The T RIA and GnRH RIA kits were obtained from Siemens Healthcare Diagnostics Inc. (Deerfield, IL) and Peninsula Laboratories (San Carlos, CA), respectively.

Animals

Adult male Wistar rats, immature female Wistar rats, and adult male Sprague-Dawley Crl:CD(SD) rats were purchased from Charles River Japan (Kanagawa, Japan). Adult male F344/N rats were purchased from Clea Japan (Tokyo, Japan). Animals were housed in a temperature- (23 ± 2°C) and

humidity- (55 ± 10%) controlled room with a 12h:12h light:dark cycle. All animals had free access to food and water. All animal experiments were approved by the Takeda Experimental Animal Care and Use Committee.

Gonadotropin release assay *in vivo*

Kp-54 or saline alone was injected s.c. into 25-day-old female Wistar rats (weighing 66 g on average) or 11-week-old male Wistar rats (weighing 350 g on average) at 0900–1000 h. The dose of Kp-54 was 6.7 nmol/0.2ml saline for immature female rats or 35 nmol/0.2 ml saline for adult male rats. In an eCG-primed model, eCG (10 IU/0.2 ml saline) was pre-injected s.c. into immature female rats at 0900–1000 h on day 23 (weighing 54 g on average). The rats were decapitated at 0, 1, 2, or 4 h after the injection and their trunk blood was collected into plastic tubes containing 1,000 U/ml aprotinin and 0.1 g/ml disodium-ethylenediaminetetraacetic acid. In Cetrorelix-treatment studies, Kp-54 (35 nmol/0.2 ml saline) or saline alone (0.2 ml) was injected s.c. into 10-week-old male Wistar rats (weighing 410 g on average) that had been pretreated s.c. with either Cetrorelix (200 nmol/0.2 ml saline) or saline (0.2 ml) 30 min before the administration of Kp-54. The animals were decapitated and their trunk blood was collected 90 min after Kp-54 administration. In Cetrorelix-treatment studies in immature female rats, Kp-54 (3 nmol/0.2 ml saline) or saline alone (0.2 ml) was injected s.c. into 25-day-old female Wistar rats, which had been administered with eCG 2 days before, that had been pretreated s.c. with either Cetrorelix (30 nmol/0.2 ml saline) or saline (0.2 ml) 30 min before the administration of Kp-54. The animals were decapitated and their trunk blood was collected 90 min after Kp-54 administration. All blood samples were prepared into plasma by centrifugation at 1,800 x *g* for 25 min at 4 °C, and subjected to radioimmunoassay (RIA).

Ovulation Assay in Immature Rats

Female Wistar rats at 23 days of age (weighing 54 g on average) were pretreated with eCG (10 IU/0.2 ml saline, s.c.) at 0900–1000 h to induce follicular maturation. After 47–48 h (on day 25), the rats were treated with hCG (10 IU/0.2 ml saline, s.c.), Kp-54 (6.7 nmol/0.2 ml saline), or saline alone (0.2 ml). After another 24 h (on day26), the rats were decapitated and their oviducts and uteri were

collected to count the number of oocytes with the aid of a stereomicroscope. The trunk blood was prepared into plasma to determine the plasma E2 and progesterone (P4) levels by RIA.

Primary Culture of Rat Anterior Pituitary Cells

The rat anterior pituitary cells were prepared as described previously (Matsumoto *et al.*, 1990). Briefly, anterior pituitaries from male (7-week-old) or female (9-week-old) Wistar rats were dispersed by incubation with 0.4% collagenase A and 10 μg/ml DNase I and further incubation with 0.25% pancreatin. After large tissue debris were removed by filtering through 100 μm nylon mesh, dispersed cells were washed three times with DMEM containing 10% FCS, 20 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin, and cultured at 5 x 10^5 cells/well in 24-well plates for 3 days. Cells were then washed twice with 0.2% BSA-DMEM, pre-incubated in 0.2% BSA-DMEM at 37 °C for 30 min, and incubated with GnRH or Kp-54 in fresh 0.2% BSA-DMEM at 37 °C for 3 h. The FSH and LH levels in the conditioned media were determined using RIA. For testing the effect of E2, the cells were incubated in phenol red free DMEM-10% charcoal/dextran treated FCS with or without 10 nM E2.

***In vitro* Ca^{++} Mobilization Assay**

Agonistic activities of TAK-448 and TAK-683 were evaluated as described previously (Ohtaki *et al.*, 2001; Terao *et al.*, 2004; Asami *et al.*, 2013). Briefly, rat KISS1R-expressing Chinese hamster ovary (CHO) cells were stimulated with various concentrations of the peptides, and intracellular Ca^{++} mobilization was monitored by means of Ca^{++} indicator Fluo-3AM in FLIPR system (Molecular Devices, Sunnyvale, CA). The EC50 values and 95% confidence intervals (CI) (n = 3) were calculated by logistic regression analysis using the SAS system version 8.2 (SAS institute Inc, Cary, NC). In this assay system I also evaluated Kp-10 as internal control; the EC50 and 95% CI were 310 (274–351) pM in a representative experiment.

***In vitro* Receptor Binding Assay**

Receptor binding affinity of TAK-448 and TAK-683 were evaluated by competitive receptor binding

assay using the membrane fraction of rat KISS1R-expressing CHO cells and 125I-labeled Kp-15 (corresponding to N-terminal 15-aa residues of human Kp-54, originally called as metastin (40-54)) (Ohtaki *et al.*, 2001). The IC50 values and 95% CI (n = 6) were calculated by logistic regression analysis using the SAS system. Kp-10 was also evaluated as internal control; the IC50 and 95% CI values were 130 (130–140) pM in a representative experiment.

Seven-Day Repeat Dosing of TAK-448 in Male Rats

Male SD rats (10 weeks of age, body weight range 372-460 g) were randomly assigned and subcutaneously injected once a day with either vehicle (5% glucose solution; Otsuka Pharmaceutical Laboratory, Tokushima, Japan) or TAK-448 (0.008, 0.08, 0.8, or 8 μmol/ml/kg) for consecutive 7 days (n = 4). Blood samples (0.4 ml) were obtained from tail vein at 0h (just before dosing) and 4h (4 h after dosing) on days 1, 4 and 7. Blood samples were immediately mixed with aprotinin solution containing 10% w/v of EDTA-2Na, and centrifuged to obtain plasma samples, and then stored below –30°C (for plasma hormone levels) or –80°C (for plasma drug concentrations) until measurement. On day 8 the prostate, the seminal vesicles, and the testes were removed and weighed.

Seven-Day Repeat Dosing of TAK-683 in Male Rats

Male SD rats (8 weeks of age, body weight range 284-308 g) were randomly assigned and subcutaneously injected once a day with either vehicle (5% glucose solution) or TAK-683 (0.008, 0.08, 0.8, or 8 μmol/ml/kg) for consecutive 7 days (n = 4). The remaining procedure was identical as described above.

Four-Week Continuous Administration of KISS1-305 or TAK-448 in Male Rats

KISS1-305 (0.2-4 nmol/h), TAK-448 (100 pmol/h), leuprolide (0.3-2 nmol/h) were administered to male SD rats (9 weeks of age). Mini-pumps were implanted subcutaneously (s.c.) and replaced weekly as needed. Rats had a bilateral orchiectomy (ORX) performed on the day of dose initiation to compare the effect of ORX to effects produced by either kisspeptin analogues or leuprolide on plasma T and genital organ weights. Blood samples were obtained via tail vein, mixed with Trasylol solution

containing 10% (w/v) of EDTA-2Na, and centrifuged to obtain plasma samples for determination of plasma hormone levels. In studies to assess the impact of leuprolide or KISS1-305 on reproductive organ weights, the weights of the prostate, seminal vesicles, and testes were recorded at the end of the study immediately following animal sacrifice and normalized to body weight (% body weight).

Four-Week Continuous Administration of TAK-448 or TAK-683 in Male Rats

Two separate experiments were conducted. In the first experiment, male SD rats (9 weeks of age, body weight range = 318-368 g) were assigned to vehicle control, bilateral orchiectomy (ORX), TAK-448 or TAK-683 (10, 30, or 100 pmol/h), or leuprolide (30, 100, or 300 pmol/h) based on the body weight (n = 10). In the second experiment, male rats (9 weeks of age, body weight range = 302-350 g) were assigned to vehicle, ORX, or TAK-448 (0.85, 3, or 10 pmol/h) based on the body weight (n = 9-10) to evaluate the effect of TAK-448 at lower doses. In both experiments, an osmotic ALZET minipump containing vehicle (50% DMSO; mixture of 1 volume of DMSO and 1 volume of distilled water) or compounds was implanted subcutaneously, and blood samples were collected before dosing (experimental day 0), 1, 2, 3 days after the initiation of dosing, and every 7 days after day 7. For ORX animals, I performed bilateral orchiectomy under anesthesia by isoflurane after blood collection on day 0. Plasma samples were prepared and stored as described above. On day 28, weights of the prostate, seminal vesicles, and testes were recorded and expressed as relative weight compared to each animal's body weight (percent body weight). In other experiments, I verified the concentrations and the stability of the dosing formulations of TAK-448, TAK-683, or leuprolide by liquid chromatography. Based on the average initial body weight (Tables 3-2, 3-3), these doses can be approximately converted to nmol/kg/day with the conversion factor of 0.07 regardless of the compounds used (eg. 100 pmol/h is approximately equivalent to 7 nmol/kg/day).

Twelve-Week Continuous Administration of TAK-683 in Male Rats

Male rats (9 weeks of age, body weight range 313 - 340 g) were assigned to vehicle, ORX, TAK-683 (30, 100, or 300 pmol/h), or leuprolide (100, 300, or 1000 pmol/h) based on the body weight (n = 4-5). Blood samples were collected before dosing (experimental day 0), 1, 2, 3 days after the initiation of

dosing, and every 7 days after day 7, and plasma samples were prepared and stored as described above. On day 84, weights of the prostate, seminal vesicles, and testes were recorded and expressed as percent body weight. Doses can be converted to "nmol/kg/day" by the conversion factor of 0.07.

Antitumor Study in JDCaP Prostate Tumor Model

The JDCaP tumor xenograft was maintained as described previously (Kimura *et al.*, 2009). A small piece of JDCaP tumor was transplanted under the renal capsule of male F344/N nude rats. Forty-two days following tumor transplantation, animals were stratified according to baseline serum PSA levels. Groups of 7 animals were subsequently treated with TAK-683, leuprolide, ORX, or vehicle only. TAK-683 (1.4 or 7 nmol/kg/day) and leuprolide (1.4 or 7 nmol/kg/day) were administered as continuous s.c. infusions using osmotic ALZET minipumps. Serum PSA level was determined before treatments over time for 240 days after the initiation of treatment by using a commercially available EIA kit (Markit-M PA, Dainippon Sumitomo Pharma, Osaka, Japan), and the lower detection limit was 0.5 ng/ml. Values below the detection limit were expressed as 0.5 ng/ml.

Assessment of LH Release after Chronic Administration of KISS1-305

Male rats were administered either vehicle, KISS1-305 (2 nmol/h), or leuprolide (2 nmol/h) for 6 days, followed by a single s.c. administration of 100 nmol/kg of KISS1-305 or leuprolide. Blood samples were collected through a jugular vein catheter that had been implanted on day 5, and on the next day blood samples were collected before the single s.c. dose, and at 5, 15, 30, 60, and 120 min after dosing to determine plasma LH levels using the commercially available RIA kit.

To explore the responsiveness of GnRH neurons to the GnRH secretagogue N-methyl-D-aspartate (NMDA, Sigma-Aldrich), male rats were treated with either vehicle or KISS1-305 (4 nmol/h) for 6 days as described above, followed by a single s.c. administration of NMDA (30 mg/kg) or vehicle. Blood samples were collected via tail artery before and 15 min after the NMDA dose to determine plasma LH levels. This blood sampling point (15 min) was chosen based on results from a pilot experiment. Briefly, to examine the time course of LH elevation after single s.c. administration of NMDA in male rats, NMDA (30 mg/kg) or saline (vehicle) was administered s.c. in

male Crl:CD(SD) rats (9 weeks of age, n = 3). Blood samples were collected before and 15, 30, and 60 minutes after the NMDA dosing to determine plasma LH levels by RIA.

Brain Fixation and Immunohistochemistry (IHC)

The brain fixation and immunohistochemical analysis of GnRH and c-Fos were performed as described previously. Briefly, following euthanasia, the brain was fixed by transcardial perfusion with Mildform 10N (Wako Pure Chemical Industries, Osaka, Japan). The brain was dissected out, and coronal sections (30-50 μm) were cut on a cryostat and mounted onto glass slides. Sections were incubated overnight with a polyclonal goat antibody raised against c-Fos (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a polyclonal rabbit antiserum raised against rat GnRH (1:250, Sanbio, Uden, The Netherlands), and then with Cy5-conjugated donkey anti-goat IgG and fluorescein-conjugated donkey anti-rabbit IgG (1:50, Jackson Immuno Research Laboratories, West Grove, PA, USA) for 3 h at room temperature. Cellular nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The sections were examined with the aid of a TCS SP2 confocal microscope (Leica, Rockleigh, NJ, USA). The number of GnRH-immunoreactive (ir) neurons in which the nucleus was stained with DAPI was counted. The hypothalamus was divided into four regions, the OVLT-aPOA, the POA, the SON-PVN, and the mediobasal hypothalamus (MBH) (positioned at 0–150, 150–900, 900–1600, and 1,600–3,000 μm caudal from the OVLT, respectively). These subdivisions include the organum vasculosum of the lamina terminalis (OVLT) and the anterior preoptic area (aPOA), the preoptic area (POA), the supraoptic nucleus (SON) and the paraventricular nucleus (PVN), and the caudal part of the forebrain, respectively.

Effect of NMDA on c-Fos-ir Expression

Male Crl:CD(SD) rats received either vehicle or KISS1-305 for 6 days as described in Methods, followed by a single s.c. administration of NMDA (30 mg/kg) or vehicle. Ninety minutes after the NMDA dose, the brain was fixed as described in Methods for immunohistochemical c-Fos evaluation. Frozen serial coronal cryomicrotome sections (50 μm) of the brain were made to detect c-Fos-ir. Using a free floating technique, sections were washed with PBS and immersed sequentially in the following

solutions: 1) 1% sodium metaperiodate in PBS for 10 min, 2); 10% normal donkey serum (Jackson Immuno Reserch, #017-000-121) and 0.3% Triton X-100 in PBS for 1 h; 3) goat anti-c-Fos polyclonal antibody (1:100; Santa Cruz Biotechnology, #SC52G) overnight; 4) biotinylated anti-Goat IgG (H+L) (1:300; Vector Laboratories [Burlingame, CA], #BA-5000) for 90 min; 5) avidin-biotinylated HRP complex (ABC Elite; Vector Laboratories, #PK-6100) for 30 min; and 6) ImmPACT DAB solution (Vector Laboratories, #SK-4105) for 3-4min. The sections were mounted, dehydrated and examined by light microscopy.

Gene mRNA and Protein Expression Analysis after Chronic Administration of KISS1-305

The hypothalamus was dissected from the brain of euthanized animals that had received chronic KISS1-305 for 3 weeks, as described previously by Kumano *et al.* (Kumano *et al.*, 2003). For protein analysis, the hypothalamic samples were weighed immediately after tissue collection, followed by boiling for 5 min in 1 mL of DW, and then chilled on ice until sample homogenization. After homogenization in DW, 1/16 volume of glacial acetic acid (Wako Pure Chemical Industries) was added to the homogenate, which was gently rotated at 4°C for 1hr, followed by centrifugation for 5 min at 15,000 rpm (MX305, Tomy, Tokyo, Japan). The supernatant from the hypothalamic homogenate was then applied to a SepPak C18 column (Waters, Milford, MA), washed once with 1M acetic acid in DW, and eluted with 60% CH_3CN containing 0.1% trifluoroacetic acid in DW. The elution was then lyophilized and stored at -30°C until GnRH determination. Hypothalamic GnRH contents were determined by RIA (as described below), normalized to the tissue weight, and expressed as relative values (arbitrary units) versus GnRH detected in vehicle control treated animals. For mRNA analysis, hypothalamic total RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, CA), followed by first strand cDNA synthesis using SuperScript III reverse transcriptase and oligo$(dT)_{20}$ primers (Invitrogen). Expression of rat mRNA for *Kiss1*, *Kiss1r*, *Gnrh* and *β*-actin were determined by quantitative polymerase chain reaction (qPCR, 7900HT Fast Real-Time PCR System, Applied Biosystems, Foster, CA). Gene-specific probes and primers used for *Kiss1*, *Kiss1r*, and β-actin have been described previously (Kinoshita *et al.*, 2005); the following primers and probe were used for *Gnrh*; forward: 5'-GCAGAACCCCAGAACTTCGA-3', reverse: 5'-TGCCCAGCTTCCTCTTCAAT-3', and probe:

5’-(FAM)-TCTGCGAGGAGCTCTGGAACGTCTG-(TAMRA)-3’. mRNA expression levels were normalized to those of *β-actin*, and then expressed as relative values (arbitrary units) versus vehicle control-treated animals.

Gene mRNA and Protein Expression Analysis after ORX or Chronic Administration of TAK-448 or lLuprolide

After 1 (n = 5) or 4 weeks (n = 10) post-ORX or treatment with TAK-448 (100 pmol/h), or leuprolide (300 pmol/h), the brain, pituitary, and blood samples were collected from euthanized animals to determine hypothalamic GnRH peptide contents by RIA, hypothalamic *Gnrh* and pituitary *Fshb* and *Lhb* mRNA expressions by qPCR, pituitary FSH and LH contents, and plasma FSH, LH, and T levels by RIA. For protein analysis, weights of the hypothalamus and pituitary glands were recorded immediately after tissue collection and the hypothalamic GnRH contents were determined by RIA as described above. The pituitary was homogenized in 0.3 mL of phosphate buffered saline containing 1× complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), followed by centrifugation for 5 min at 15,000 rpm. The supernatants were stored at -80°C until determination of FSH and LH contents by RIA (see below). The GnRH, FSH, and LH contents were expressed as relative values (%) versus vehicle control. For qPCR analysis, total RNA from the hypothalamus or pituitary was purified as described above, and mRNA expressions were determined by qPCR using TaqMan® Gene Expression Assays (Applied Biosystems), for *β-actin* (*Actb*: Rn00667869_m1), for *Gnrh* (*Gnrh1*: Rn00562754_m1), for *Fshb* (Rn01484594_m1), and for *Lhb* (Rn00563443_g1). Expression levels of mRNA were analyzed by the ΔCt method using *β-actin* as the internal control, and expressed as relative expression levels (arbitrary units) versus vehicle controls.

Radioimmunoassay (RIA)

Plasma levels of FSH and LH were determined using rat FSH (rFSH-RP-2 for reference peptide) and LH (rLH-RP-3 for reference peptide) RIA kits, which were used according to the instruction from NIDDK. The detection limits for FSH and LH were 0.8 and 0.08 ng/mL, respectively, in 100μL of reaction volume. Intra-assay (n = 9) and inter-assay (n = 3) coefficients were 3.3% and 0.63% at 21

ng/mL for FSH and 4.2% and 1.4% at 5.7 ng/mL for LH, respectively. In some experiments, I also used commercially available rat FSH and LH RIA (radioimmunoassay) kits, respectively (Amersham Biosciences, NJ, USA). The detection limits of these RIA kits were 1.6 ng/ml (FSH) and 0.8 ng/ml (LH), and the values under the detection limits were determined as 0 ng/ml. My pilot experiment on the cross-reactivity of these commercially available RIA kits to 1 IU/ml of eCG revealed that it was equivalent to 5.4 ng/ml of rat LH, while it was undetectable using rat FSH RIA kit. Plasma T, E2 and progesterone (P4) were determined using a DPC total testosterone kit, DPC estradiol kit and a DPC progesterone kit, respectively (Diagnostic Products, CA, USA). The detection limits of these RIA kits were 0.04 ng/mL (T), 8 pg/ml (E2) and 0.02 ng/ml (P4). GnRH levels were determined using a commercially available RIA kit with a detection limit of 0.01 ng/mL. Values less than the lower limit of detection were set equal to the detection limit. All procedures were performed according to the manufacturer's instructions.

Determination of Plasma Drug Concentrations

Plasma TAK-448 and TAK-683 concentrations were determined either by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or EIA (Yoshida *et al.*, 2012). For LC-MS/MS, the plasma samples were deproteinized by adding methanol, formic acid and acetone, followed by centrifugation to obtain supernatant. The supernatant was then evaporated to dryness under a stream of nitrogen at 50°C, and the residue was reconstituted in ultra pure water containing 1/500 volume of formic acid and centrifuged to obtain supernatant. The supernatant was applied to a solid phase cartridge (OASIS HLB, Waters, Milford, MA) and then eluted by methanol, followed by evaporation under a stream of nitrogen at 50°C. The residue was reconstituted in ultra pure water containing 0.2% formic acid/methanol (4:1, v/v), then filtered (0.22 μm; Ultra-free MC, Millipore, Billerica, MA), and finally injected into the LC-MS/MS system. Plasma leuprolide concentrations were determined by RIA as described previously (Yamazaki & Okada, 1980). The detection limit for each assay was 50 pg/ml for TAK-683 by LC-MS/MS, 5 pg/ml for TAK-683 by EIA, 3 pg/ml for TAK-448 by EIA, and 50 pg/ml for leuprolide by RIA.

Statistics

Statistical significance was analyzed using the SAS system version 8.2 (SAS institute Inc, Cary, NC). Differences were considered to be significant when *P* values were ≤0.05 (two-tailed test) or ≤0.025 (one-tailed test). The homogeneity of variance for multiple groups was analyzed by the F test (two groups) or Bartlett's test (multiple groups). If the results were not significant, the data were analyzed by parametric statistics. If the results were significant, the data were analyzed by nonparametric statistics. Specific statistical method used for each data will be described in each Figure or Table legends.

Chapter 1: Peripheral Administration of Kisspeptin Induces Marked Gonadotropin Release and Ovulation in the Rat

Results

In this Chapter, I will describe the functional analysis of kisspeptin in the rat by focusing on the effect of subcutaneous administration of Kp-54 or a kisspeptin agonist analogue (KISS1-305) on reproductive functions.

Ovulation-inducing Activity in Prepubertal Intact Female Rats

Kp-54 administration to the eCG-primed rats promoted the ovulation of a large population of oocytes, comparable to that attained by hCG administration (Table 2). On day 25, untreated rats showed low E2 and low P4 levels (8.9 pg/ml and 1.8 ng/ml on average, respectively) whereas the eCG-treated rats showed increased E2 and low P4 levels (164.4 pg/ml and 2.2 ng/ml on average, respectively). The increase in E2 levels is a sign of the maturation of follicles. On day 26 (24 h after the injection of Kp-54 or hCG), the E2 levels in the Kp-54- and hCG-treated rats returned to similarly lower levels, while those in saline-treated rats remained high (Table 2). In contrast, the P4 levels in the Kp-54- and hCG-treated rats were elevated, but there was some difference in the increase of P4 levels between these two groups (Table 2).

Table 2 Number of ovulated oocytes, and plasma E2 and P4 levels.

Treatment	Oocytes (number/rat)	E2 (pg/ml)	P4 (ng/ml)
Saline	1.8 ± 1.1^{a}	87.0 ± 17.6^{a}	2.5 ± 0.8^{a}
Metastin	27.8 ± 3.2^{b}	32.4 ± 8.2^{b}	6.8 ± 0.8^{b}
hCG	28.2 ± 1.6^{b}	18.3 ± 5.3^{b}	13.3 ± 1.1^{c}

eCG-primed 25-day-old female Wistar rats were treated with Saline, kisspeptin, or hCG, and sacrificed on 26 days. Data shown are mean ± SEM. Significant differences are observed between the different letters ($P < 0.01$, Tukey's test, n = 5).

I also tested another kisspeptin agonist, KISS1-305, and demonstrated that the pituitary is indispensable for ovulation-inducing activity by kisspeptin agonist (Data not shown).

Gonadotropin-releasing Activity in eCG-primed Immature Female Rats

I next investigated the effect of Kp-54 on plasma gonadotropin levels in eCG-primed 25-day-old rats having maturated follicles. Though basal FSH levels (saline-treated group) in the eCG-primed rats showed slight temporal changes (statistically significant), Kp-54 administration significantly elevated plasma FSH and LH levels compared to each zero-time level and also compared to saline-treated group at each time point (Fig. 2). The result affirms that the follicles in Kp-54-treated rats received surge-like LH stimulation; the preovulatory LH surge is known to decrease the levels of E2 and to enhance the synthesis of P4 in follicles (Fortune & Hansel, 1985). The higher P4 levels in hCG-treated rats may be due to the excessive stimulation of luteal cells because of its higher stability compared to LH (European Recombinant LH Study Group, 2001)

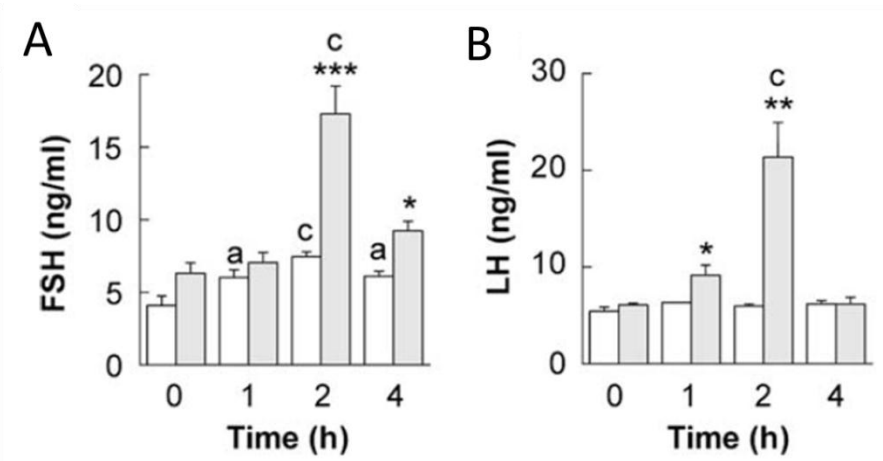

Fig. 2 Kp-54 administration elevates plasma gonadotropin levels in eCG-primed prepubertal female rats.

Plasma FSH (A) and LH (B) levels in eCG-treated 25-day-old rats after saline (open) or Kp-54 (shaded) administration (n = 5). Data shown are mean + SEM. The letters a, b, and c indicate $P <$ 0.05, 0.01, and 0.001 versus time 0 control of each group by Dunnett's test. The symbols *, **, and *** indicate $P < 0.05$, 0.01, and 0.001 versus saline-treated control at each time by Student's *t*-test.

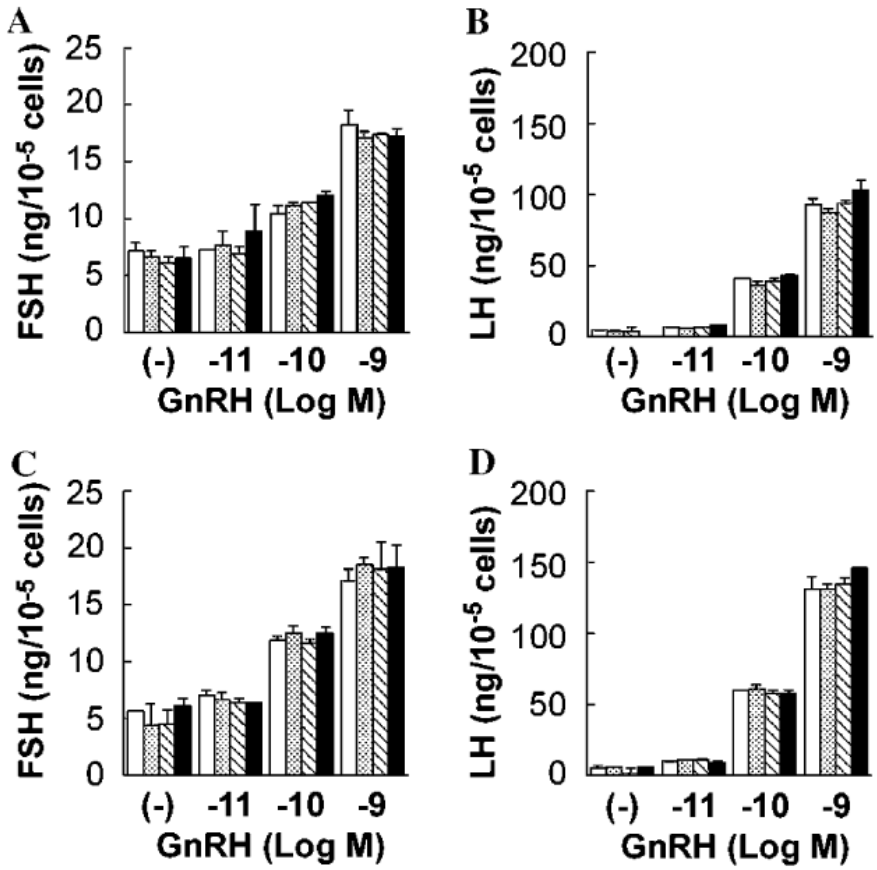

Fig. 3 The effect of GnRH or Kp-54 on gonadotropin release from female rat anterior pituitary cells *in vitro*.

FSH (A,C) and LH (B,D) levels in the conditioned medium of female pituitary cells cultured in the presence (A,B) or absence (C,D) of 10nM E2 in phenol red-free DMEM are shown versus GnRH concentrations. Open bars, 0M; dotted bars, 10^{-10} M; hatched bars, 10^{-8} M; and closed bars, 10^{-6} M of Kp-54. Data shown are mean + SEM.

Gonadotropin-releasing Assay in Primary Culture of Rat Anterior Pituitary Cells

Kp-54 did not induce gonadotropin release in any dose, while GnRH successfully induced LH release in a dose-dependent manner from the primary cultured male or female pituitary cells (Data not shown). Because it has been shown that phenol red had estrogenic effect on the rat pituitary cells (Dumesic *et al.*, 1989), I next cultured female rat anterior pituitary cells in the presence or absence of 10 nM E2 in phenol red-free medium, and the cells were incubated with GnRH, Kp-54, or both (Fig. 3). Kp-54 did not induce gonadotropin release nor affect the GnRH-induced gonadotropin release.

Gonadotopin-releasing Activity in Prepubertal Female Rats and Adult Male Rats

Subcutaneous administration of 6.7 nmol/body (ca. 100 nmol/kg) of Kp-54 markedly elevated plasma FSH and LH levels compared to each zero-time level and also compared to saline-treated group at

each time point in 25-day-old female rats without eCG treatment (Fig. 4). The maximal increase was found 2 h after administration. Saline-treatment did not induce any significant changes in these hormone levels.

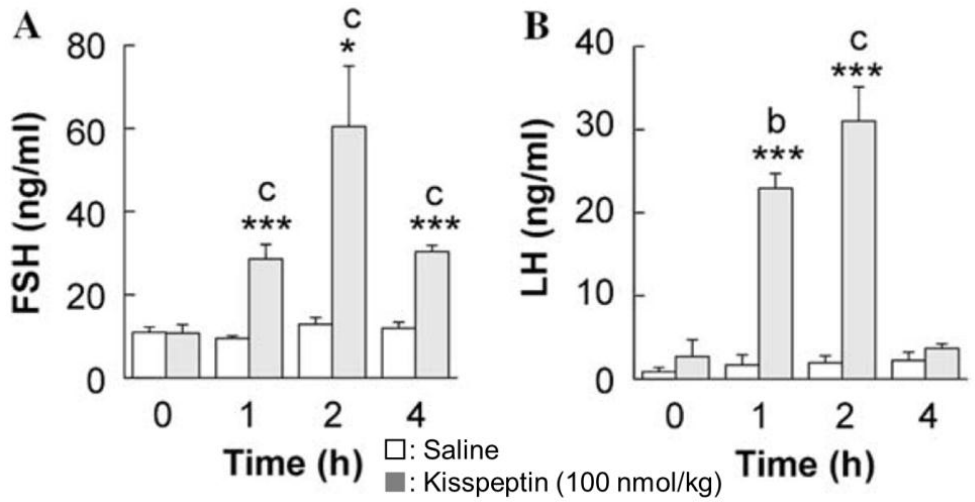

Fig. 4 Kp-54 administration elevates plasma gonadotropin levels in prepubertal female rats without eCG treatment.

Plasma FSH (A) and LH (B) levels in 25-day-old rats after saline (open) or Kp-54 (shaded) administration (n = 5). Data shown are mean + SEM. The letters a, b, and c indicate $P < 0.05$, 0.01, and 0.001 versus time 0 control of each group by Dunnett's test. The symbols *, **, and *** indicate $P < 0.05$, 0.01, and 0.001 *vs.* saline-treated control at each time by Student's *t*-test.

Next I investigated the gonadotropin releasing effect in adult male rats. Subcutaneous administration of Kp-54 to adult male Wistar rats markedly elevated the plasma levels of FSH and LH, with the maximum increase occurring 2 h after administration (Fig. 5). The pre-treatment with Cetrorelix, a GnRH antagonist, suppressed both FSH and LH elevations in response to Kp-54 (Fig. 5).

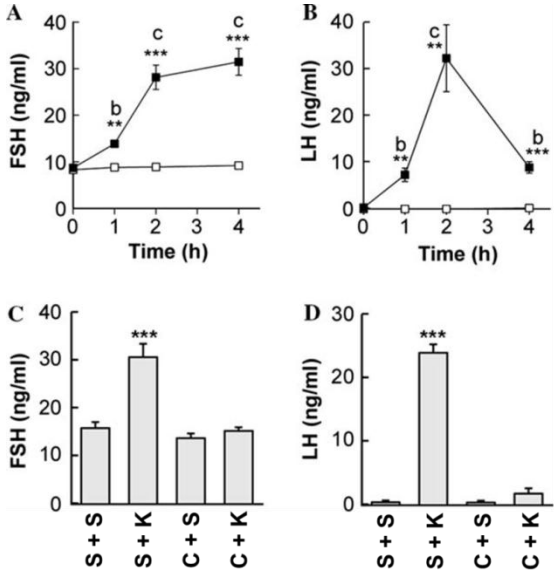

Fig. 5 The effect of kisspeptin on gonadotropin release in adult male Wistar rats
Data shown are mean + SEM. Plasma FSH (A) and LH (B) levels in 10-week-old rats after treatment with kisspeptin (closed square) or saline (open square) (n = 5). The letters b and c indicate < 0.01 and 0.001 versus time 0 control of each group by Dunnett's test, respectively. The symbols ** and *** indicate $P < 0.01$ and 0.001 versus saline-treated control at each time by Student's *t* test, respectively. Plasma FSH (C) and LH (D) levels in 10-week-old rats 90 min after treatment with either Kp-54 (+K) or saline (+S), following pretreatment with Cetrorelix (C+) or saline (S+) (n = 5). ***P < 0:001 versus the other three groups by Tukey's test.

Induction of c-Fos Immunoreactivity in GnRH Neurons

Kp-54-treated rats (n = 2) exhibited a large population of c-Fos and GnRH-double-immunoreactive (ir) neurons in the hypothalamus, whereas saline-treated rats (n = 2) exhibited no c-Fos-ir GnRH neurons (Fig. 6). The proportion of c-Fos-ir GnRH neurons in the total population of GnRH neurons was highest in the OVLT-aPOA, moderate in the POA and in the MBH, and low in the SON-PVN (Table 3). These results suggest that GnRH neurons in the hypothalamus are activated after Kp-54 s.c. administration.

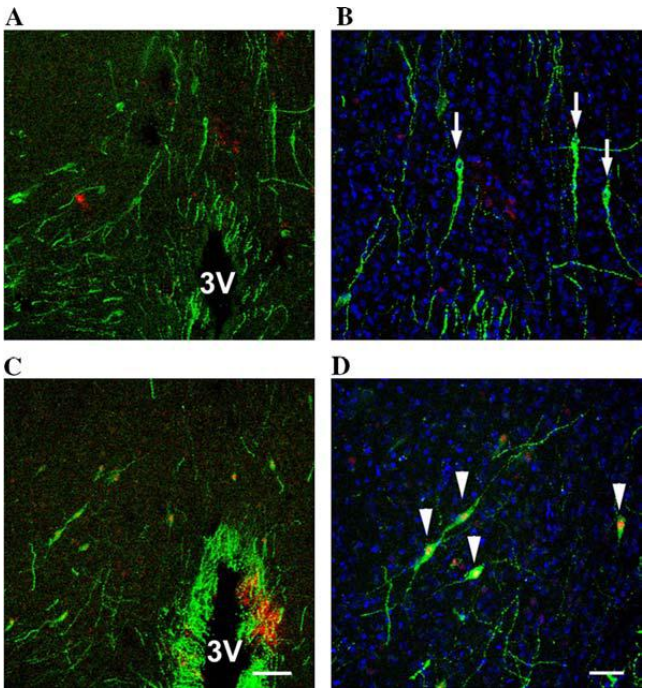

Fig. 6 Double-labeling immunohistochemistry.
c-Fos (red) and GnRH (green) immunoreactivity in the aPOA at the level of the OVLT in saline-treated (A,B) or kisspeptin-treated (C,D) male Wistar rats. Blue signals indicate DAPI-stained nuclei. (B,D) Higher-magnification images of the fields shown in (A,C), respectively. Arrows and arrowheads indicate c-Fos-negative and positive GnRH neuronal bodies, respectively. 3V, the third ventricle. Bars; 100 μm in (A,C), and 40μm in (B,D).

Table 3 The proportion of c-Fos-immunoreactive GnRH neurons in kisspeptin-treated adult male rats.

Subdivision	c-Fos-ir GnRH			GnRH			c-Fos-ir/GnRH		
	#1	#2	Sum	#1	#2	Sum	#1 (%)	#2 (%)	Sum (%)
OVLT-aPOA	30	31	62	37	32	69	81.1	96.9	88.4
POA	35	18	53	69	35	104	50.7	51.4	51.0
SON-PVN	1	5	6	16	16	32	6.3	31.3	18.8
BMH	10	7	17	18	9	27	55.6	77.8	63.0
Total	76	61	137	140	92	232	54.3	66.3	59.1

Values are the total from two rats. The definitions of subdivisions are described in material and method.

Chapter 2: Pharmacologic Profiles of Investigational Kisspeptin/Metastin Analogues, TAK-448 and TAK-683, in Adult Male Rats in Comparison to the GnRH Analogue Leuprolide

Results

In this Chapter, I will describe the pharmacological profiles of kisspeptin agonist analogues especially focusing on potential application of kisspeptin agonist analogues for hormone-related disease treatment.

In vitro Properties of TAK-448 and TAK-683

The *in vitro* properties of KISS1-305 has been previously published (Asami *et al.*, 2013). *In vitro*, TAK-448 and TAK-683 were both found to be potent and full KISS1R agonists; IC50 values (95% CI) from receptor binding assays were 460 (420 – 500) pM for TAK-448 and 170 (150 – 180) pM for TAK-683 (n = 6), and EC50 values (95% CI) from Ca^{++} mobilization assays were 632 (558 – 716) pM for TAK-448 and 180 (159 – 203) pM for TAK-683 (n = 3). TAK-683 showed approximately three times more potent receptor binding and agonistic activity compared to TAK-448.

Four-Week Continuous Administration of KISS1-305 in Male Rats

I studied the dose dependency and time course of T lowering produced by KISS1-305. Chronic administration of KISS1-305 (0.2, 0.5, 1 nmol/h) transiently elevated plasma T levels (Fig. 7A). KISS1-305 (1 nmol/h) reduced plasma T levels to the lower limit of detection (0.04 ng/mL) at day 3 and day 28 (Fig. 7B), but the efficacy was not sustained. I also studied the effect of KISS-305 at higher doses (1, 2, or 4 nmol/h) in comparison to leuprolide. Consistent with the above findings, chronic administration of KISS1-305 (1, 2, or 4 nmol/h) resulted in a temporal rise in plasma T levels and reduction in T levels to the lower limit of detection by day 3 at every dose evaluated (Fig. 7C). T levels at day 28 were also undetectable and significantly lower than vehicle controls (Fig. 7D). In groups receiving 2 and 4 nmol/h, plasma T levels were depleted for the entire 4-week administration period (Fig. 7C), suggesting that 2 nmol/h of KISS1-305 could be considered the minimum effective dose. In contrast, while chronic leuprolide administration (1 nmol/h) did result in T flare of approximately the same magnitude as that observed following KISS1-305 dosing, chronic leuprolide dosing did not result in maximal T lowering until 2 weeks following the initiation of chronic dosing (Fig. 7A). Dose

dependency of leuprolide was also examined. The results revealed that the T-lowering effect of leuprolide appeared to plateau at a chronic dose ≥0.3 nmol/h (data not shown). These results suggest that T lowering with KISS1-305 is more rapid and of greater magnitude than that produced by leuprolide. Prostate, seminal vesicles, or testes tissue weights (a direct reflection of circulating T or gonadotropins) on day 28 were significantly reduced both in KISS1-305- and leuprolide-treated animals but the administration of KISS1-305 resulted in a greater reduction in genital organ weight than leuprolide under these conditions (Fig. 7E).

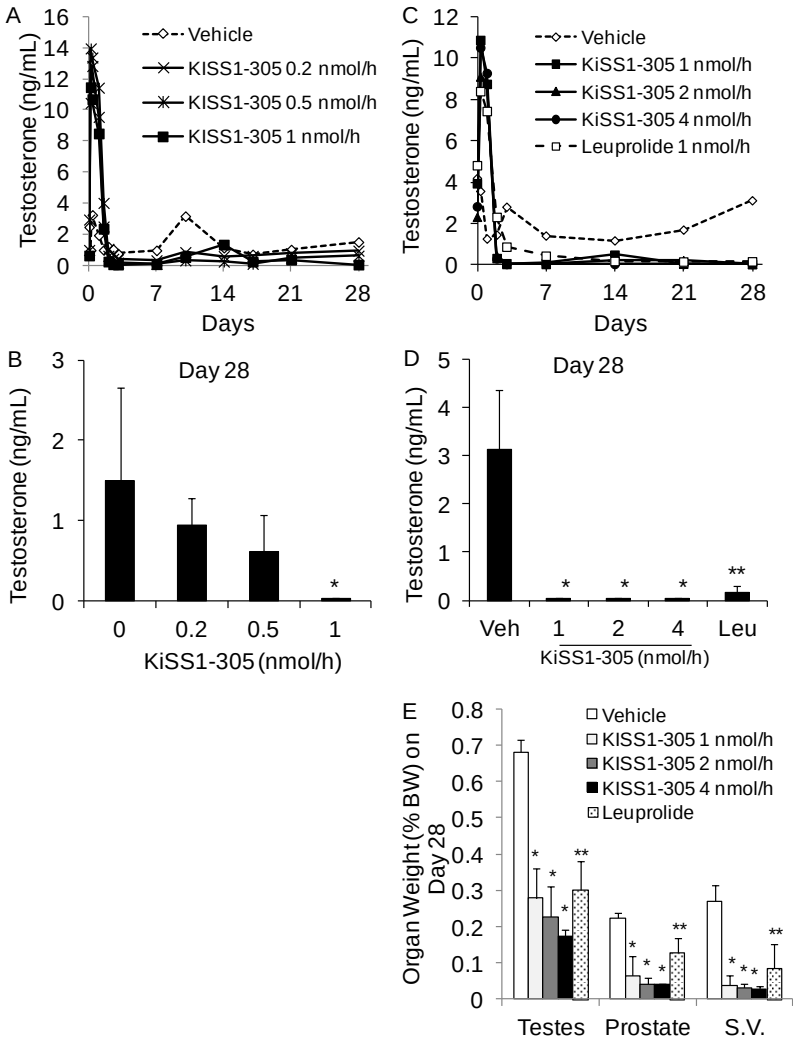

Fig. 7 Effect of chronic s.c. administration of KISS1-305 or leuprolide in male rats
(A) KISS1-305 was chronically administered to male rats, and mean plasma T levels at day 0 (pre), 4, 8, 24, 36, 48, 60, 72 h, and 7, 10, 14, 17, 21, and 28 days after treatment are shown. (B) Plasma T levels on day 28. (C) KISS1-305 or leuprolide were chronically administered to male rats, and mean plasma T levels at day 0 (pre), 8 h, and 1, 2, 3, 7, 14, 21, and 28 days after treatment are shown. (D) Plasma T levels on day 28. (E) Relative weights of the testes, prostate, and seminal vesicles (S.V.) on day 28. Data shown in B, D, and E are mean + SD (n = 5). * and ** indicate $P < 0.025$ and < 0.01 versus vehicle by one-tailed Shirley-Williams' test and Aspin-Welch *t*-test, respectively.

Seven-Day, Once-Daily Repeat Dosing of TAK-448 or TAK-683.

The first dosing of TAK-448 induced substantial elevation in plasma LH and testosterone levels at 4h (Fig. 8A-B). The baseline plasma LH and testosterone levels either or both on days 4 and 7 (i.e. at 0h on each experimental day) were decreased below the vehicle control levels; especially the latter was as low as below the detection limit (0.04 ng/ml). Responses to a new TAK-448 daily injection were not apparent on days 4 and 7 in terms of LH release. Testosterone levels were slightly increased in response to a new daily injection on days 4 and 7 especially at doses of 0.08 μmol/kg and less; while the testosterone levels after a new daily injection were still significantly lower compared to the vehicle control at doses of 8 μmol/kg (Day 4) and ≥0.08 μmol/kg (Day 7). The weights of the prostate, the seminal vesicles, and the testes on day 8 were significantly reduced compared to vehicle-treated group (Fig. 8C). Similar results were obtained with TAK-683 (Fig. 9), and the testosterone levels on day 7 at 4 h after a new TAK-683 administration were significantly lower than vehicle control at doses of 0.8 μmol/kg and above (Fig. 9B). It appears that TAK-448 produced more profound suppression of the baseline LH and testosterone levels observed at 0h on days 4 and 7 and the weights of testis, the differences between TAK-448 and TAK-683 were not apparent in terms of the weights of prostate and seminal vesicles (Fig. 9C).

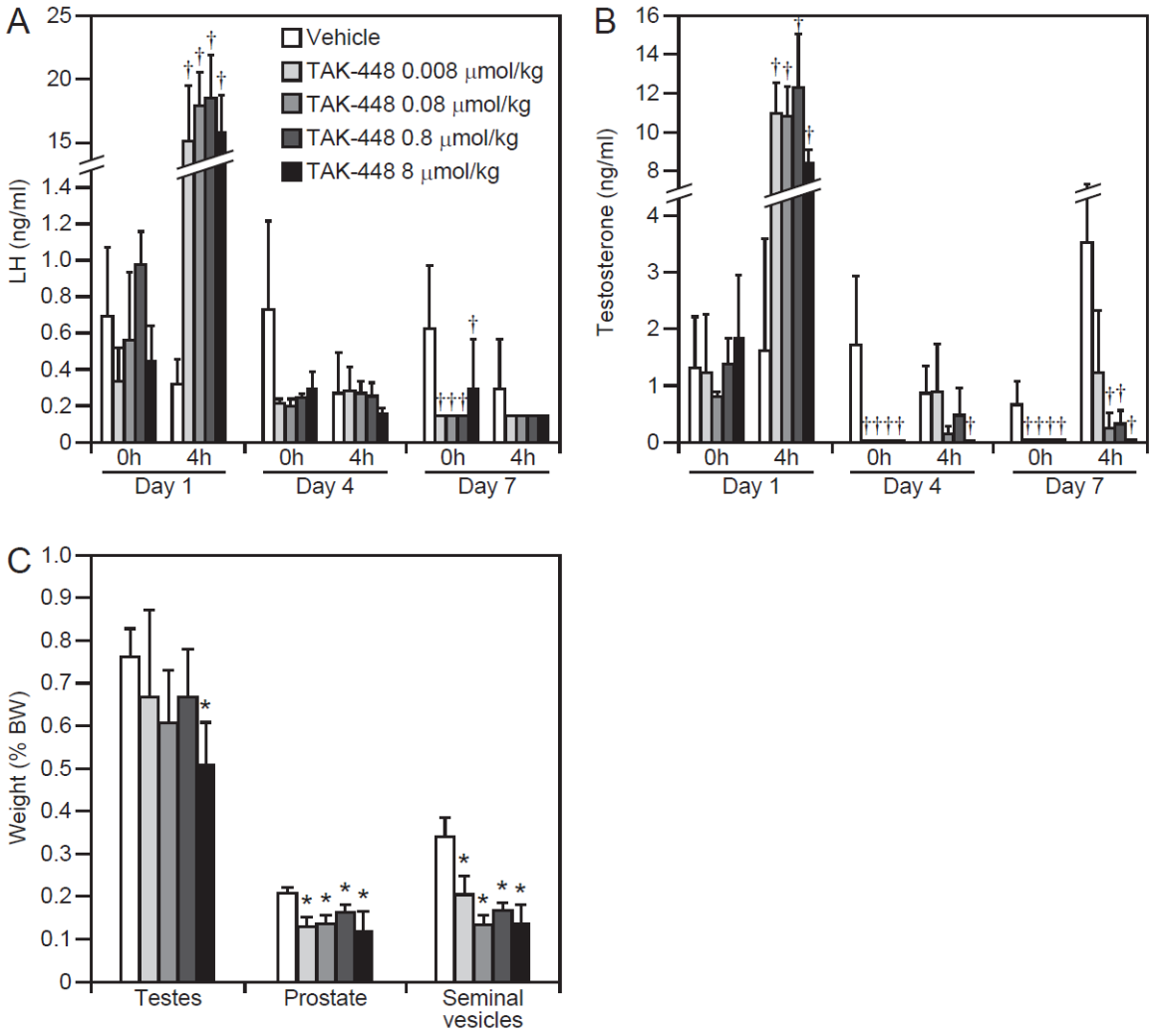

Fig. 8 Effect of repetitive administration of TAK-448 in male rats.
Plasma LH (A) and testosterone (B) levels at 0 and 4 h on experimental days 1, 4, and 7, showing suppressions of LH and testosterone after repeated dosing. These hormone profiles were first analyzed by repeated measures 2-way analysis of variance (ANOVA), and the main effect of dose (LH), main effect of time (LH and testosterone) and interaction effect (LH and testosterone) were considered to be significant ($P < 0.05$). The data were then analyzed by two-tailed Willams' or Shirley-Williams test, and † indicates $P < 0.05$ versus vehicle control. (C) The weights of the prostate, the seminal vesicles and the testes on day 8 were reduced in a dose-dependent manner. Data shown are mean + S.D. (n = 4). * indicates $P < 0.025$ (one-tailed Williams' or Shirley-Williams test) versus vehicle control.

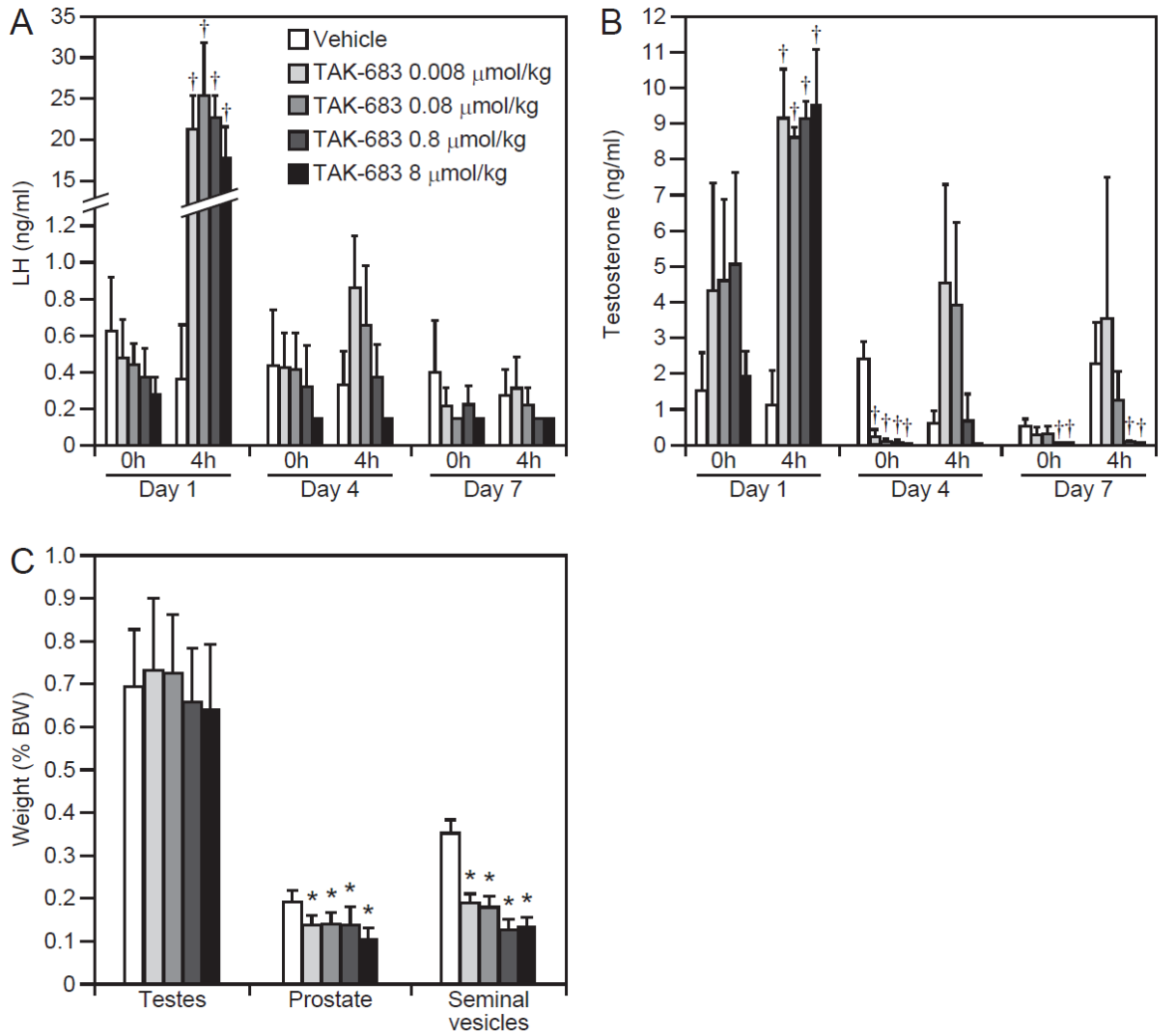

Fig. 9 Effect of repetitive administration of TAK-683 in male rats.
Plasma LH (A) and testosterone (B) levels at 0 and 4 h on experimental days 1, 4, and 7, showing suppressions of LH and testosterone after repeated dosing. These hormone profiles were first analyzed by repeated measures 2-way analysis of variance (ANOVA), and the main effect of dose, main effect of time and interaction effect were considered to be significant for both hormone profiles ($P < 0.05$). The data were then analyzed by two-tailed Willams' or Shirley-Williams test, and † indicates $P < 0.05$ versus vehicle control. (C) The weights of the prostate, the seminal vesicles and the testes on day 8 were reduced in a dose-dependent manner. Data shown are mean + S.D. (n = 4). * indicates $P < 0.025$ (one-tailed Williams' or Shirley-Williams test) versus vehicle control.

Four-Week and 12-Week Continuous Administration of Kisspeptin Analogues.

I then evaluated the effect of continuous s.c. administration of TAK-448 (0.7, 2.1, or 7 nmol/kg/day; doses are described in the converted doses as mentioned in the Materials and Methods), TAK-683 (0.7, 2.1, or 7 nmol/kg/day), or leuprolide (2.1, 7, or 21 nmol/kg/day) in male rats, and the effects were compared to ORX. Plasma concentrations of all three peptides were dose-dependent, confirming appropriate and stable drug release during this study period (Table 4). Mean plasma concentrations during this 4-week dosing periods showed that at the same dose TAK-448 produced about 50% higher plasma drug concentrations than TAK-683, suggesting that TAK-448 had better pharmacokinetic profiles in this subcutaneous infusion setting (Table 4).

Table 4 Plasma drug concentrations in subcutaneous infusion studies

Mean ± SD (pg/ml) (n = 10)

Dose (nmol/kg/day)	TAK-448	TAK-683	Leuprolide
0.06[a]	3.6 ± 2.9	-	-
0.21[a]	16.1 ± 5.8	-	-
0.7[b]	36.7 ± 12.9	21.1 ± 6.0	-
2.1[b]	101.2 ± 36.3	65.8 ± 18.8	145.0 ± 33.5
7[b]	359.6 ± 87.7	212.1 ± 64.0	443.0 ± 117.9
21[b]	-	-	1192.8 ± 279.9

a: PK values from the infusion study shown in Fig. 11
b: PK values from the infusion study shown in Fig. 10

Both TAK-448 and TAK-683 administration induced an increase in plasma testosterone levels only on day 1, followed by abrupt reduction of plasma testosterone levels by day 3. In this study, continuous administration of 0.7 nmol/kg/day of TAK-448 or 2.1 nmol/kg/day of TAK-683 was sufficient to maintain complete suppression of testosterone (below the detection limit; 0.04 ng/ml) for 4 weeks (Fig. 10A-B). I defined the castrate level as “mean + 2 S.D. of the ORX group”, and found that TAK-448 (≥ 0.7 nmol/kg/day) and TAK-683 (≥ 2.1 nmol/kg/day) reduced the weights of the

prostate or seminal vesicles to, or close to, the castrate level (Fig. 10C-E). In contrast, while continuous leuprolide administration resulted in testosterone flare of approximately the same magnitude as that observed following administration of TAK-448 or TAK-683, continuous leuprolide dosing took 2 weeks to induce maximal plasma testosterone reduction (Fig. 10A-B). Though leuprolide treatment significantly reduced the weights of the prostate, seminal vesicles, and testes, both TAK-448 and TAK-683 showed more pronounced reduction in weights of these organs (Fig. 10C-E).

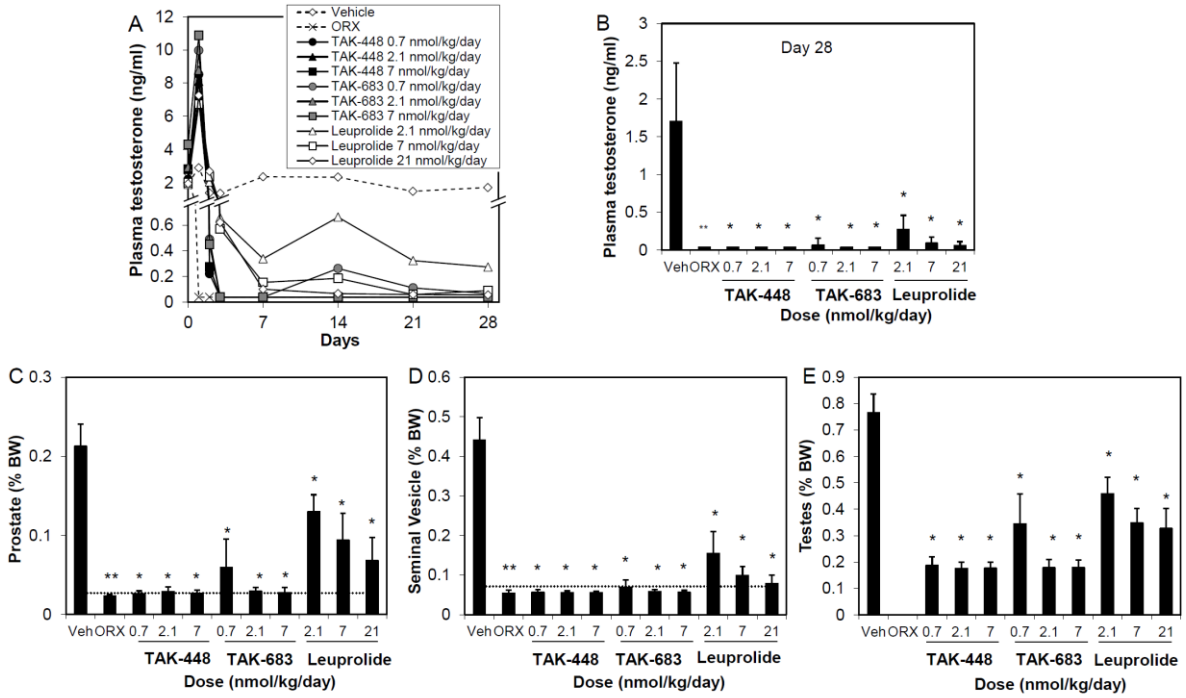

Fig. 10 Effect of continuous administration of TAK-448, TAK-683, and leuprolide in male rats. (A) Mean plasma testosterone levels at indicated time points. Plasma testosterone levels (B), relative weights of the prostate (C), the seminal vesicles (D), and the testes (E) on day 28. Data shown are mean + S.D. unless otherwise stated. The lower limit of quantification of testosterone was 0.04 ng/ml, and values below the limit were expressed as 0.04 ng/ml. Dashed lines in figures (C) and (D) indicate castrate levels (mean + 2 S.D. of ORX). As stated in the section 3.2, the doses can be converted to μg/kg/day with the conversion factor of 0.09. * indicates $P < 0.025$ (one-tailed Williams' test; ORX) or $P < 0.05$ (Wilcoxon rank sum test; TAK-448, TAK-683, leuprolide) versus vehicle control.

At the end of the study little difference in body weights were observed between treatments (Table 5).

Table 5 Body weights in the experiment shown in Fig. 10

Mean ± SD

Group	Day 0	Day 28
Vehicle	346 ± 11	459 ± 17
ORX	346 ± 7	445 ± 11
TAK-448 0.7 nmol/kg/day	345 ± 14	454 ± 30
TAK-448 2.1 nmol/kg/day	346 ± 13	443 ± 29
TAK-448 7 nmol/kg/day	345 ± 10	453 ± 18
TAK-683 0.7 nmol/kg/day	347 ± 8	458 ± 17
TAK-683 2.1 nmol/kg/day	346 ± 12	456 ± 25
TAK-683 7 nmol/kg/day	342 ± 10	447 ± 24
Leuprolide 2.1 nmol/kg/day	346 ± 11	448 ± 20
Leuprolide 7 nmol/kg/day	345 ± 9	451 ± 21
Leuprolide 21 nmol/kg/day	346 ± 12	448 ± 23

Since this first study could not clarify dose-dependent efficacy of TAK-448, I conducted another experiment with lower dose of TAK-448 (0.06, 0.21, and 0.7 nmol/kg/day) to estimate the minimum effective dose. Stability studies revealed that in the stock solution used for the lowest dose, the actual concentration of TAK-448 was 85% of the nominal concentration, probably due to absorption to the container. Therefore, the lowest administered dose of TAK-448 was calculated to be 0.06 nmol/kg/day rather than the nominal value of 0.07 nmol/kg/day. The concentrations of TAK-448 in the stock solutions used for the 0.21, and 0.7 nmol/kg/day doses were found to be accurate and stable. The results demonstrated that 0.7 nmol/kg/day of TAK-448 was sufficient to completely suppress plasma testosterone levels below the detection limit for 4 weeks with well-maintained plasma drug concentrations (Table 6 and Fig. 11A-B). All three dose levels reduced the weights of the prostate, seminal vesicles, and testes; at 0.7 nmol/kg/day the weights of the prostate and seminal vesicles were reduced to the castrate level (Fig. 11C-E). Little differences in body weights between treatments were observed at the end of the study (Table 6).

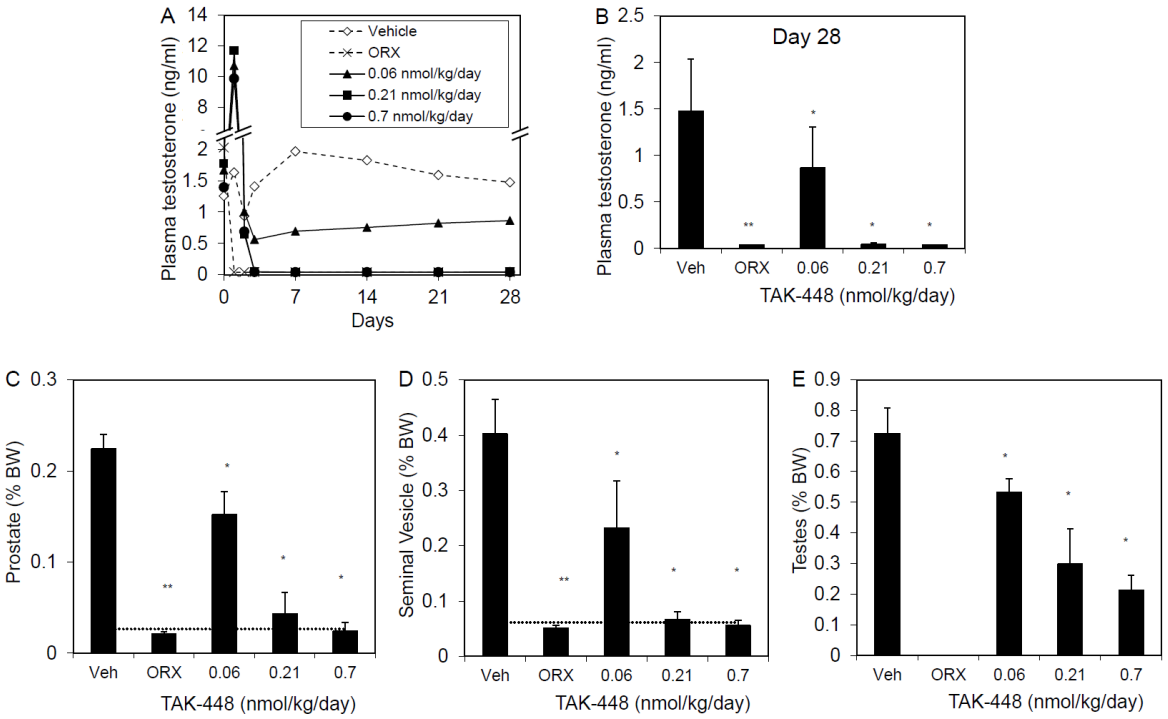

Fig. 11 Effect of continuous administration of TAK-448 in male rats.
(A) Mean plasma testosterone levels at indicated time points. Plasma testosterone levels (B), relative weights of the prostate (C), the seminal vesicles (D), and the testes (E) on day 28. Data shown are mean + S.D. unless otherwise stated. The lower limit of quantification of testosterone was 0.04 ng/ml, and values below the limit were expressed as 0.04 ng/ml. Dashed lines in figures (C) and (D) indicate castrate levels. As stated in the section 3.2, the doses can be converted to μg/kg/day with the conversion factor of 0.09. * indicates $P < 0.025$ (one-tailed Williams' test; ORX) or $P < 0.05$ (Wilcoxon rank sum test; TAK-448) versus vehicle control.

Table 6 Body weights in the experiment shown in Fig. 11

Mean ± SD

	Day 0	Day 28
Vehicle	330 ± 11	460 ± 40
ORX	326 ± 11	443 ± 35
TAK-448 0.06 nmol/kg/day	328 ± 12	459 ± 27
TAK-448 0.21 nmol/kg/day	330 ± 10	444 ± 14
TAK-448 0.7 nmol/kg/day	331 ± 9	434 ± 23

I also evaluated the effect of continuous infusion of TAK-683 in male rats for 12 weeks (Fig. 12), and confirmed the testosterone suppressive effect was maintained at least for 12 weeks in male rats.

A

Drug	Dose nmol/kg/d	Time	pg/ml Mean ± S.D.
TAK-683	2.1	4W	0.05 ± 0.03
		12W	0.08 ± 0.05
	7	4W	0.24 ± 0.04
		12W	0.30 ± 0.05
	21	4W	0.96 ± 0.17
		12W	0.64 ± 0.36
Leuprolide	7	4W	0.50 ± 0.04
		12W	0.44 ± 0.03
	21	4W	1.51 ± 0.33
		12W	1.47 ± 0.23
	70	4W	3.72 ± 1.19
		12W	3.78 ± 0.72

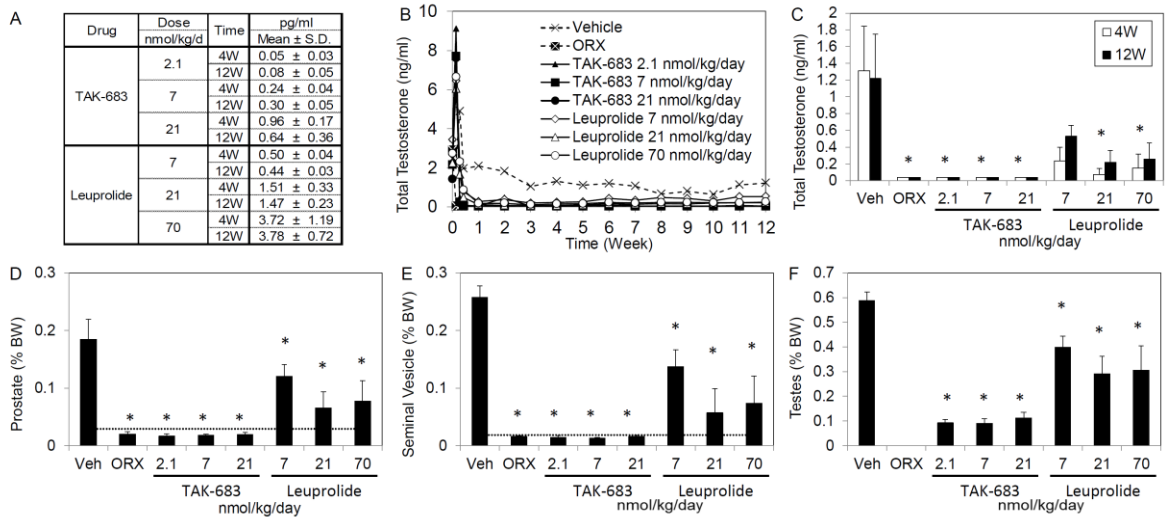

Fig. 12 Effect of continuous administration of TAK-683 for 12 weeks in male rats.
(A) Plasma drug concentrations at 4 and 12 weeks. (B) Mean plasma testosterone levels at indicated time points. Plasma testosterone levels at 4 (open bars) and 12 weeks (solid bars) (C), relative weights of the prostate (D), the seminal vesicles (E), and the testes (F) at 12 weeks. Data shown are mean + S.D. unless otherwise stated. The lower limit of quantification of testosterone was 0.04 ng/ml, and values below the limit were expressed as 0.04 ng/ml. Dashed lines in figures (D) and (E) indicate castrate levels. * indicates $P < 0.025$ (one-tailed Williams' test; ORX) or $P < 0.05$ (Wilcoxon rank sum test; TAK-683) versus vehicle control. Note that differences in plasma testosterone levels were considered to be significant only when the values were significantly reduced at both 4 and 12 weeks.

Antitumor Effect of TAK-683 in JDCaP Prostate Tumor Model.

In male nude rats bearing JDCaP prostate tumor, I evaluated antitumor effect of TAK-683, as a representative of kisspeptin analogues, in comparison to leuprolide. Doses were 1.4 and 7 nmol/kg/day for both compounds. Serum concentrations of PSA were reduced in rats treated with TAK-683, leuprolide, or ORX (Fig. 13), compared with rats treated with vehicle control. Leuprolide (1.4 or 7 nmol/kg/day) treatment resulted in elevated serum PSA levels on day 3, but neither treatment with TAK-683 nor ORX induced PSA elevation on day 3 (Fig. 13A). PSA concentrations were reduced to below the limit of detection (0.5 ng/ml) in all rats by day 7 (ORX), day 14 (TAK-683, 1.4 and 7 nmol/kg/day), day 42 (leuprolide, 7 nmol/kg/day), or day 56 (leuprolide 1.4 nmol/kg/day) after

treatment initiation (Fig. 13B). At the doses studied, both ORX and TAK-683 induced a significantly more rapid reduction in serum PSA levels to below the limit of detection compared to leuprolide ($P \leq$ 0.01, log-rank test).

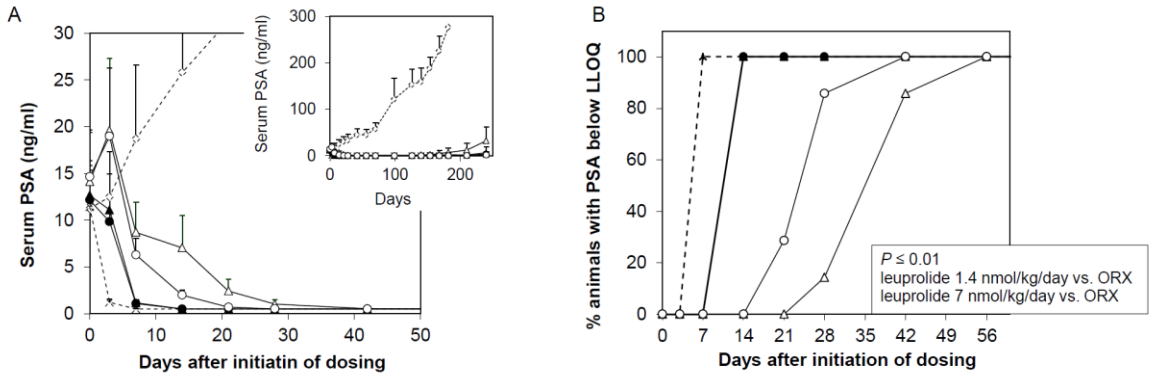

Fig. 13 Effect of continuous administration of TAK-683 on serum PSA levels in JDCaP tumor-bearing rats.

(A) Serum PSA levels over time with the magnified y-axis to clearly show PSA changes in each treated group. The inset shows the overall view, only vehicle-treated gonad-intact animals exhibited continual PSA increases over time. (B) Onset of PSA reduction below the limit of detection (0.5 ng/ml) by each treatment is shown and the differences are analyzed by log-rank multiple comparison test versus ORX treatment. Vehicle control (dashed diamond), ORX (dashed cross), TAK-683 1.4 nmol/kg/day (closed triangle), TAK-683 7 nmol/kg/day (closed circle), leuprolide 1.4 nmol/kg/day (open triangle), leuprolide 7 nmol/kg/day (open circle). Data in A and B are shown in mean + S.D.

Chapter 3: Chronic administration of the Kisspeptin Analogue KISS1-305 or the Investigational Agent TAK-448 Suppresses Hypothalamic Pituitary Gonadal Function and Depletes Plasma Testosterone in Adult Male Rats

Results

In this chapter, I will describe the mechanism of the testosterone lowering effect of kisspeptin agonist analogues under chronic administration.

Effect of Chronic Administration of KISS1-305 in Male Rats

I studied the effect of chronic administration of the kisspeptin analogue KISS1-305 (4 nmol/h) in male rats. Both plasma T and LH levels were markedly increased on day 1 following initiation of dosing and dramatically decreased thereafter to the lower limit of detection (LH: 0.08 ng/mL, T: 0.04 ng/mL) by day 6 (Fig. 14A-B), suggesting suppression of pituitary-gonadal functions. Similarly, 4 and 8 hours following KISS1-305 administration c-Fos-ir expression in GnRH neurons within the region of the OVLT and aPOA was significantly increased but returned to normal levels by 24 h (Fig. 14C-J and Table 7), suggesting that the GnRH neurons became desensitized to KISS1-305 stimulation as a consequence of chronic exposure. I also found that chronic administration of KISS1-305 reduced the number of GnRH-ir neurons both at 24 h and day 6 (Table 7), suggesting the depletion of releasable GnRH pools as a consequence of the chronic administration of the kisspeptin analogue.

Table 7 Summary of GnRH and c-Fos immunoreactivity after the initiation of chronic administration of KISS1-305

	GnRH-ir cells		Fos-ir GnRH cells		Fos-ir GnRH (%)	
Time	Vehicle	KISS1-305	Vehicle	KISS1-305	Vehicle	KISS1-305
4 h	88 ± 22	112 ± 19	3 ± 1	91 ± 20[a]	3.1 ± 0.9	82.0 ± 10.9[b]
8 h	88 ± 8	74 ± 23	2 ± 1	54 ± 13[a]	1.8 ± 1.1	74.2 ± 6.7[b]
24 h	89 ± 11	21 ± 8[b]	3 ± 2	2 ± 1	3.4 ± 2.0	11.3 ± 7.2
6 d	92 ± 7	41 ± 21[a]	5 ± 3	3 ± 3	5.6 ± 3.0	5.1 ± 4.8

Mean ± SD (n = 3).

[a] $P \leq 0.05$ *vs.* vehicle (Student's or Aspin-Welch *t* test).

[b] $P \leq 0.01$ *vs.* vehicle (Student's or Aspin-Welch *t* test).

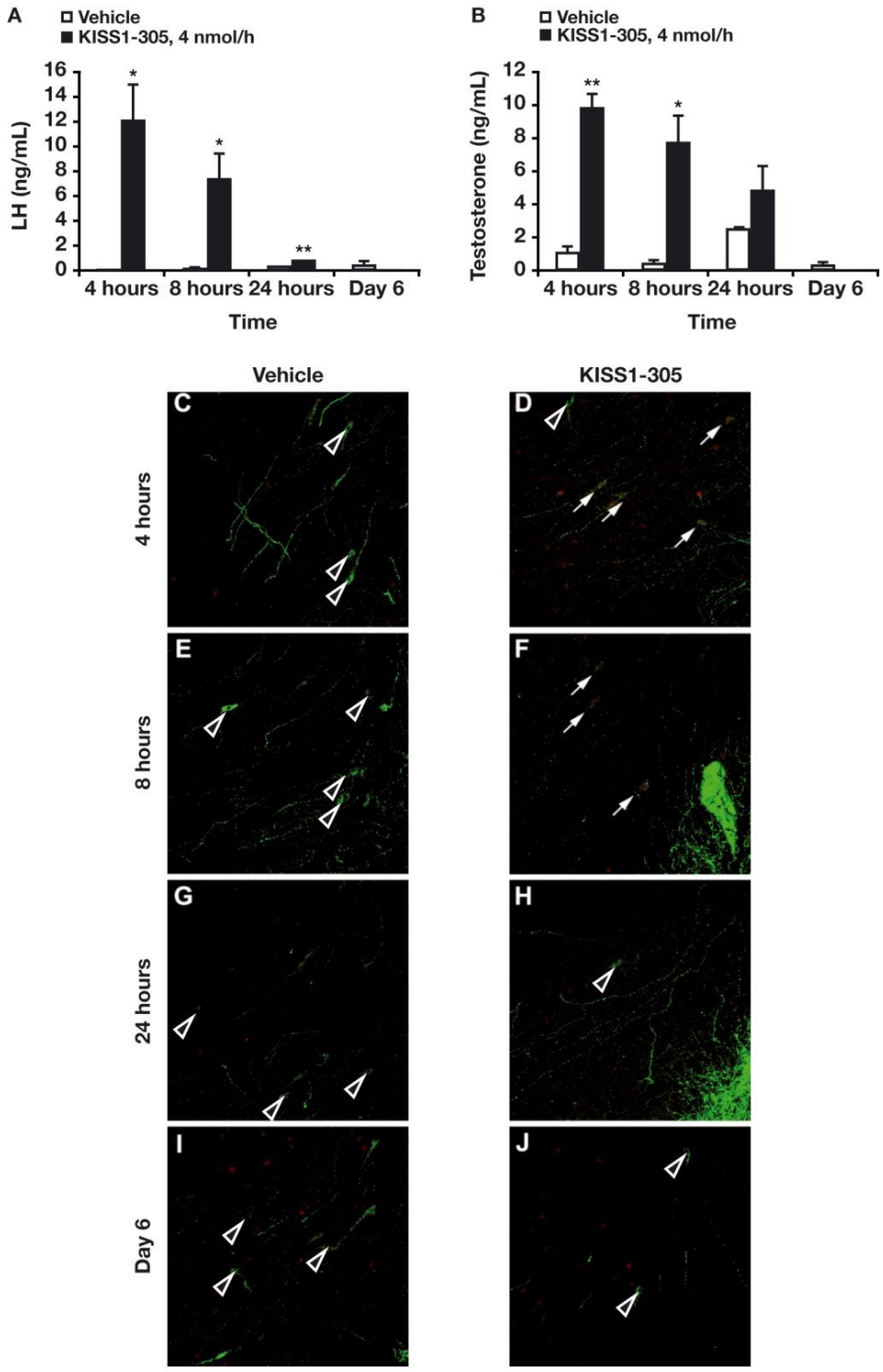

Fig. 14 Effect of chronic s.c. administration of KISS1-305 in male rats
KISS1-305 or vehicle was chronically administered to male rats, and plasma LH (A) and T (B) levels were determined at 4, 8, 24 h, and 6 days after the initiation of dosing. Data shown are mean + SD (n = 3). * and ** indicate $P < 0.05$ and 0.01 versus vehicle either by Student's or Aspin-Welch *t*-test. (C-J) Representative photographs of GnRH (green) and c-Fos (red) IHC. Observation at 4 h (C, D), 8 h (E, F), 24 h (G, H), and 6 days (I, J) after the initiation of either vehicle (C, E, G, I) or KISS1-305 (D, F, H, J). Arrows and arrowheads indicate c-Fos positive and c-Fos negative GnRH neuronal bodies, respectively.

LH Release after Chronic Administration of Kisspeptin Analogues

To explore the mechanism of action underlying the effect of lowering T and gonadotropin by kisspeptin analogues, I first studied the LH-releasing response to a bolus administration of KISS1-305 (100 nmol/kg) or leuprolide (100 nmol/kg) in rats that had received a 6-day chronic administration of KISS1-305 (2 nmol/h), leuprolide (2 nmol/h), or vehicle. Bolus KISS1-305 induced LH release in vehicle-treated rats, but not in rats treated with KISS1-305 or leuprolide (Fig. 15A). In contrast, bolus leuprolide increased LH levels in both vehicle- and KISS1-305-treated rats, but not in leuprolide-treated rats (Fig. 15A). These findings suggest that chronic administration of the GnRH analogue leuprolide desensitizes the pituitary to further GnRH exposure, while chronic KISS1-305 does not desensitize the pituitary to GnRH stimulation. This also suggests that the gonadotropin-lowering effect of kisspeptin analogues is not due to desensitization of the pituitary to GnRH but rather reflects diminished GnRH release from the hypothalamus in response to chronic exposure to kisspeptin analogues. Following 6-day chronic KISS1-305 administration, I also evaluated the effect of another GnRH secretagogue, NMDA, on plasma LH levels, and found that a single bolus s.c. administration of NMDA (30 mg/kg) failed to elevate plasma LH levels (Fig. 15B) at 15 minutes after NMDA dosing.

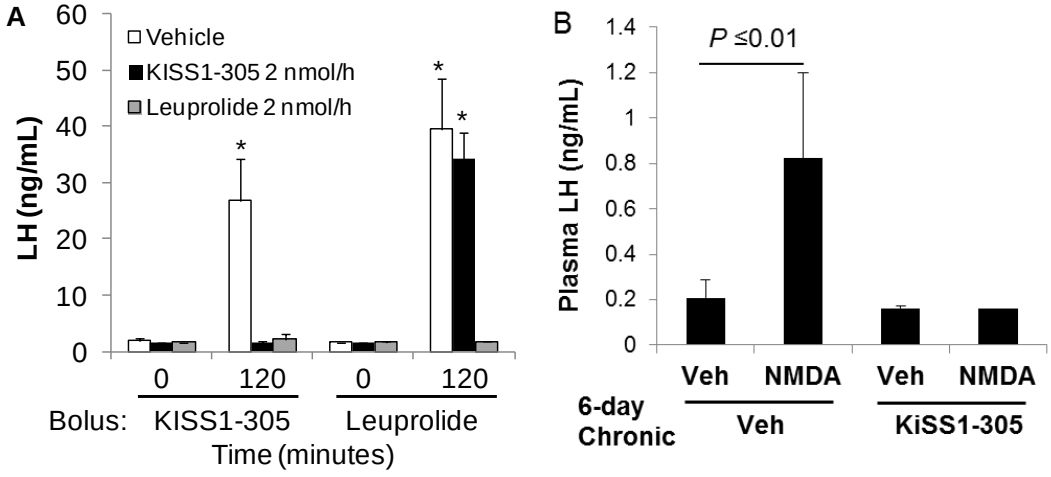

Fig. 15 LH release after 6-day chronic administration of KISS1-305
(A) After 6-day chronic administration of vehicle, KISS1-305 (2 nmol/h), or leuprolide (2 nmol/h), animals received a bolus s.c. administration of KISS1-305 (100 nmol/kg) or leuprolide (100 nmol/kg) at time 0. Data shown are mean + SD (n = 6-8). * indicates $P < 0.01$ by paired *t*-test (0 versus 120 minutes). (B) After 6-day chronic administration of vehicle or KISS1-305, animals received a bolus s.c. administration of NMDA (30 mg/kg) or saline. Plasma LH levels were determined 15 minutes after the NMDA administration (n = 8). The differences between vehicle- and NMDA-treatment were analyzed by Aspin-Welch *t*-test.

This blood sampling point (15 min) was chosen based on results from a pilot experiment (Fig. 16). NMDA induces c-Fos-ir proteins in the OVLT-aPOA area as shown in KISS1-305 naïve animals (Fig. 17).

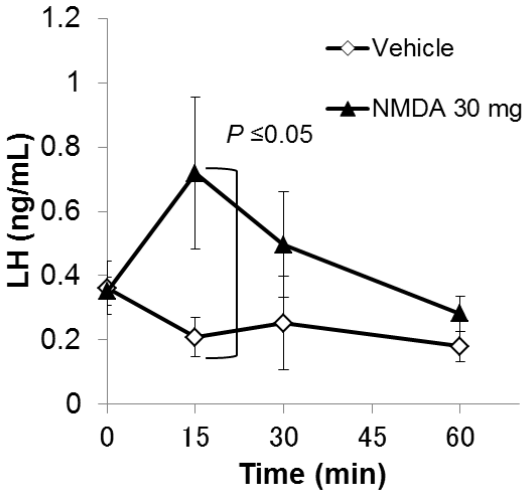

Fig. 16 Effect of NMDA on plasma LH levels in intact male rats
Plasma LH levels peaked at 15 min after s.c. NMDA administration (30 mg/kg) in male Crl:CD(SD) rats. Data shown are mean ± SD (n = 3).

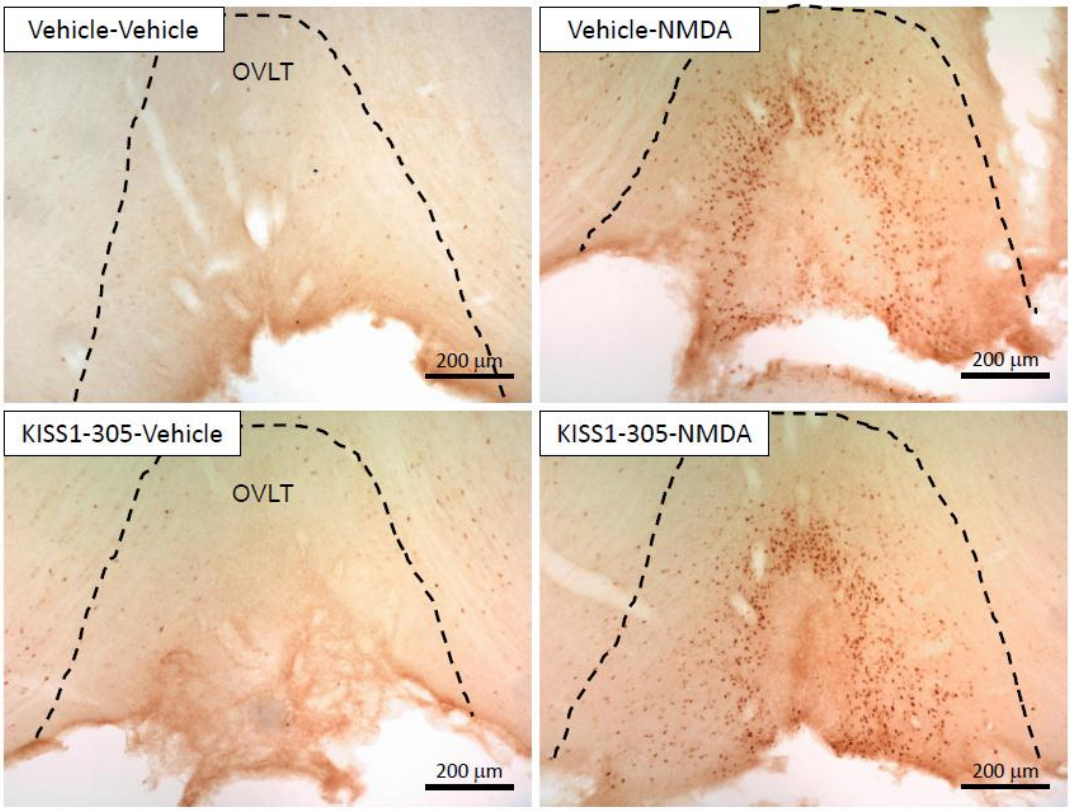

Fig. 17 Effect of NMDA on c-Fos-ir expression.
Representative photographs (n = 3) showing c-Fos-ir expression around the region of the OVLT. NMDA administration induced c-Fos-ir expression in both 6-day vehicle and 6-day KISS1-305 treated rats.

Effect of Chronic Administration of Kisspeptin Analogues on the Hypothalamic GnRH Neuron Activity

My data in Fig. 14 suggested that chronic exposure of GnRH neurons to kisspeptin analogues transiently induced c-Fos expression. Therefore, I studied whether GnRH neurons in rats that were chronically treated with KISS1-305 remained responsive to high-dose bolus KISS1-305. These studies employed IHC techniques to monitor c-Fos expression in target neurons. After a 3-week chronic administration of KISS1-305 (2 nmol/h), a single s.c. injection of KISS1-305 (100 nmol/kg) induced expression of c-Fos-ir in GnRH neurons, while injection of vehicle alone did not (representative photomicrographs are shown in Fig. 18). Similar results were obtained from multiple independent experiments (data not shown). These findings suggested that GnRH neurons still maintained their responsiveness to excessive kisspeptin stimuli in terms of c-Fos induction following chronic KISS1-305 administration.

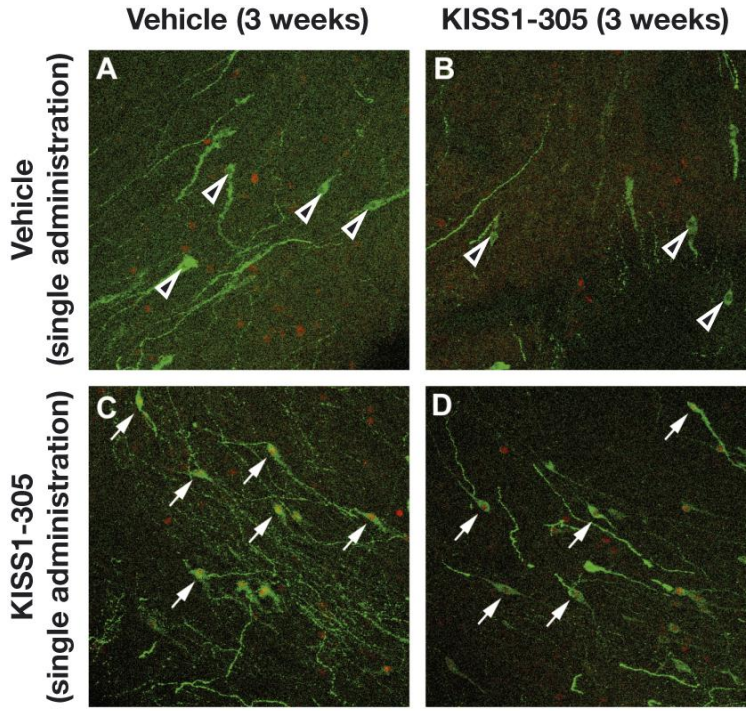

Fig. 18 Effect of bolus KISS1-305 injection on IHC of c-Fos and GnRH in animals treated with KISS1-305 for 3 weeks

Representative photographs of c-Fos (red) and GnRH (green) immunoreactivity in the aPOA at the level of the OVLT are shown. Male rats were chronically administered either vehicle (A, C) or KISS1-305 (B, D) for 3 weeks, followed by a single administration of either vehicle (A, B) or KISS1-305 (C, D). Arrows and arrowheadindicate c-Fos positive and c-Fos negative GnRH neuronal bodies, respectively.

I evaluated *Kiss1*, *Kiss1r*, and *Gnrh* mRNA expression in rats that were treated with chronic KISS1-305 to determine if mRNA levels of these associated proteins were altered. Hypothalamic *Kiss1* mRNA levels were significantly upregulated after chronic administration of KISS1-305 for 3 weeks (Fig. 19A), most likely reflecting the reduction of circulating T (13). Of interest, *Kiss1r* mRNA and *Gnrh* mRNA levels were essentially unchanged under these conditions (Fig. 19B and C). Finally, I assessed hypothalamic protein levels of GnRH, and found a significant reduction in hypothalamic GnRH at 3 weeks (Fig. 19D). This reduction in hypothalamic GnRH supports my finding that there is a reduction of the number of GnRH-ir neurons as a consequence of chronic dosing with KISS1-305 (Table 7 and Fig. 14).

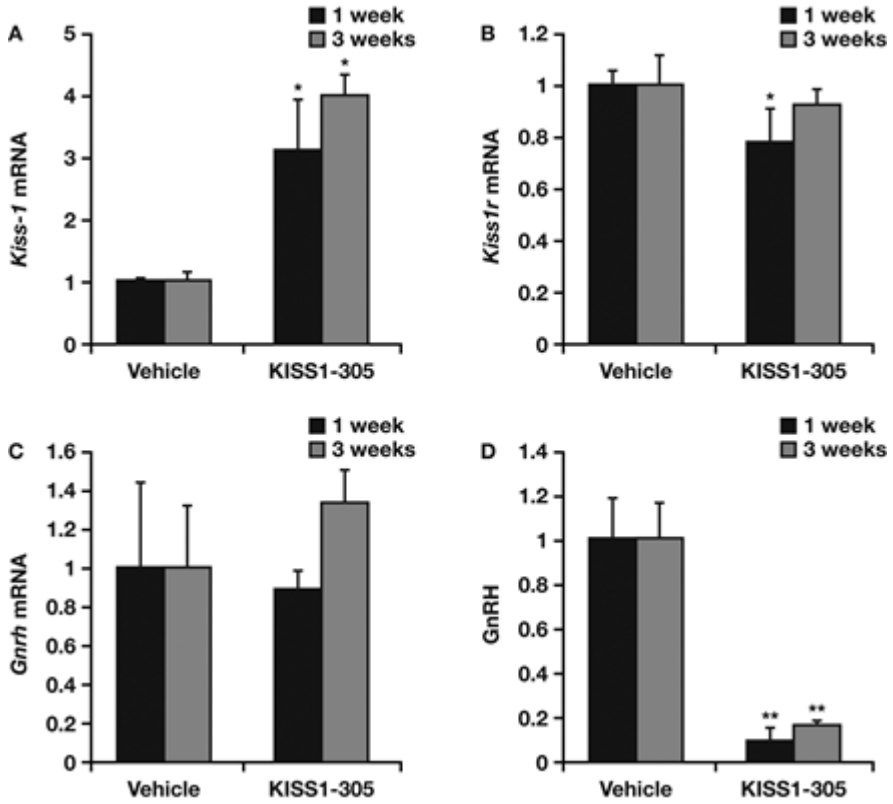

Fig. 19 Effect of chronic administration of kisspeptin analogues on hypothalamic *Kiss-1*, *Kiss1r*, *Gnrh* mRNA expression levels and GnRH peptide contents

Effect of chronic administration of KISS1-305 for 3 weeks on *Kiss-1* (A), *Kiss1r* (B), *Gnrh* (C) mRNA levels and GnRH peptide contents (D). Data shown are mean + SD (n = 4) for relative values versus vehicle (vehicle = 1), * and ** indicate $P < 0.05$ and 0.01 versus vehicle, respectively, either by Student's or Aspin-Welch *t*-test.

Effect of Chronic Administration of the Novel Kisspeptin Analogue TAK-448 on the HPG Axis

I have designed and developed a novel, investigational, nonapeptide derivative of KISS1-305, designated TAK-448 (30). Chronic administration of TAK-448 (0.1 nmol/h) depleted plasma T levels below 0.04 ng/mL in male rats both at 1 and 4 weeks (Fig. 20A), demonstrating that this dose of TAK-448 was as effective at lowering T levels as KISS1-305 (2 nmol/h) and readily achieved chemical castration status in male rats. Chronic administration of TAK-448 achieved testosterone depletion as rapid as KISS1-305 but at a lower dose. Consequently, studies were initiated employing TAK-448 to better define the impact of kisspeptin analogue exposure on hypothalamic-pituitary functions. In these experiments I compared hypothalamic GnRH peptide content, *Gnrh* mRNA expression levels, plasma FSH and LH levels, and pituitary protein or mRNA levels for FSH and LH in rats treated with TAK-448 (0.1 nmol/h) to that observed following exposure to leuprolide (0.3 nmol/h) or ORX (Fig. 20B-I). In agreement with my observations following the chronic administration of KISS1-305, chronic administration of TAK-448 significantly reduced hypothalamic GnRH content at both 1 and 4 weeks. In contrast, ORX or leuprolide treatment showed a significant reduction only after 4 weeks of therapy (Fig. 20B). *Gnrh* mRNA expression levels were unchanged by any treatment (Fig. 20C).

ORX elevated plasma FSH and LH levels both at 1 and 4 weeks (Fig. 20D and G), and pituitary gonadotropin protein as well as mRNA levels were also elevated by 4 weeks (Fig. 20E, F, H and I), suggesting modulation of steroidal negative feedback and compensatory activation of hypothalamic-pituitary functions. In contrast, chronic TAK-448 administration significantly reduced plasma levels of FSH and LH at both 1 and 4 weeks, which were lower than those by leuprolide treatment (Fig. 20D and G). This effect of TAK-448 was associated with significant reduction of pituitary FSH contents and *Fshb* and *Lhb* mRNA expression levels both at 1 and 4 weeks, and that of LH contents at 4 weeks (Fig. 20E, F, H and I). Leuprolide treatment significantly reduced plasma LH levels only at 4 weeks, and FSH levels at 1 and 4 weeks (Fig. 20D and G). Further, leuprolide administration dramatically depleted both FSH and LH protein and mRNA levels at 1 and 4 weeks (Fig. 20E, F, H and I). In other words, TAK-448 treatment decreased plasma LH and FSH levels more rapidly and effectively, with comparative reduction in the pituitary *Fshb* and *Lhb* mRNA levels with less apparent

reduction in the pituitary FSH and LH contents, than leuprolide treatment.

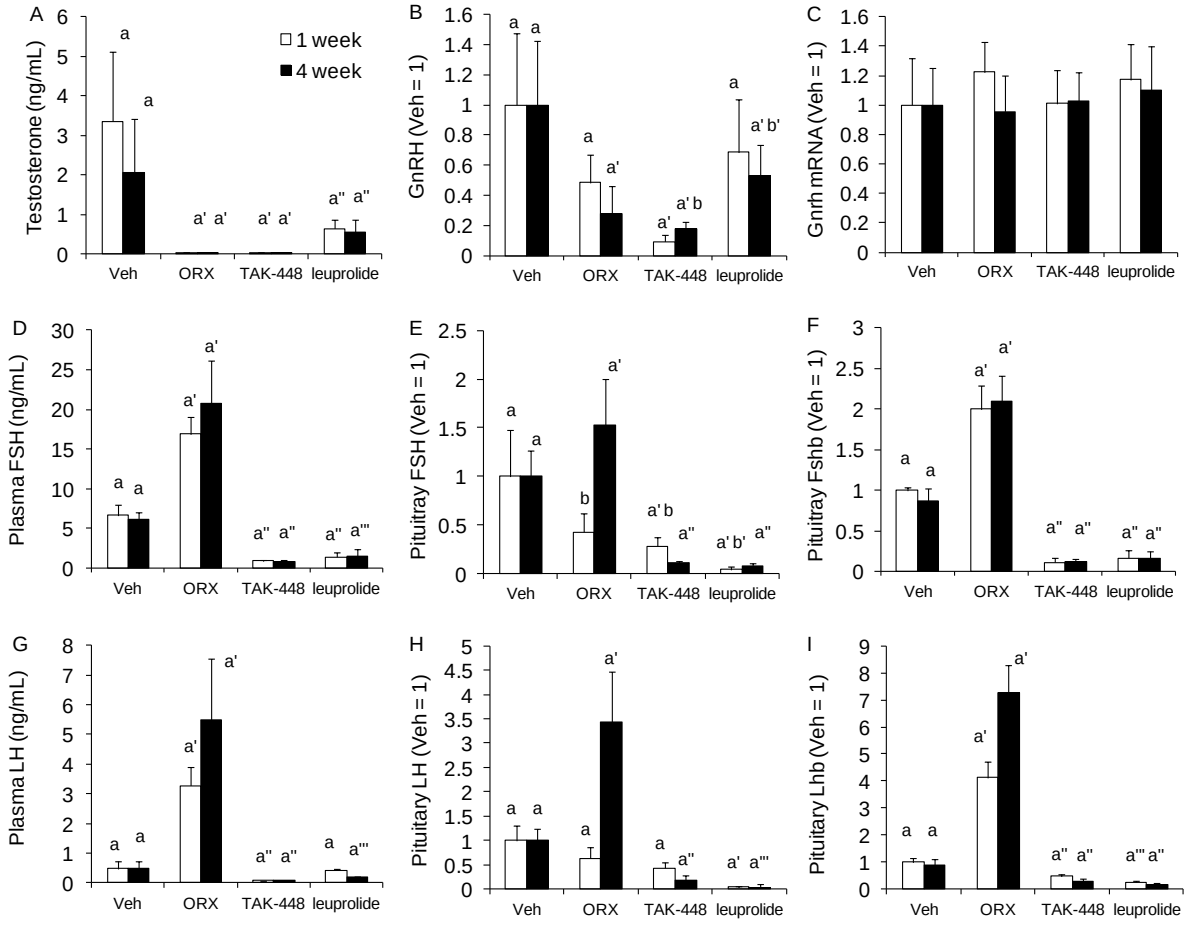

Fig. 20 Effect of chronic administration of TAK-448 on HPG axis functions
After 1 or 4 weeks post-ORX, or treatment with vehicle, TAK-448 (0.1 nmol/h), or leuprolide (0.3 nmol/h), plasma T levels (A), relative hypothalamic GnRH contents (B) *Gnrh* mRNA levels (C), plasma FSH (D) and LH (G) levels, relative pituitary FSH (E) and LH (H) contents, and relative pituitary *Fshb* (F) and *Lhb* (I) mRNA levels were examined. Data shown are mean + SD (n = 5 for 1 week, n = 10 for 4 weeks). Statistically significant differences at each time point were observed between same letters with different numbers of apostrophe (e.g. a versus a' versus a'' are significantly different) by Steel-Dwass multiple comparison test.

Discussion

In this study, I first revealed the robust stimulatory effect of kisspeptin agonist on ovulation induction in the rat. The gonadotropin releasing effect of kisspeptin was potent enough to induce full ovulation in female rats. This result suggests that Kp-54 induces biologically active LH release, and that the amount of released LH is sufficiently high to induce ovulation in the model. Such an explosive LH release is rather surprising and physiologically uncharacteristic in the normal male, and to my knowledge Kp-54 is the first reported peptide to be capable of producing this effect. This stimulatory effect was observed regardless gender differences (male and immature female rats) or hormonal milieu (immature female rats with or without follicular maturation). Immunohistochemical analysis revealed that almost 60% of GnRH neurons were c-Fos-positive. This is comparable to data reported for the preovulatory or steroid-induced LH surge that occurs in female rats (Lee *et al.*, 1990; Attardi *et al.*, 1997), suggesting that the observed numbers of activated GnRH neurons account for the Kp-54-induced increase in plasma LH. On the other hand, Kp-54 did not potentiate gonadotropin release in monolayer-cultured rat pituitary cells. Therefore, I hypothesize that in rats, peripherally administered Kp-54 induces the secretion of hypothalamic GnRH, which in turn elicits gonadotropin release.

In accordance with these findings, three independent studies from de Roux *et al.*, Seminara *et al.*, and Funes *et al.* demonstrated functional defects in KISS1R in human or mice resulted in the defective onset of puberty and suggested critical contribution of kisspeptin/KISS1R system in controlling the reproductive functions (de Roux *et al.*, 2003; Funes *et al.*, 2003; Seminara *et al.*, 2003). Following these reports including my own, numbers of evidences demonstrated that 1) the existence of kisspeptin neurons (Kinoshita *et al.*, 2005; Smith *et al.*, 2005a; Smith *et al.*, 2005b; Adachi *et al.*, 2007; Ramaswamy *et al.*, 2008), 2) the expression of KISS1R in the GnRH neurons (Irwig *et al.*, 2004; Han *et al.*, 2005; Messager *et al.*, 2005; Liu *et al.*, 2008; Pielecka-Fortuna *et al.*, 2008), 3) depolarization and neural firing of GnRH neurons by kisspeptin in ex vivo experiments (Han *et al.*, 2005; Liu *et al.*, 2008; Pielecka-Fortuna *et al.*, 2008), and all of which strongly suggests that kisspeptin directly activate GnRH neurons, and supports my hypothesis that the stimulatory effect of

kisspeptin reflects the important feature of its physiological functions. It has not been still elucidated whether KISS1R is expressed on GnRH neural terminals, which locate outside of the blood brain barrier (BBB), or on GnRH perikarya, which locate inside of the BBB, or both due to the lack of reliable antibody against KISS1R. Available evidences have suggested that both can be considered, and further studies are required to adequately reveal the anatomical distribution of KISS1R in the brain (Kinoshita *et al.*, 2005; d'Anglemont de Tassigny *et al.*, 2008; Herde *et al.*, 2011). Also, kisspeptin has shed light on the mechanism of steroid hormonal positive and negative feedback machinery within the hypothalamus. As described in the Introduction section, the activity of GnRH neurons is regulated by steroid hormones in a positive (i.e. pre-ovulatory GnRH surge) and negative (i.e. GnRH pulses) manner. Given that GnRH neurons do not express reliable steroid hormone receptors (Nishihara *et al.*, 1999), historical evidences have strongly suggested the existence of GnRH neural afferents which express steroid hormone receptors and critically regulate GnRH release. Subsequent studies have revealed that, especially in rodents, there are two populations of kisspeptin neurons; one is within the arcuate nucleus (ARC) and the other is around the anteroventral periventricular nucleus (AVPV) (Fig. 1). The kisspeptin mRNA and protein expressions within the ARC Kp neurons have been shown to be increased and decreased by steroid hormone withdrawal and supplementation, respectively, suggesting ARC Kp neurons are the acceptor of steroid hormone negative feedback effect (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Adachi *et al.*, 2007). On the other hand, the AVPV kisspeptin neurons have been predominantly determined in female rats/mice and kisspeptin expressions are increased/decreased by the supplementation/withdrawal of estradiol, suggesting that the AVPV kisspeptin neurons are the acceptor of estradiol positive feedback effect and also female specific (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Adachi *et al.*, 2007). Also, the expression of steroid hormone receptors (ER, AR) has been demonstrated in kisspeptin neurons (Smith *et al.*, 2005a; Smith *et al.*, 2005b; Adachi *et al.*, 2007). Thus, the currently most accepted hypothesis is that kisspeptin neurons critically regulate both GnRH pulses and surge as GnRH afferents (Fig. 21).

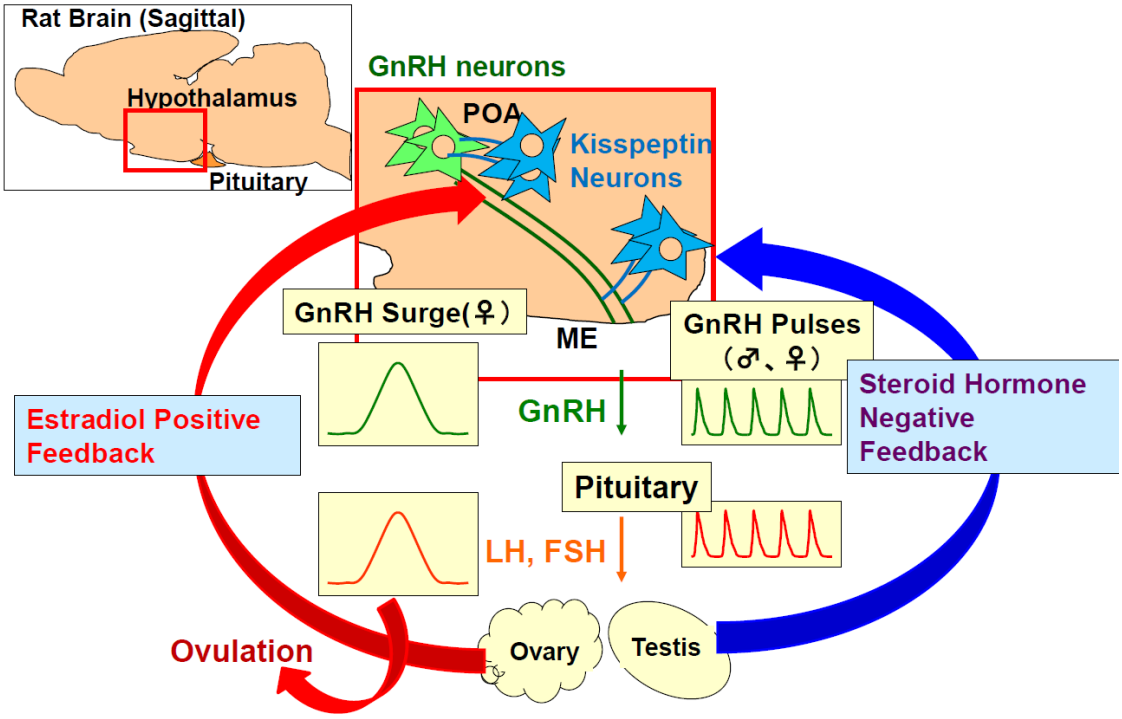

Fig. 21　Kisspeptin play a pivotal role in controlling GnRH release
In rodents, two distinct populations of kisspeptin neurons have been identified, one is the ARC kisspeptin neurons which are believed to receive steroid hormone negative feedback. The other is the AVPV kisspeptin neurons which are specific to female animals and receiving estradiol positive feedback.

To more fully understand the effects of kisspeptin analogues, I then characterized pharmacological profiles of kisspeptin agonist analogues, and the effect of both repeated daily s.c. injections and continuous s.c. administrations in male rats. *In vitro* experiments demonstrated that both TAK-448 and TAK-683 are potent agonists for rat KISS1R, and TAK-683 showed slightly more potent *in vitro* receptor binding and agonistic activities than TAK-448. Recently it has been recognized that kisspeptin also interacts with neuropeptide FF receptors especially with NPFFR1 rather than NPFFR2, and RF-amide related peptide (RFRP)-NPFFR1 system has been suggested to act as GnRH inhibitory hormone (GnIH) (Oishi *et al.*, 2011). Several studies have demonstrated that C-terminal RF-amide structure is important for interacting with NPFFR1/2 and substitution of the C-terminal Phe with other amino acids such as Tyr or Trp attenuated agonist activity or receptor binding affinity(Mollereau *et al.*, 2002; Engstrom *et al.*, 2003; Yoshida *et al.*, 2003; Findeisen *et al.*, 2011). I also observed a similar

attenuated agonistic effect of rat Kp-10 with RY-amide structure compared to human Kp-10 with RF-amide structure (data not shown). Though it is very unlikely that TAK-448 and TAK-683, both having Arg(Me)-Trp-amide structure, potently interact with NPFFRs, additional *in vitro* studies are required to address this possibility. *In vivo* settings, TAK-448 showed better pharmacokinetic profiles than TAK-683 in my subcutaneous infusion protocol, and required dose of TAK-448 for complete testosterone suppression and profound reductions in weights of genital organs was lower than that of TAK-683. This superiority of TAK-448 over TAK-683 *in vivo* may be attributed to this better pharmacokinetic profiles or target engagement (i.e. GnRH neurons) given that TAK-683 showed better *in vitro* profiles than TAK-448. Further studies especially focusing on pharmacokinetic profiles of these compounds including tissue distribution such as the hypothalamus would be helpful to answer to this question.

In this study, I evaluated the effect of both repeated daily subcutaneous injections and continuous subcutaneous administrations in male rats, and found that both regimens eventually suppressed male reproductive functions as evidenced by reductions in testosterone and weights of genital organs. In a once-daily s.c. injection study, the animals became insensitive to bolus TAK-448 and TAK-683 injection in terms of LH and testosterone releases at 4h on Days 4 and 7, suggesting the desensitization to exogenous kisspeptin analogues. Reductions in baseline testosterone levels (i.e. 0h on Days 4 and 7) were also observed both by TAK-448 and TAK-683 treatments. At 8 μmol/kg of TAK-448 and TAK-683, both 0h and 4h testosterone levels were suppressed below the limit of quantification (0.04 ng/ml), suggesting that the 8 μmol/kg was the minimum effective once-daily dose of TAK-448 and TAK-683 for complete suppression of plasma testosterone levels in male rats. In this study I could not observe statistically significant reductions in baseline plasma LH levels, except for at 0h on Day 7 with 8 μmol/kg of TAK-448 the dose which was associated with significantly reduced testicular weights. Additional studies such as observations of LH pulses are required to clearly demonstrate the effect on baseline plasma LH levels, as recently observed in goats after chronic infusion of TAK-683 (Tanaka *et al.*, 2013). Chronic administration of the kisspeptin analogue, KISS1-305, at doses of 2 nmol/h or greater, induced an initial brief elevation in plasma LH and T levels,

followed by rapid reduction in plasma T that remained at decreased levels throughout the 4-week study period. Genital organ weights also were markedly reduced as a consequence of chronic KISS1-305 administration. The infusion rates of TAK-448 and TAK-683 required to reduce plasma testosterone to below the limit of detection (0.04 ng/ml) throughout the 4-week dosing period were ≥ 0.7 nmol/kg/day and ≥ 2.1 nmol/kg/day; at those infusion rates, weights of the genital organs were reduced to the castrate levels. Therefore these doses can be considered as the minimum effective continuous doses of TAK-448 and TAK-683 in male SD rats, and the results suggest that the continuous administration regimen is a simpler and more convenient method to suppress male reproductive functions in rats. Thus, TAK-448 and TAK-683 have basically the same *in vivo* profiles but required dose of TAK-448 is smaller than TAK-683 for testosterone suppression. Currently both TAK-448 and TAK-683 have been evaluated in human male, and continuous subcutaneous infusion of TAK-448 (≥ 0.1 mg/day) achieved castration (i.e. serum testosterone levels ≤ 50 ng/dL) at lower doses than TAK-683 (2 mg/day) (Scott *et al.*, 2013; MacLean *et al.*, 2014). In this study I did not determine pharmacokinetic profiles of TAK-448 and TAK-683 after single subcutaneous administration. I need to evaluate other dosing regimens such as twice daily administration to compare repeated dosing regimen with continuous infusion regimen precisely.

Others have previously conducted repetitive or continuous administration of the natural kisspeptins Kp-10 and Kp-54 in rodents, monkeys, and humans. In rodents, continuous 13-day administration of Kp-54 (50 nmol/day) in male rats suppressed plasma testosterone levels and genital organ weights (Thompson *et al.*, 2006). Hourly injections of Kp-10 (2 μg/injection, [approximately 1.5 nmol/injection]) in juvenile male monkeys led to a train of repetitive LH elevations, while continuous infusion of Kp-10 (400 μg/h, [approximately 300 nmol/h]) in adult male monkeys desensitized the responsiveness of the hypothalamic-pituitary system against exogenous Kp-10 (Plant *et al.*, 2006; Seminara *et al.*, 2006; Ramaswamy *et al.*, 2007). In the present study, both once-daily s.c. injection and continuous administration of TAK-448 and TAK-683 suppressed the pituitary-gonadal functions in male rats. Testosterone reduction by continuous administration of TAK-448 and TAK-683 was more evident than that caused by continuous 13-day Kp-54 infusion (Thompson *et al.*, 2006). Also, Tanaka

et al. previously have shown that continuous administration of kisspeptin analogues in male monkeys results in more pronounced testosterone suppression than continuous administration of Kp-10 (Ramaswamy *et al.*, 2007; Tanaka *et al.*, 2010). Better metabolic stability of TAK-448 and TAK-683 compared to the natural kisspeptins may explain the analogue's more pronounced suppression of the hypothalamic-pituitary-gonadal functions.

In comparison to the GnRH agonist analogue leuprolide, kisspeptin agonist analogues produced more rapid and profound reductions in plasma testosterone levels as well as weights of genital organs. Clinically, GnRH analogue-based ADT is widely used in prostate cancer therapy, but T flare, which can be observed in the first week of therapy, may be associated with unfavorable therapeutic outcomes (Bubley, 2001). It has also been suggested that lower serum testosterone levels leads to better prognosis including overall survival (Perachino *et al.*, 2010). My study shows that chronic administration of kisspeptin analogues was more effective than leuprolide in terms of rapid and dramatic reductions in plasma T and weights of genital organs. Therefore, by virtue of their ability to profoundly reduce T, kisspeptin analogues such as TAK-448 may hold promise as a novel, more efficacious ADT than the current GnRH analogue-based prostate cancer therapies. In addition to GnRH agonist analogues, a peptidic GnRH antagonist analogue, degarelix, has been approved for prostate cancer treatment, but I did not compare the efficacy of kisspeptin analogues to GnRH antagonists. Additional studies would be helpful to understand the therapeutic potential of kisspeptin analogues in comparison to GnRH agonist and antagonist analogues.

Next I evaluated the antitumor effect of kisspeptin analogues in the JDCaP androgen-dependent prostate tumor model (Kimura *et al.*, 2009) in nude rats, and TAK-683 was used as a representative kisspeptin analogue in this study because of technical availability at the time of study conducted. I used serum PSA as a biomarker for tumor growth instead of direct measurement of tumor size since the tumor was implanted under renal capsule and I could not observe the tumor volume directly. Bilateral orchiectomy rapidly reduced serum PSA levels below the lower detection limit; the PSA nadir was observed in all rats within 7 days after treatment, suggesting that this JDCaP model showed good androgen dependency as expected from the original observation in mice (Kimura

et al., 2009). Analysis of the time course of PSA reduction in this model showed that TAK-683 and ORX result in more rapid PSA reduction compared with leuprolide at the doses tested. Although I did not measure testosterone levels in this study, it is plausible that the differences in the time to onset of PSA reduction arose from differences in time to onset of testosterone suppression. Since I did not evaluate objective tumor responses as described above, additional examinations such as tumor morphology, AR expressions are required to further understand potential superiority of TAK-683 over leuprolide. Recently it has been shown that the JDCaP tumor line can acquire hormone resistance in mice (Nakata *et al.*, 2010). Longer-term evaluation or studies in other prostate cancer model would provide better understanding of potential therapeutic benefits of kisspeptin analogues in prostate cancer treatment. Thus, I have demonstrated that repetitive or continuous administration of kisspeptin analogues, TAK-448 and TAK-683, rapidly suppresses male reproductive functions in rats. Compared to leuprolide, continuous administration of TAK-448 and TAK-683 resulted in shorter testosterone flare, more rapid onset of testosterone suppression, and greater reduction of both testosterone levels and weights of genital organs in male rats. Continuous administration of kisspeptin analogues also produced more rapid PSA suppression than leuprolide in a rat model of androgen-dependent prostate cancer. These results suggest that kisspeptin analogues may hold promise as a novel treatment option for several hormone-related diseases including prostate cancer. Further studies, especially in humans, will provide more precise understanding of the clinical significance and therapeutic potential of TAK-448 and TAK-683.

The last part of this Thesis was aimed to elucidate the neuroendocrine and physiological effects associated with the chronic administration of kisspeptin analogues or a GnRH analogue in male rats. To clarify the cellular events associated with these observations, I examined c-Fos-ir and GnRH-ir within the hypothalamic region as well as pituitary responsiveness to exogenous GnRH analogue stimulation. The administration of KISS1-305 was associated with transiently-increased expression of c-Fos-ir in a large proportion of GnRH neurons within the region of the OVLT and aPOA, anatomical regions possessing a large number of neurons responsible for the release of GnRH from the median eminence(Silverman *et al.*, 1987; Merchenthaler *et al.*, 1989). Following chronic kisspeptin analogue

dosing, c-Fos expression and the number of GnRH-ir neurons decreased in parallel with a reduction in plasma LH and T levels. These results highlight two important mechanistic impacts of kisspeptin analogue treatment on the hypothalamus. First, the initiation of chronic kisspeptin analogue administration strongly but transiently activates GnRH neurons and appears to induce marked GnRH release, which correlates with elevated LH and T (flare) observed on the first day. Secondly, within 24 hours, GnRH neurons become desensitized to further kisspeptin stimulation, at least in terms of analogue-induced c-Fos expression. This second effect is also associated with drastic reduction in GnRH-ir positive neurons. This observation suggests that chronic exposure to kisspeptin analogues desensitizes GnRH neurons to chronic stimuli from kisspeptin analogues and exhausts GnRH neuronal pools. Either of these effects, alone or combined, induces a rapid reduction in plasma T and LH levels.

On the other hand, following chronic kisspeptin analogue administration, pituitary responsiveness to the GnRH analogue leuprolide was maintained. Indeed, following a 1-week exposure to KISS1-305, a single bolus injection of leuprolide (but not KISS1-305) elevated plasma LH levels. I also examined pituitary and plasma gonadotropin levels. TAK-448 and leuprolide reduced pituitary and plasma FSH and LH levels in different ways. Leuprolide rapidly depleted pituitary gonadotropin mRNA and protein levels as early as 1 week but required longer exposure (4 weeks) to decrease plasma LH levels. FSH and LH depletion from the pituitary by leuprolide at 1 week can be primarily attributed to a substantial and prolonged gonadotropin release, followed by desensitization of the pituitary. It can also be speculated that leuprolide caused continuous low but detectable FSH and LH release at 4 weeks as evidenced by detectable plasma FSH, LH, and T levels. In contrast to leuprolide, TAK-448 caused a less apparent but still significant reduction in pituitary FSH content at 1 and 4 weeks and LH content at 4 weeks, while pituitary *Fshb* and *Lhb* mRNA levels as well as plasma gonadotropin levels were downregulated by 1 week. Since both gradual reduction in gonadotropin contents and apparent decrease in *Fshb* and *Lhb* mRNA levels are well explained by reduced GnRH pulses (McDowell *et al.*, 1982; Young *et al.*, 1983; Kaiser, 1997), my findings in kisspeptin analogue-treated animals supports the contention that pulsatile GnRH release from the hypothalamus is reduced or disrupted by chronic TAK-448 treatment by 1 week, leading to inhibition of pituitary function.

As discussed above, GnRH neurons appear to be desensitized following continuous stimulation by kisspeptin analogues. These findings support previous studies in monkeys (Seminara *et al.*, 2006; Ramaswamy *et al.*, 2007) suggesting that the endocrinologic effects of chronic kisspeptin analogue administration may be similar in different species. Several lines of evidences have suggested that the desensitization of GPCR may involve receptor internalization and subsequent reduction in cell surface GPCR, uncoupling to G-proteins, altered downstream signaling, or ubiquitination (Bianco *et al.*, 2011; Millar *et al.*, 2012). In addition to these proposed mechanism, my study in Chapter 3 also proposes a hypothesis that the desensitization does not mean "the complete abolishment of the KISS1R responsiveness to kisspeptin" but actually suggest "the attenuation of the KISS1R responsiveness to kisspeptin", as c-Fos expression was induced in GnRH neurons by bolus KISS1-305 administration despite the fact that these animals had been chronically treated with lower dose KISS1-305. Indeed, *Kiss1r* mRNA levels were not downregulated following the 3-week chronic administration regimen. These findings imply that the chronic administration of kisspeptin analogues probably attenuates, but does not completely abolish, GnRH neuronal responsiveness to exogenous kisspeptin analogue stimulation, and, in turn, hinders endogenous kisspeptin signaling, while endogenous kisspeptin neuronal activity was likely enhanced after chronic administration of kisspeptin analogues as evidenced by upregulation of *Kiss1* mRNA levels and its consequent impact on the endogenous neural pathway. This concept may be generalized to the other GPCRs. For example, as also discussed above, it can also be speculated that continuous administration of the GnRH analogue leuprolide caused continuous low but detectable FSH and LH release at 4 weeks as evidenced by detectable plasma FSH, LH, and T levels. This hypothesis requires remaining KISS1R on the GnRH neural surface under the continuous exposure to kisspeptin agonists, and may be supported by the recent findings which suggest dynamic KISS1R recycling and intracellular KISS1R pool which may enable the cells to be responsive to the ligand under the chronic exposure (Bianco *et al.*, 2011; Babwah *et al.*, 2012).

Chronic administration of kisspeptin analogues reduced hypothalamic GnRH peptide contents during the entire 4-week dosing regimen. This may explain why KISS1-305-treated rats showed no LH release even after high doses of KISS1-305 or NMDA. However, it is unclear whether the decreased

GnRH content alone has a significant impact on GnRH pulse generation, because highly decreased GnRH levels were also observed in ORX rats that had elevated pituitary function as evidenced by increased pituitary and plasma gonadotropin levels. Indeed, reduction of GnRH after castration can be explained by maximized pulsatile GnRH release due to the lack of T negative feedback on GnRH neurons. In contrast, the more rapid and sustained GnRH reduction with suppressed pituitary function associated with kisspeptin analogue treatment can be explained by two processes. The first process is an acute effect: Depletion of releasable GnRH resulting from substantial GnRH release at dosing initiation, as discussed above. The second process is a long-term effect: As *Gnrh* mRNA levels were unchanged after the chronic administration of kisspeptin analogues, and it is unlikely that kisspeptin analogues affect GnRH translation or processing since both *Kiss1* and *Kiss1r* disruption do not affect hypothalamic GnRH content (Messager *et al.*, 2005; d'Anglemont de Tassigny *et al.*, 2007), the most plausible explanation accounting for decreased hypothalamic GnRH content without changing Gnrh mRNA expression is that GnRH neurons are continuously releasing GnRH, albeit at very low levels without detectable increase of c-Fos expression in GnRH neurons, in response to continuously-administered kisspeptin analogues. This continuous stimulation of GnRH neurons and the consequent continuous low-level release of GnRH may also disrupt GnRH pulses caused by redundant neuronal pathways, as suggested by Chan *et al.* (Chan *et al.*, 2009). In this case, these proposed redundant pathways cannot recover or participate in hypothalamic-pituitary functioning. Direct and highly sensitive determination of GnRH release from the hypothalamus or monitoring of GnRH neuronal electrophysiological activity would be required to confirm this explanation, since c-Fos-ir induction in GnRH neurons may not always correlate with GnRH release.

Thus, my findings suggest that chronic administration of kisspeptin analogues suppresses intrinsic GnRH pulses and downstream pituitary-gonadal functions. This may be due to the attenuation of the responsiveness of GnRH neurons to endogenous kisspeptin stimulation and the stimulation of GnRH neurons to release low levels of GnRH continuously (Fig. 22), however further studies are required to exactly elucidate the status of GnRH neurons.

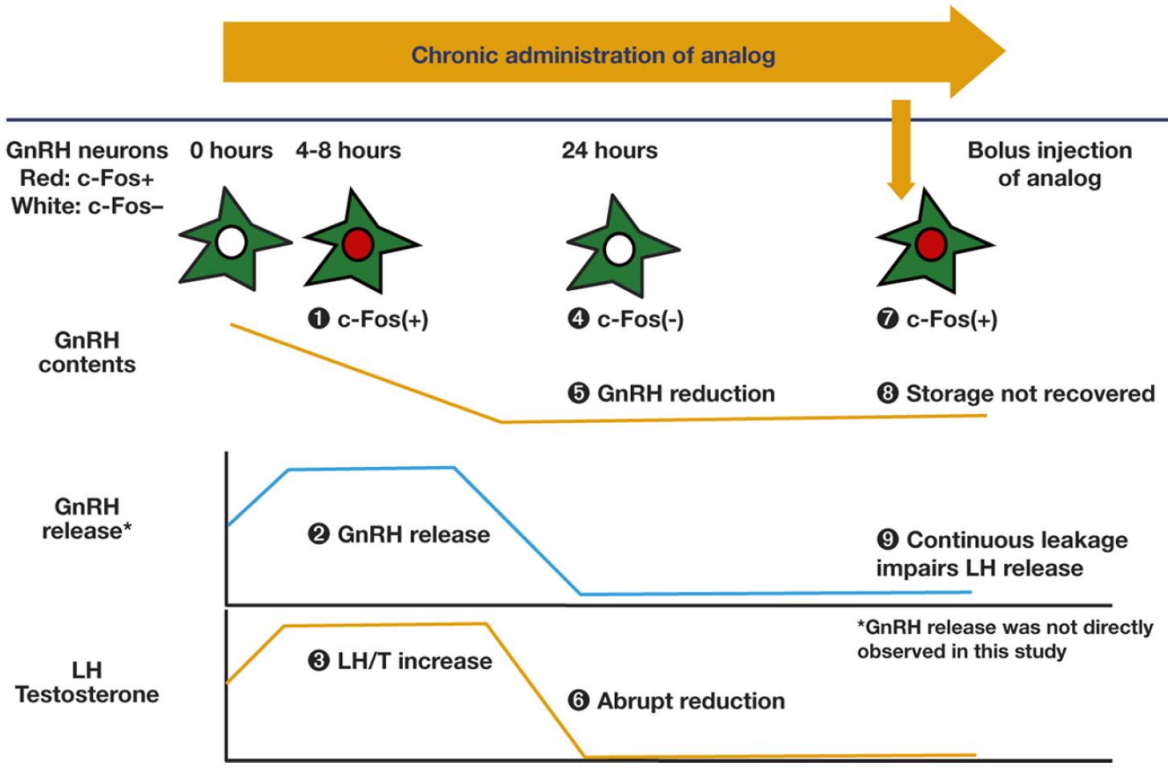

Fig. 22 Proposed mechanism of action
Kisspeptin analogue treatment results in strong activation of GnRH neurons ❶, and substantial GnRH and LH/T release ❷❸. Subsequent rapid attenuation of GnRH neuron responsiveness, which hinders endogenous kisspeptin input ❹, and/or rapid reduction of GnRH contents ❺ may explain abrupt LH/T reduction ❻. GnRH neuron responsiveness is not fully abolished ❼, GnRH contents are not recovered ❽, suggesting continuous leakage of GnRH ❾.

Conclusions

In conclusion, this study demonstrated that the novel peptide kisspeptin acts on the hypothalamic GnRH neurons and stimulates robust release of gonadotropins (FSH and LH), suggesting that kisspeptin/KISS1R system is the critical component of the HPG-axis by controlling the GnRH neurons. My study also demonstrated the potential application of kisspeptin agonist analogues as androgen dependent prostate cancer agents through the mechanism of desensitization of the GnRH neurons; more precisely attenuation of the responsiveness of KISS1R on the GnRH neurons to kisspeptin. Further studies are warranted to more precisely elucidate the physiological roles of kisspeptin and its clinical benefits.

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