Neurokinin B receptor agonist and Dynorphin receptor antagonist stimulated luteinizing hormone secretion in fasted male rodents

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Abstract. Kisspeptin/neurokinin B (NKB)/dynorphin (Dyn) (KNDy) neuron in hypothalamic arcuate nucleus plays a key role in GnRH/LH pulsatile secretion. We aimed to determine whether stimulation of NKB/neurokinin 3 receptor (NK3R) signaling and inhibition of Dyn/kappa-opioid receptor (KOR) signaling recover LH secretion that is suppressed by acute fasting in male rats. Furthermore, we determined dose dependent effect of NKB/NK3R signaling on serum LH level under acute fasting condition in male mice. Mature male rats were injected saline (0.1 mL) and senktide (20 μg/kg), a NK3R agonist, or nor-BNI (800 μg/kg), a KOR antagonist intraperitoneally (ip) after 72 h fasting. And mature male mice were injected multiple doses of senktide, ip after 48 h fasting. Blood and brain sample were collected 90 min after injections for LH measurement and hypothalamic mRNA expressions. All three studies showed significantly lower LH concentration in fasted groups than non-fasted groups. Senktide did not recover LH suppressed by acute fasting in male rats, whereas nor-BNI injected male rats showed significantly higher LH than 72 h fasted male rats (p < 0.05). Mice study showed significantly higher LH concentration in higher doses senktide groups than 48 h fasted group and one of lower doses senktide group. These results suggest that stimulation of NKB/NK3R signaling and attenuation of Dyn/KOR signaling could recover suppressed LH secretion under acute fasting condition in male rodents.

Key words: Hypothalamus, Kisspeptin, Kisspeptin/neurokinin B/dynorphin neuron, Kappa-opioid receptor antagonist, Neurokinin B receptor agonist
for reproduction. In women with functional hypothalamic amenorrhea (FHA) or congenital hypothalamic hypogonadism have low estrogen and decreased pulsatile LH or apulsatile LH secretion [16]. They also have less body fat and low serum leptin concentration.

In rodent, fasting reduces the kiss1 mRNA expression level in ARC and serum LH concentration and prolonged estrous cycle [17]. In this study, we tried to determine whether stimulation of NKB/neurokinin 3 receptor (NK3R) signaling and inhibition of Dyn/kappa-opioid receptor (KOR) signaling recover LH secretion suppressed by negative energy in male rats in order to evaluate the efficacy of such drugs in the treatment for the ovulatory disorder. Furthermore, we determined dose dependent effect of NKB/NK3R signaling on serum LH level under acute fasting condition in male mice.

Materials and Methods

Animals

Eight week male Sprague-Dawley (SD) rats and 10-week C57BL/6J male mice were purchased from Charles River Laboratories, Inc. (Tokyo, Japan). The rats and mice were housed in a temperature-controlled room (24°C) under a daily photoperiod of 14 h light: 10 h darkness (lights on at 0700) and were given food and tap water ad libitum. This study was conducted in accordance with Institutional Animal Care and Use Committee of Tokushima University.

Experiment 1. Influence of neurokinin B receptor (NK3R) agonist, senktide on fasting suppressed LH in male rats

Eight week male SD rats were randomly divided into three experimental groups; Fed, Fast and Fast + Senktide (n = 10–11). Fed group was allowed free for eat ad libitum. Fast and Fast + senktide groups were fasted for 72 h. Fed and fast groups were injected with 0.1 mL saline and fast + senktide group was injected with senktide (Santa Cruz Biotechnology, Inc. California, USA) 20 μg/kg, a NK3R agonist dissolved with saline into intraperitoneally (ip) after 72 h fasting, respectively. Blood and brain tissue were collected after 90 min from injection. Hypothalamic tissue blocks were cut as reported previously and based on rat brain atlas [17, 18]. The hypothalamic tissues were snap frozen and stored –80°C until use. Serum was isolated by centrifugation and stored at –20°C for LH measurement.

Experiment 2. Influence of kappa opioid receptor (KOR) antagonist, nor-binaltorphimine (nor-BNI) on fasting suppressed LH in male rats

Eight week male SD rats were randomly divided into three experimental groups; Fed, Fast and Fast + nor-BNI (n = 18–20). Fed group was allowed free for eat ad libitum. Fast and Fast + nor-BNI groups were fasted for 72 h. Fed and fast groups were injected with 0.1 mL saline and fast + nor-BNI group was injected with nor-BNI (Sigma-Aldrich, St. Louis, MO, USA) 800 μg/kg, a KOR antagonist dissolved with saline into ip after 72 h fasting. Blood and brain tissue were collected after 90 min from injection. Hypothalamic tissue blocks were cut as reported previously and based on rat brain atlas [17, 18]. The hypothalamic tissues were snap frozen and stored –80°C until use. Serum was isolated by centrifugation and stored at –20°C for LH measurement.

Experiment 3. Influence of multiple doses neurokinin B receptor (NK3R) agonist, senktide on fasting suppressed LH in male mice

Ten week male C57BL/6J mice were randomly divided into six experimental groups according to senktide, NK3R agonist, doses; Fed, Fast, Fast + Senktide 1, Fast + Senktide 2, Fast + Senktide 3 and Fast + Senktide 4 (n = 21–22). Fed group was allowed free for eat ad libitum. Fast and Fast + senktide groups were fasted for 48 h. Fed and fast groups were injected saline (0.1 mL) ip, respectively. Senktide groups were injected with multiple doses of senktide; Fast + senktide 1 (2 μg/kg), Fast + senktide 2 (20 μg/kg), Fast + senktide 3 (200 μg/kg) and Fast + senktide 4 (2 mg/kg) dissolved with saline ip after 48 h fasting. Blood sample was collected after 90 min from injection. Serum was isolated by centrifugation and stored at –20°C for LH measurement.

Hormone assays

Serum LH concentration was determined using an I-125 radioimmunoassay kit (Rat LH [I-125] RIA kit; Institute of Isotopes. Co., Ltd., Tokyo, Japan). The analytical sensitivity of LH assay was 0.09 ng/tube and intra-assay of LH was 6.5% by manufacturer protocol [19].

Real time reverse-transcription PCR analysis

Total RNA was extracted from hypothalamic tissue blocks using the TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® Mini kit (Qiagen...
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GmbH, Hilden, Germany). cDNA was synthesized with an Oligo (deoxythymidine) primer at 50°C using the SuperScript III First-Standard Synthesis System and the real-time polymerase chain reaction (RT-PCR) (Invitrogen™, Thermo Fisher K.K., Tokyo, Japan). RT-PCR analysis was performed by using the StepOnePlus™ RT-PCR system (PE applied Biosystems, Foster City, CA, USA) and the Fast SYBR® Green Master Mix (Thermo Fisher K.K., Tokyo, Japan). Expression levels were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level, which is the most stable housekeeping gene in the brain [20]. The forward and reverse primers used were as follows: GAPDH: F: 5′-ATG GCA CAG TCA AGG CTG AGA-3′, R: 5′-CGC TCC TGG AAG ATG GTG AT-3′ [21]; Kiss1: F: 5′-ATG ATC TCG CTG GCT TCT TGG-3′, R: 5′-GGT TCA CCA CAG TCA GTG CCA CTT TTT T-3′; Kiss1r: F: 5′-TGT GCA ATC TCG CTG GCT TCT TGG-3′, R: 5′-AGC ACC GGG GCG GAA ACA GCT GC-3′; GnRH: F: 5′-GCA GAA CCC CAG AAC TTC GA-3′, R: 5′-TGC CCA GAA CCC CAG AAC TTC GA-3′ [22]; Tac3: F: 5′-ATA GGC CAG CAG TGC AGA AA-3′, R: 5′-AGC CAA CAG GAG GAC CTT G-3′ [22]; Tacr3: F: 5′-AGC AGC TGA AGG CTA AAC GA-3′, R: 5′-GGT AGA TCG CAG TGA TGT GGA C-3′ [13]; Pdyn: F: 5′-GTT CCC TGG TGG TGA GGA C-3′ [13]; Oprk1: F: 5′-TAG GTG ATG TCC TGG GCC CAG CGG TGT TCT A-3′ [13]; Oprk1: F: 5′-GAT GTC ATT GAA TGC TCC TTG C-3′, R: 5′-CAG GAT CAT CAG GGT GTA GCA G-3′. The PCR conditions were as follows: initial denaturation and enzyme activation at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s and annealing at 62°C for 30 s (Tac3, Tacr3, Pdyn); 64°C for 30 s (GAPDH, GnRH, Oprk1); 65°C for 30 s (Kiss1, Kiss1r) and then extension at 72°C for 1 min. The copy numbers of the transcripts were normalized against those of GAPDH transcripts for each sample.

Statistical analysis

Serum LH concentration and hypothalamic Kiss1, Kiss1r, GnRH, Tac3, Tacr3, Pdyn, Oprk1 mRNA expressions were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer, Bonferroni and Dunnett’s post hoc tests. All data are presented as mean ± SE values. p < 0.05 and p < 0.01 were considered significant in all analysis.

Results

Experiment 1. Influence of neurokinin B receptor (NK3R) agonist, senktide on fasting suppressed LH in male rats

Serum LH concentration was significantly lower in Fast group than Fed group (p < 0.01) (Fig. 1). Fast + senktide group also had significantly lower LH concentration than Fed group, and did not show significant difference with Fast group (0.36 ± 0.02 ng/mL vs. 0.34 ± 0.01, mean ± SE, Fast + senktide vs. Fast, p = 0.29). Hypothalamic Kiss1r mRNA expression level was significantly higher in Fast + senktide group than other two groups (p < 0.01 vs. Fed and Fast) (Fig. 2). Hypothalamic Oprk1 mRNA expression levels was significantly higher, while hypothalamic Pdyn mRNA expression level was lower in Fast + senktide group than Fed group (p < 0.01 vs. Fed). Kiss1, GnRH, Tac3 and Tacr3 mRNA expression levels were not difference among three groups.

Experiment 2. Influence of kappa opioid receptor (KOR) antagonist, nor-BNI on fasting suppressed LH in male rats

Serum LH concentration was significantly lower in Fast and Fast + nor-BNI groups than Fed group (p < 0.01 vs. Fed) (Fig. 3). Fast + nor-BNI group has significantly higher LH concentration than fast group (0.47 ± 0.02 ng/mL vs. 0.40 ± 0.01, mean ± SE, Fast + nor-BNI vs. Fast, p < 0.05). Hypothalamic Kiss1, Kiss1r, GnRH,
Serum LH concentration was decreased by 72 h fasting in male rats in experiment 2. Even in lower level of LH concentration in Fast + nor-BNI group, nor-BNI (800 μg/kg) could partly recover fasting induced LH suppression. Data were expressed as mean + SE. 

\[ **p < 0.01 \]

vs. Fed group, \[ # p < 0.05 \] vs. Fast group.

Experiment 3. Influence of multiple doses neurokinin B receptor (NK3R) agonist, senktide on fasting suppressed LH in male mice

Serum LH concentration was significantly lower in Fast and Fast + senktide 1 groups than Fed group (\( p < 0.05 \) vs. Fed) (Fig. 5). Fast + senktide 3 and Fast + senktide 4 groups had significantly higher LH concentration than Fast group (\( p < 0.05 \) vs. Fast). Furthermore, Fast + senktide 3 and Fast + senktide 4 groups also had higher LH concentration than Fast + senktide 1 group (\( p < 0.01 \) vs. Fast + senktide 1).

Discussion

In the present study, we found that attenuation of Dyn/KOR signaling in male rat and stimulation of NKB/...
Fig. 4  Hypothalamic Kiss1, Kiss1r, GnRH, Tac3, Tacr3, Pdyn and Oprk1 mRNA expression levels in male rats in experiment 2. There was no significant difference between three groups. Values were normalized by GAPDH as an internal control. Data were expressed as mean + SE values.

Fig. 5  Serum LH concentration was decreased by 48 h fasting in male mice in Fast and Fast + senktide 1 and Fast + senktide 2 groups in experiment 3. Therefore, senktide could recover fasting induced LH suppression in higher doses of senktide including Fast + senktide 3 (200 μg/kg) and Fast + senktide 4 (2 mg/kg). Fast + senktide 1 and Fast + senktide 2 doses were 2 μg/kg and 20 μg/kg, which is similar doses of senktide could not recover LH suppression. Data were expressed as mean + SE. n = 21–22 per group. * p < 0.05 vs. Fed group, # p < 0.05 vs. Fast group, ++ p < 0.01 vs. Fast + senktide 1 group.
NK3R signaling with high dose of senktide in male mice recovered the suppressed LH secretion under acute fasting condition. The fasted groups in all experiments showed significantly lower LH concentrations than non-fasted groups. Nor-BNI injection could partly recover LH level suppressed by 72 h fasting. Although senktide injection could not recover suppressed LH in male rats, higher doses of senktide showed completely recovered serum LH concentration in male mice.

Basic secretion of LH is maintained by pulsatile GnRH/LH secretion that is originated from KNDy neuron in hypothalamic ARC. Negative energy balances such as weight loss or fasting easily suppress gonadotropin secretion and female menstrual cycle especially in rodents [17]. We previously reported that Kiss1 mRNA expression level in ARC was suppressed by acute fasting in gonadally intact mature female rats [17], indicating that suppression of KNDy neuron activity was involved in suppressed gonadotropin secretion and ovulatory disorder under weight loss or negative energy balance condition. Therefore, stimulating KNDy neuron activity seemed to have a possibility to improve gonadotropin secretion and female reproductive function in fasted condition. NKB is a stimulator of KNDy neuron through its cognitive receptor, NK3R. Selective NK3R agonist, senktide increased LH secretion in follicular phase and anestrous activity is suppressed. In our rats experiment, although senktide decreased Dyn mRNA expression in fasted adults and was increased by KOR antagonist, nor-BNI in OVX goats [9]. In addition, chronic peripheral administration of senktide increases pulsatile LH secretion and advanced puberty onset in female rat [23]. Furthermore, repeated central administration of senktide advanced puberty onset that was suppressed by negative energy balance in female rats [24]. In the present study, senktide recovered LH concentration in fasted male mice although lower doses of senktide did not show significant effect in rat and mice. Several issues should be addressed to conclude effectiveness of senktide. Senktide dose used in rat experiment was just similar to the dose of senktide 2 group in the mice experiment; approximately 20 μg/kg. This dose seems lower than effective dose, and that would be the reason why senktide could not recover LH suppression in the rat experiment. In addition, senktide seemed to have potential to increase LH secretion only in normo- or hypogonadotropic condition just like our experiments. For example, senktide stimulated LH secretion and triggered puberty onset in normal developing rats [24]. Senktide also increases LH secretion and percentage of Kiss1 neuron expressing c-fos in the presence of physiological estradiol (E2), such as intact mature female rats on diestrus 1, proestrus day of estrous cycle and ovariectomized (OVX) rats with E2 replacement [10]. Conversely, intracerebroventricular administration of senktide reduced serum LH levels in OVX rats. Moreover, senktide also prolonged LH pulse interval in OVX rats with or without gonadal steroids [25, 26]. Taken together, NKB/NK3R signaling has both stimulatory and inhibitory effects depending on physiological states and range of species and doses of administrations. NKB/NK3R signaling could have stimulatory effect on KNDy neuron under hypogonadotropic condition such as acute fasting, in which KNDy neuron activity is suppressed.

In our rats experiment, although senktide decreased Dyn mRNA expression in fasted condition, this change was not reflected in the serum LH levels and hypothalamic Kiss1 expression levels. Thus, physiological roles of such action of senktide remain unclear.

LH secretion was inhibited by KOR agonist, U50488 in adult WT female mice OVX with or without E2 [14] and was increased by KOR antagonist, nor-BNI in OVX goats [9]. In addition, KOR antagonist advanced pulsatile LH secretion in prepubertal female rats [23]. Furthermore, central nor-BNI administration significantly increased LH secretion in OVX with E2 replacement in female rats [13]. In our present study, even there was no significant difference in mRNA expression levels of Kiss1, Kiss1r, GnRH, NKB, NK3R, Dyn and KOR in hypothalamus. However, LH secretion was suppressed by fasting in both fast and nor-BNI groups, there was significant (p < 0.05) increase in nor-BNI group than fast group even there was lower level of LH secretion than fed group. Thus, nor-BNI could partly recover fasting induced LH suppression in male rats. It was reported that, nor-BNI administration alone has no effect on LH pulse frequency, but pretreatment with nor-BNI could block senktide induced suppression of LH pulse frequency in OVX with or without sex steroids supplementation in female rats [25, 26]. These phenomenon suggest that inhibitory Dyn/KOR signal might be more potently regulate GnRH/LH pulse secretion than stimulatory NKB/NK3R signal. In the present study, fasting suppressed LH secretion was recovered by senktide dose dependently in male mice and by nor-BNI in male rats. These findings suggest that stimulation of NKB/NK3R and attenuation of Dyn/KOR signalings stimulate GnRH/LH secretion in male rodents.

In this study, neither fasting, senktide nor nor-BNI did
not alter hypothalamic GnRH mRNA expressions. These data suggest that they might affect the secretion, but not production, of GnRH in hypothalamus and such changes induced the increase or decrease of LH secretion.

Women with weight loss-related amenorrhea [16], hypogonadotropic hypogonadism [1, 6], have symptoms of menstrual disorder, anovulation and infertility. Gonadotropin therapy has side effects such as multiple pregnancy and ovarian hyperstimulation syndrome, and pulsatile GnRH therapy is methodologically complex. The present results may contribute to apply alternative therapeutic approach of NK3R agonism and/or KOR antagonism to restore GnRH/LH secretion in hypogonadotropic ovulatory disorder.

In conclusion, stimulation of NKB/NK3R signaling and attenuation of Dyn/KOR signaling play a key role on the ARC KNDy neuron for recovery of suppressed LH secretion under acute fasting in male rodents. These results might indicate a therapeutic tactics modulating KNDy neuron activity in human ovulatory disorders in the future.

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Disclosure

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References


