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Chronic Peripheral Administration of Kappa-Opioid Receptor Antagonist Advances Puberty Onset Associated with Acceleration of Pulsatile Luteinizing Hormone Secretion in Female Rats

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Running head: KOR antagonism advanced puberty in rats

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Abstract

Puberty in mammals is timed by an increase in gonadotropin-releasing hormone (GnRH) secretion. Previous studies have shown involvement of the two neuropeptides kisspeptin and neurokinin B (NKB) in controlling puberty onset. Little is known about the role of other key neuropeptide dynorphin in controlling puberty onset, although these three neuropeptides colocalize in the arcuate kisspeptin neurons. The arcuate kisspeptin neuron, which is also referred to as the KNDy neuron, has recently been considered to play as an intrinsic source of the GnRH pulse generator. The present study aimed to determine if attenuation of an inhibitory dynorphin-kappa-opioid receptor (KOR) signaling triggers the initiation of puberty in normal developing female rats. The present study also determined if stimulatory NKB-neurokinin 3 receptor (NK3R) signaling advances puberty onset. Wistar-Imamichi female rats were weaned and intraperitoneally implanted with osmotic minipumps filled with nor-binaltorphimine (nor-BNI), a KOR antagonist, or senktide, a NK3R agonist, at 20 days of age. Fourteen-day intraperitoneal infusion of nor-BNI or senktide advanced puberty onset, manifested as vaginal opening and the first vaginal estrus in female rats. Frequent blood sampling showed that nor-BNI significantly increased luteinizing hormone (LH) pulse frequency at 29 days of age compared with vehicle-treated controls. Senktide tended to increase this frequency but its effect was not statistically significant. The present results suggest that the inhibitory input of dynorphin-KOR signaling plays a role in the prepubertal restraint of GnRH/LH secretion in normal developing female rats and that attenuation of dynorphin-KOR signaling and increase in NKB-NK3R signaling trigger the onset of puberty in female rats.

Key words: metastin, GPR54, sexual maturation

Introduction

Puberty, a complex sequence of maturational events, is driven by the activation of the hypothalamo-pituitary-gonadal axis. The secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus represents the first well-known step in the initiation of puberty [1, 2]. GnRH-induced gonadotropin secretion from the anterior pituitary stimulates gametogenesis and gonadal steroidogenesis in the ovary [3]. Increase in circulating level of estrogen leads opening of the vaginal cavity as a sign of the puberty onset in female rodents, and then female rodents consequently show estrous cyclicity. The potential mechanism underlying pubertal increase in GnRH secretion has been sought by pinpointing the loss-of-functional mutations of genes coding kisspeptin, neurokinin B (NKB), or their cognate receptors, GPR54 and neurokinin 3 receptor (NK3R), in patients with hypogonadotropic hypogonadism [4-8]. Previous rodent studies showed pubertal increase in kisspeptin and NKB expression in the hypothalamus [9-12], and central administration of kisspeptin advanced puberty onset in normal developing female rats [13]. Central administration of senktide, an NK3R agonist, induced puberty onset in 5 of 11 underfed female rats [12], suggesting that NKB-NK3R signaling at least partly contribute to pubertal increase in GnRH secretion in underfed rats. On the other hand, central administration of SB222200, an NK3R antagonist, failed to affect puberty onset in normal-fed rats [12]. This inconsistency in the involvement of NKB-NK3R signaling in puberty onset leads us to evaluate the roles of NKB-NK3R signaling on puberty onset in normal-fed developing female rats.

Dynorphin, a member of endogenous opioids derived from the prodynorphin gene [14], has emerged as a key inhibitory neuropeptide in controlling reproductive function [15-18]. Dynorphin neurons are abundantly distributed in several regions of the hypothalamus [19, 20] including the ARC, where dynorphin is coexpressed in a group of neurons with kisspeptin and NKB in mice [21], rats [22], sheep [23], and goats [16]. The group of ARC neurons, referred to as KNDy neurons [23], was recently considered to play a critical role in pulsatile GnRH secretion. A series of studies recording the chronic multiple unit activity (MUA) in close proximity to KNDy neurons in the ARC of goats demonstrated that periodic bursting (MUA volleys) were accompanied by luteinizing hormone (LH) pulses with regular intervals [16, 24], suggesting that ARC KNDy neurons may be the intrinsic source

of the GnRH pulse generator and that the MUA volleys may represent the rhythmic release of kisspeptin from the KNDy neurons. Central administration of NKB or a synthetic antagonist for kappa-opioid receptor (KOR), the preferred receptor for dynorphin, facilitated the MUA volleys [16]. Combined with the fact that kisspeptin governs GnRH/LH secretion in a variety of mammals [24-29], these findings suggest that dynorphin and NKB expressed in ARC KNDy neurons are involved in the process of generating the rhythmic discharge of kisspeptin that drives pulsatile GnRH/LH secretion. More specifically, dynorphin and NKB exert an inhibitory and stimulatory role, respectively, to generate pulsatile GnRH/LH secretion [16].

To date, no mutation of gene coding dynorphin or KOR has been reported in human. Little is known about involvement of dynorphin-KOR signaling in the regulation of pubertal increase in GnRH/LH pulses. Our recent study indicated that central administration of the KOR antagonist facilitates pulsatile LH secretion in estrogen-treated ovariectomized rats, but not ovariectomized animals, suggesting that dynorphin is involved in mediating the negative feedback action of estrogen on GnRH/LH secretion in female rats [18]. Taken together with estrogen-dependent suppression of LH secretion in prepubertal rats [10], we hypothesized that dynorphin-KOR signaling plays a central and critical role in the negative regulation of the prepubertal restraint GnRH/LH secretion in rats. A single intracerebroventricular injection of KOR antagonist had no effect on LH secretion in prepubertal female rats [30], suggesting that dynorphin-KOR signaling had lower importance on the prepubertal restraint of LH secretion in rats. Chronic KOR-antagonizing action, however, may be required for advancement of puberty onset in female rats.

The present study aimed to determine the roles of dynorphin-KOR and NKB-NK3R signaling on initiation of puberty in normal developing female rats. To this end, we examined the effects of chronic peripheral administration of KOR antagonist and NK3R agonist on puberty onset and LH pulses in peripubertal female rats to evaluate if attenuation of dynorphin-KOR signaling triggers puberty onset and if increase in NKB-NK3R signaling advances puberty onset. The peripheral infusion was chosen because of the possibility that those treatments in patients with a disruption of the normal pulsatile GnRH/LH secretion may have been therapeutic.

Materials and Methods

Animals

Wistar-Imamichi strain rats were kept under a 14:10 h light/dark cycle (lights on at 05:00 h) and 22 ± 2 C with free access to food (CE-2; CLEA Japan Inc., Tokyo, Japan) and water. Female rats (8-9 weeks old of age) having at least two consecutive regular 4-day estrus cycles were mated with males overnight on the day of proestrus, and then the pregnant females were housed individually. The day on which a newborn litter was found at noon was designated postnatal day 0. The litter size was adjusted to eight on day 1 to minimize the growth variation within litters. The pups were weaned on day 20. The surgical procedure was performed under anesthesia with inhaled isoflurane or ketamine-xylazine mixture.

The care of the animals and all of the experimental procedures used in these experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Effects of chronic intraperitoneal administration of KOR antagonist or NK3R agonist on puberty onset

Female rats (n = 6 per treatment group) were weaned on day 20 and randomly assigned to one of three treatment groups. Alzet osmotic minipumps (model 2002; flow rate 0.5 µl/h, 200 µl capacity; Durect Corporation, Cupertino, CA, USA) filled with nor-binaltorphimine (nor-BNI; 800 nmol/100 µl; Sigma-Aldrich, St. Louis, MO, USA), a KOR antagonist, or senktide (800 nmol/100 µl; synthesized at Kyoto University, Japan), an NK3R agonist, were surgically implanted into the abdominal cavity of female rats on day 20. Control animals were implanted with the pump filled with a vehicle (17 mM $NaHCO_3$) in the same manner. The dose of senktide (4 nmol/h/40-110 g BW) was chosen according to the previous study, in which continuous subcutaneous infusion of senktide (2400 nmol/h/20-35 kg BW) produced intermittent bursts of the GnRH pulse generator with corresponding LH pulses in goats [31]. Equimolar dose of nor-BNI was used. Female rats were housed in groups of three in plastic cages, and their body weight and vaginal openings were

monitored daily. After the vaginal opening, vaginal smears were examined daily to monitor estrous cyclicity. The first day when the cornified cells were dominant was designated as the day of the first vaginal estrous.

Effects of chronic intraperitoneal administration of KOR antagonist or NK3R agonist on pulsatile LH secretion in peripubertal rats

Female rats were weaned at day 20 and randomly assigned to one of three treatment groups: nor-BNI (n=5), senktide (n=4) and vehicle (n=5). On day 29, blood samples (50 µl) were collected from the free-moving animals for 3 h at 6-min intervals, starting at 13:00 h through a silicone catheter inserted into the right atrium through the jugular vein on the previous day of blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the cannula after each blood collection to keep the hematocrit constant. Plasma was separated by an immediate centrifugation and stored at -20 C until LH assay.

At the end of the blood sampling, rats were sacrificed and ovaries and uteri were collected and weighed. Five-hundred µl blood samples were also collected to determine plasma estradiol-17β levels.

LH and estradiol-17β assay

LH contents in 25-µl plasma samples were measured by a double antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Baltimore, MD). Plasma LH concentrations were expressed in terms of the NIDDK rat LH RP-3. Values below the level of detectability for the assay were assigned the minimum detectable concentration of the assay. The least detectable level was 7.8 pg/tube and the intra- and inter-assay coefficients of variation were 6.2% and 7.2% at 45 pg/tube, respectively.

Estradiol-17β contents in 35-µl plasma samples were measured by electrochemiluminescence using ECLusys E2II kit (Roche Diagnostics Japan, Tokyo, Japan) in a commercial laboratory (SRL, Inc., Tokyo, Japan). Values below the level of detectability for the assay were assigned the minimum detectable concentration of the assay. The least detectable level was 0.35 pg/tube and the intra- and

inter-assay coefficients of variation were 4.5% and 3.1% at 1.26 pg/tube, respectively.

Data analysis

LH pulses were identified by the PULSAR computer program [32]. Mean LH concentrations and the frequency and amplitude of LH pulses were calculated for the 3-h sampling period in each individual. Averages of these parameters were then calculated for each group. Before statistical analysis, frequency of LH pulses was square-root transformed to normalize variability across a range of values. Statistical differences in the mean LH concentrations, in the frequency of LH pulses, and in the age and body weight at the vaginal opening and first estrus, were determined by one-way ANOVA followed by the post hoc Tukey's Honest Significant Difference (HSD) test. Statistical differences in amplitude of LH pulses were not determined, because only one vehicle-treated animal showed an LH pulse.

Results

Effects of chronic intraperitoneal administration of KOR antagonist or NK3R agonist on puberty onset

The intraperitoneal chronic infusion of nor-BNI, a KOR antagonist, between days 20 and 34 advanced the vaginal opening and the first estrus. Half of the nor-BNI -treated animals showed the vaginal opening on day 29, when no vehicle-treated controls showed the vaginal opening (Fig. 1A). In addition, all individuals showed the first estrus by day 37, when no vehicle-treated controls showed first estrus (Fig. 1B). The average age at the vaginal opening tended to be younger in nor-BNI-treated animals (P=0.052, Tukey's HSD test) than in the vehicle-treated controls (Fig. 1C), and average age at the first estrus was significantly younger in nor-BNI-treated animals (P<0.05, Tukey's HSD test) than in the vehicle-treated controls (Fig. 1D). The nor-BNI treatment had no significant effect on the body weight at any age (Fig. 2A). The average body weight at the vaginal opening (Fig. 2B) and first estrus (Fig. 2C) were significantly lower (P<0.05, Tukey's HSD test) in nor-BNI-treated animals than in vehicle-treated controls.

The intraperitoneal chronic infusion of senktide, an NK3R agonist, also advanced the first estrus, but was slightly less effective than nor-BNI. Half of the senktide-treated animals showed the first estrus on day 37, when no vehicle-treated controls showed the first estrus (Fig. 1B). The average age (Fig. 1D) and body weight (Fig. 2C) at the first vaginal estrus were significantly (P<0.05, Tukey's HSD test) younger and lower in senktide-treated animals than in vehicle-treated controls, respectively, but the age at the vaginal opening was comparable to the vehicle-treated animals.

Effects of chronic intraperitoneal administration of KOR antagonist or NK3R agonist on pulsatile LH secretion in peripubertal rats

Intraperitoneal chronic infusion of nor-BNI or senktide between days 20 and 29 enhanced LH secretion on days 29 of age as shown in the representative animals in Fig. 3A. All five nor-BNI-treated animals and three out of four senktide-treated animals showed pulsatile LH secretion, while four out of five vehicle-treated animals showed no LH pulse. The frequency of LH pulses in the nor-BNI-treated rats was significantly higher (P<0.05, Tukey's HSD test) compared with the vehicle-treated controls, while the frequency in the senktide-treated rats was comparable to the vehicle-treated rats (Fig. 3B). There was no significant difference in mean LH concentrations at days 29 between experimental and vehicle-treated animals (Fig. 3B).

The chronic administration of nor-BNI tended to increase plasma estradiol levels and uteri weight, but both of nor-BNI and senktide treatments failed to show a significant effect on plasma estradiol levels and weights of ovaries and uteri at 29 days of age (Fig. 4).

Discussion

The present study demonstrates that dynorphin-KOR signaling plays a critical inhibitory role in the pubertal restraint of GnRH/LH secretion in female rats, because chronic peripheral administration of nor-BNI, a KOR antagonist, advanced pulsatile LH secretion and hence puberty onset in developing female rats. Dynorphin neurons are abundantly distributed in several regions of the hypothalamus

[19, 20] including the ARC, wherein dynorphin is coexpressed in a group of neurons with kisspeptin and NKB in mammals [16, 21-23]. Increasing evidence indicates that a group of ARC neurons, referred to as KNDy neurons [23], may play a critical role in a pulsatile GnRH secretion [16, 24]. Navarro et al. [21] showed that KOR mRNA was found in 20% KNDy neurons in the ARC of female mice, suggesting that recurrent collaterals of KNDy neurons could signal through a dynorphin-KOR signaling pathway. Thus, dynorphin-KOR signaling in the ARC KNDy neurons, a putative intrinsic source for driving the GnRH pulse generator, may play a critical role in prepubertal restraint of GnRH/LH secretion. Recently, Navarro et al. [35] suggested that some other KOR-expressing interneurons (not KNDy neurons) may mediate the action of dynorphin on GnRH pulse generation. Therefore, the current KOR antagonist may directly or indirectly act on ARC KNDy neurons to enhance GnRH/LH pulse in prepubertal rats. It is unlikely that the KOR antagonist directly act on GnRH neurons, because previous studies showed few KOR expressions in rat GnRH neurons [33, 34]. Further studies are needed to clarify the site(s) of KOR antagonism, which advanced puberty onset in the present study.

The present study demonstrates that NKB-NK3R signaling also plays a role in regulating pubertal increase in GnRH/LH secretion in normal developing female rats, because the administration of senktide, a NK3R agonist, advanced pulsatile LH secretion in 75% of animals and hence puberty onset in normal developing female rats. Navarro et al. [12] have shown that repeated central administration of senktide advanced puberty onset in underfed female rats. Taken together with the facilitatory effect we found with senktide on LH pulses, the stimulatory role of NKB-NK3R signaling was at least partly involved in the increase in pubertal GnRH/LH secretion, and thus puberty onset, in normal developing rats as well as underfed rats. KNDy neurons have been reported to express KN3R in mammals including rats [20, 21, 36], suggesting that the current senktide may directly act KNDy neurons to advance puberty onset in female rats.

Based on the roles of dynorphin and NKB in gating pubertal initiation of GnRH/LH pulsatile secretion, we propose a possible mechanism for the pubertal activation of the GnRH/LH pulse generator in rats. The present study exhibited that the KOR antagonist had a more potent effect on relieving LH pulses from the prepubertal restraint in female rats, compared with the NK3R agonist.

This leads us to an assumption that the GnRH pulse generator may be mainly downregulated by inhibitory dynorphin-KOR signaling during the prepubertal period, be rid of this inhibition at the onset of puberty, and then be upregulated by stimulatory input of NKB. The assumption is consistent with the previous finding that NK3R antagonist SB222200 administration had no effect on puberty onset in intact developing peripubertal female rats [12]. The present study used only a single dose of the nor-BNI and senktide to evaluate the role of dynorphin and NKB signaling on the onset of puberty. Higher dose of senktide or more potent other NK3R agonists may overcome prepubertal restraint of GnRH/LH secretion. It is also needs to be addressed that several queries such as effective doses, stability in circulation, and binding affinity for its receptors. Future investigations are required to uncover those issues.

The present results may lead to a chance to apply a KOR antagonism and/or NK3R agonism to restore GnRH/LH secretion for patients bearing a hypogonadotropic hypogonadism. Exogenous gonadotropin therapy, the current major approach for human infertility, bears the risk of hyperstimulation of gonadal function, such as a multiple follicular development leading to cycle cancellation, ovarian hyperstimulation syndrome, and multiple pregnancies, all of which require an intense monitor of hormonal profiles. Administration of pulsatile GnRH is another therapeutic method for human infertility with a low risk of a multiple pregnancies. However, this method could be borne by patients because of its methodological complexity, such as maintenance of an infusion pump attached to an intravenous or subcutaneous catheter. Thus, chronic administration of a KOR antagonist or NK3R agonist with a sustained-release capsule could be an alternative therapeutic approach for stimulating gonadal function based on the physiological function of GnRH/LH pulse generation.

In conclusion, the present study suggests that the inhibitory input of dynorphin on the ARC KNDy neurons plays a role in the prepubertal restraint of GnRH/LH secretion in normal developing female rats. The present study also suggests that attenuation of dynorphin-KOR signaling and increase in NKB-NK3R signaling trigger the onset of puberty in normal developing female rats. This study expands our understanding of the mechanism controlling pubertal changes in GnRH/LH pulse generation and provides a therapeutic possibility of KOR antagonism and/or NKB agonism, which

restores GnRH/LH secretion in patients with a disruption in normal pulsatile GnRH/LH secretion.

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408

409

410 **Figure Legends**

411 **Fig. 1.** Advanced puberty onset in female rats treated with nor-binaltorphimine (nor-BNI), a
412 kappa-opioid receptor antagonist, or senktide, a neurokinin 3 receptor agonist. Vaginal openings,
413 vaginal smears and body weights were monitored daily. Ages at the vaginal opening and first estrus
414 (expressed as a percentage of the total number of animals per experimental group) are shown in
415 panels A and B. Ages at the vaginal opening and first estrus are shown in panels C and D.
416 Numbers in each column indicate numbers of animals used. Values are means ± SEM. Values with
417 different letters are significantly different ($P < 0.05$, one-way ANOVA followed by Tukey's HSD test)
418 from each other in each panel.

419

420 **Fig. 2.** Body size at the puberty onset in female rats treated with nor-binaltorphimine (nor-BNI), a
421 kappa-opioid receptor antagonist, or senktide, a neurokinin 3 receptor agonist. Vaginal openings,
422 vaginal smears and body weights were monitored daily. Growth curves and timing of vaginal
423 opening and of first estrus in female rats are shown in panel A. Body weights at vaginal opening and
424 first estrus are shown in panels B and C. Numbers in each column indicate numbers of animals used.
425 Values are means ± SEM. Values with different letters are significantly different ($P < 0.05$, one-way
426 ANOVA followed by Tukey's HSD test) from each other in each panel.

427

428 **Fig. 3.** Advanced increase in plasma luteinizing hormone (LH) levels in female rats treated with a
429 kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI), or a neurokinin 3 receptor agonist,
430 senktide. Plasma LH profiles in representative animals were shown in panel A. Arrowheads
431 indicate peaks of LH pulses identified by PULSAR computer program. Mean LH concentrations
432 and the frequency and amplitude of LH pulses were shown in panel B. The numbers in each column
433 of mean LH concentrations and the frequency of LH pulses indicate the numbers of animals used, and
434 the numbers in each column of the amplitude of LH pulses indicate the number of animals showing
435 LH pulses. Values are means ± SEM. Values with different letters are significantly different ($P <$

436 0.05, one-way ANOVA followed by Tukey's HSD test) from each other. Statistical differences in
437 amplitude of LH pulses were not determined, because only one vehicle-treated animal showed an LH
438 pulse.
439
440 **Fig. 4.** Effect of a kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI), or a neurokinin
441 3 receptor agonist, senktide, on plasma estradiol concentrations and weights of ovaries and uteri at 29
442 days of age. The numbers in each column indicate the numbers of animals used. Values are means
443 ± SEM. Values were not significantly different from each other in each panel.
444

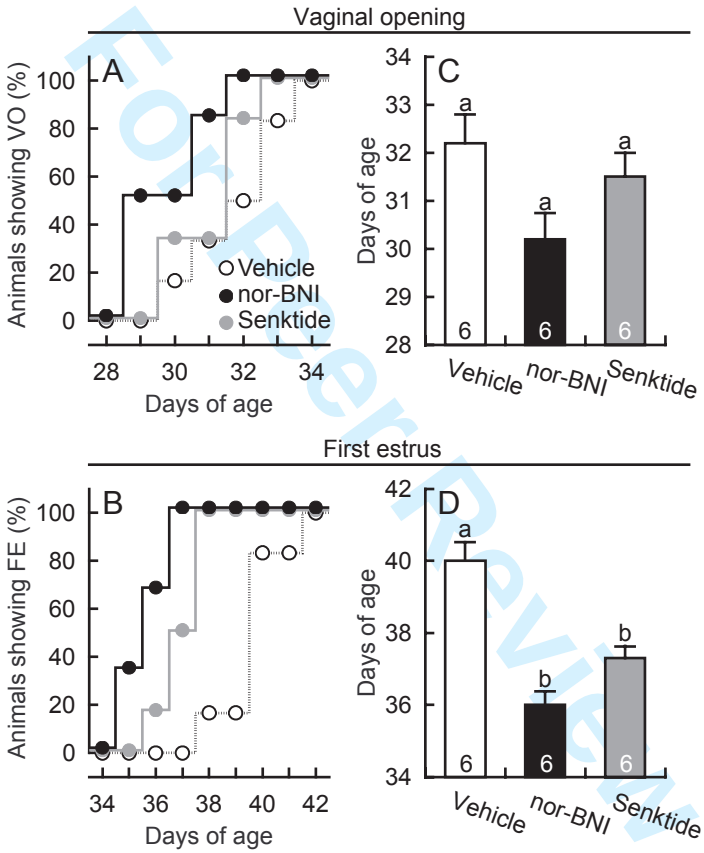

Figure 1, Nakahara et al.

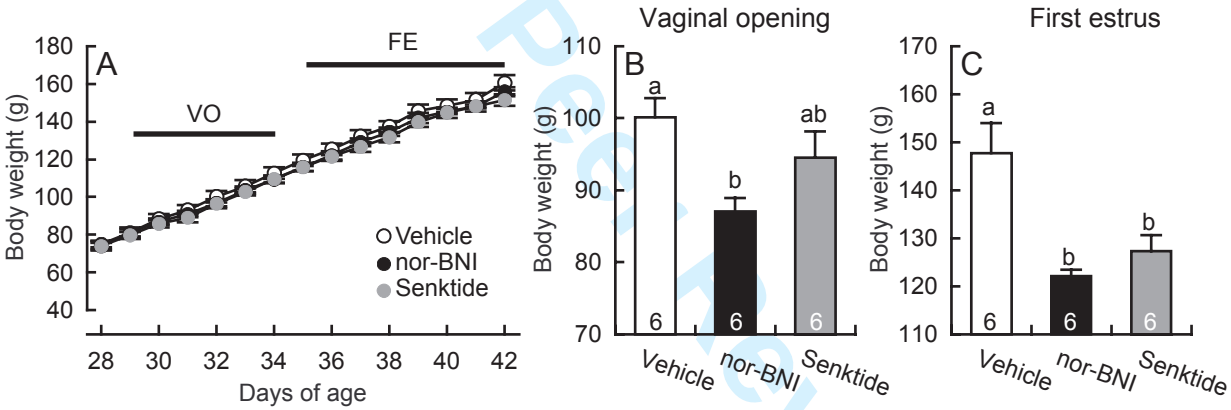

Figure 2, Nakahara et al.

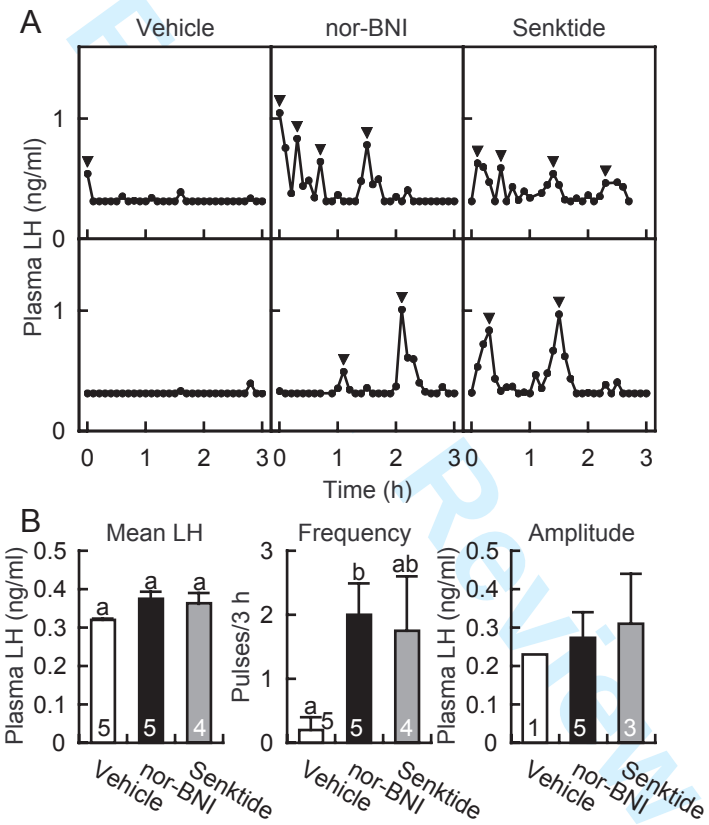

Figure 3, Nakahara et al.

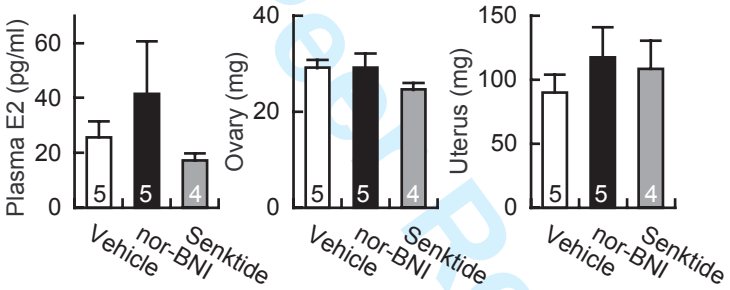

Figure 4, Nakahara et al.