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Effect of neurokinin 3 receptor agonist senktide on the activation of hypothalamic nNOS neurons in cycling female rats

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ABSTRACT

Objective: To examine the effect of an neurokinin 3 receptor (NK3R) agonist, senktide, on neuronal nitric oxide synthase (nNOS) activation in the median eminence-arcuate nucleus (ME-ARC) and preoptic area (POA) regions of the hypothalamus across proestrus, diestrus, and ovariectomized states in female rats and its correlation with luteinizing hormone (LH) secretion.

Methods: Adult female Sprague-Dawley rats were examined for proestrus and diestrus phases of the estrous cycle. Female rats were categorized into proestrus and diestrus groups, and each was further divided into four subgroups ($n=4$). In both the diestrus and proestrus categories, Group 1 was the control group. Groups 2, 3, and 4 received senktide ($100 \mu\text{g}/\text{kg}^{-1}$), NK3R antagonist SB222200 ($10 \text{ mg}/\text{kg}^{-1}$), and SB222200 followed by senktide, respectively. To evaluate the effect of sex steroids on NK3R agonist-induced nNOS activation, female rats underwent bilateral ovariectomy and were divided into four groups ($n=3$). Group 1 served as the control. Group 2 received a subcutaneous injection of 17β -estradiol 3-benzoate (E2, $3 \mu\text{g}/\text{rat}$). Group 3 received E2 and progesterone ($30 \mu\text{g}/\text{rat}$). Group 4 was administered senktide ($100 \mu\text{g}/\text{kg}$). Female rats from each group were sacrificed, blood was collected for LH ELISA, and hypothalamic tissues were collected for Western blotting.

Results: Senktide increased nNOS phosphorylation in the ME-ARC during both the proestrus and diestrus phases. In the POA, senktide increased nNOS phosphorylation only during the diestrus phase. In ovariectomized rats, senktide activated nNOS independent of sex steroid levels. Senktide also increased serum LH concentration in diestrus and ovariectomized female rats.

Conclusions: Senktide, an NK3R agonist, activates nNOS in the POA and ME-ARC regions of the hypothalamus in a phase dependent manner. The activation of nNOS by senktide suggests a potential mechanism by which neurokinin B triggers nNOS activation in the ARC and POA regions and regulates GnRH/LH secretion.

KEYWORDS: Neurokinin B; Neuronal nitric oxide synthase; Luteinizing hormone; Neurokinin 3 receptor; Ovariectomized; Preoptic area; Median eminence-arcuate nucleus

Key Points

Question: Does senktide, an neurokinin 3 receptor agonist, activate neuronal nitric oxide synthase (nNOS) in specific hypothalamic regions and modulate luteinizing hormone (LH) secretion in cycling and ovariectomized female rats?

Results: Senktide administration activated nNOS in the median eminence-arcuate nucleus (ME-ARC) and preoptic area (POA) hypothalamic regions of cycling female rats in phase dependent manner and in ovariectomized female rats independent of steroid hormones. Increased serum LH concentration was observed after senktide treatment in diestrus and ovariectomized female rats.

Meaning: Senktide activates nNOS in the ME-ARC and POA regions in a phase-dependent manner, influencing LH secretion in cycling female rats, stimulating nNOS and increasing serum LH levels in ovariectomized female rats independent of sex steroids hormones.

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1. Introduction

Reproduction is a highly sophisticated physiological process regulated by coordination of the hypothalamus-pituitary-gonadal (HPG) axis[1]. Various neuronal and non-neuronal cell populations regulate puberty onset at the central nervous system level and make organisms competent for reproduction. Gonadotrophin-releasing hormone (GnRH) is vital for making organisms capable of reproduction and is secreted by GnRH neurons located primarily in the preoptic area (POA) of the hypothalamus[2]. These neurons are the final common targets of multiple hormonal and neuropeptide signals for the secretion of GnRH from the median eminence (ME) and onset of puberty[3].

GnRH secreted from these neurons causes the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. The trophic hormones LH/FSH act on their target organs, the gonads (ovaries and testes), and are essential for the regulation of ovulation, folliculogenesis, and spermatogenesis in the normal reproductive cycle. GnRH neurons are not directly responsive to most signals required for normal reproductive cycles[4]. Kisspeptin neurons in adjacent areas transmit exogenous and endogenous signals to GnRH neurons and regulate GnRH secretion during different phases of the estrous cycle[5]. Kisspeptin neurons in rodents are located in two regions of the hypothalamus, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), which regulate the bimodal secretion (tonic and surge) of GnRH[6,7]. ARC Kiss1 neurons co-express kisspeptin, neurokinin B (NKB), and dynorphin, known as KNDy (Kisspeptin/Neurokinin-B/Dynorphin) neurons[8]. Kiss1 neurons from the AVPV and Kiss1 ARC (KNDy) neurons play an essential role in GnRH surge (ovulation) and in the generation of GnRH pulses (folliculogenesis), respectively[9]. During diestrus phase, a low estradiol level provides negative feedback to KNDy neurons. This leads to the release of NKB, which acts through its neurokinin 3 receptor (NK3R) within the KNDy circuits, and leads to the secretion of kisspeptin from KNDy neurons, resulting in the initiation of GnRH pulses required for folliculogenesis and spermatogenesis[10,11]. Loss of function mutations in *TAC3* (encoding NKB) and *TAC3R* (encoding NK3R) result in hypogonadotropic hypogonadism and abnormal pubertal development in humans[12]. *Tac1/Tac2* knockout male and female mice showed low LH pulse frequency, delayed puberty, and no signs of puberty, respectively[13]. *Tacr3*^{-/-} mice exhibit irregular estrous cycles, decreased uterine volume, reduced testicular weight, and reproductive impairments[14]. NKB's critical roles have been well documented in various species through multiple studies on the regulation of LH pulses[15]. Sex steroid hormones provide negative (low estradiol levels) and positive (high estradiol levels) feedback to these neuronal populations during different phases of the reproductive cycle for the standard release of GnRH[16]. Neuronal nitric oxide synthase (nNOS) neurons play an

essential role in GnRH secretion[17]. nNOS neurons are crucial for generating pre-ovulatory surges and are present in the POA and ARC regions of the hypothalamus[17,18]. Most nNOS neurons are closely associated with the cell bodies of GnRH neurons and dendrites in the medial preoptic nucleus (MePO)/organum vasculosum laminae terminalis (OVLT) of the hypothalamus[19]. nNOS immunoreactivity was also found in the ARC region of mice[19] and rats[20,21], where KNDy neurons are present. Kisspeptin modulates the activity of nNOS during various phases of the estrous cycle[2]. AVPV kisspeptin neurons during estradiol positive feedback cause phosphorylation at the Ser-1417 active site of nNOS and lead to its activation, which helps initiate the GnRH surge[22]. Nitric oxide (NO) has also been found to regulate the activity of GnRH neurons in the ME region of the hypothalamus[23]. The release of NO from the ME region plays an important role in the regulation of GnRH release from the GnRH neuron terminals region[23]. The complete loss of Nos1 catalytic activity in mice results in infertility and hypogonadism[24]. The mechanism of GnRH pulse and surge generation is under the strict control of sex steroid hormones. Impairment in the sex steroid feedback mechanism is associated with endocrine pathologies such as polycystic ovarian syndrome (PCOS)[25] and infertility[26]. The prenatal androgen-treated (PNA) mouse model of PCOS showed that elevated levels of androgen that directly act on KNDy neurons disrupted the progesterone-mediated negative feedback to these neurons[25]. The critical role of KNDy and nNOS neuronal populations in normal physiology and neuroendocrine pathologies, together with the presence of nNOS neurons in the ME-ARC and POA areas, underscores the importance of investigating the interaction between NKB and nNOS during the different phases of the estrous cycle.

In this study, we investigated whether senktide (an NK3R agonist) could activate nNOS in the POA and ME-ARC during the proestrus and diestrus phases of the estrous cycle and help understand the role of NKB in the activation of nNOS in the ME-ARC and POA regions during these phases in female rats, as well as their correlation with LH secretion. In this study, we also explored the role of sex steroid hormones in the NKB-nNOS interaction regulating LH secretion in the ME-ARC and POA region of the hypothalamus.

2. Methods

2.1. Experimental design

To investigate the effects of NKB, senktide was administered during the proestrus and diestrus phases in adult female rats. To investigate whether nNOS activation is influenced by the NKB/NK3R signaling pathway in the ME-ARC and POA regions, we administered SB222200 to rats during both the proestrus and diestrus

phases. Senktide is a selective agonist of NK3R receptor. A recent study provided evidence that interactions between the N-terminal region of senktide and the second extracellular loop (ECL) of NK3R are responsible for potent activation of NK3R[27]. SB222200 is a selective potent antagonist of the NK3R[28]. To determine whether senktide induces nNOS phosphorylation *via* NK3R while excluding alternative pathways, one group was administered SB222200, followed by senktide injection. During the diestrus and proestrus phases of female rats, the phosphorylation of nNOS was detected using a specific antibody against Ser-1417 phosphorylation of nNOS in the ME-ARC and POA regions of the hypothalamus during the diestrus and proestrus phases of the estrous cycle.

2.2. Animals

Sprague–Dawley female rats aged 2–3 months, weighing 250–300 g, were used in the experiments. The rats were given a 14-day acclimatization period before the study began. The animals were housed in a room with a controlled photoperiod (12 h of light and 12 h of darkness) and temperature (21 °C–23 °C), with *ad libitum* access to food and water.

2.3. Assessment of estrous cyclicity

Daily vaginal lavage samples were collected between 8:30 a.m. and 9:30 a.m. to monitor the various phases of the estrous cycle. Through microscopic observation, the proestrus phase is identified by the presence of round nucleated cells, whereas the abundance of cornified epithelial cells distinctly characterizes the estrous phase. The diestrus phase shows prominent leukocytes and a few epithelial and cornified cells. Female rats that showed consistency across two consecutive estrous cycles were selected for experimental investigations.

2.4. Drugs

Senktide (1068) was purchased from Tocris Bioscience. SB222200 (S5192), 17 β -Estradiol 3-benzoate (E8515), progesterone (P0130), and sesame oil (S3547) were purchased from Sigma-Aldrich. Tocris bioscience and Sigma Aldrich is the manufacturer.

2.5. Antibodies

The mouse monoclonal anti-nNOS antibody (#sc-5302; 1:300) was obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-phosphorylated nNOS antibody (Ser1417; #bs-19301R; 1:200) procured from Thermo Fisher Scientific. Alexa Fluor 568 conjugated anti-mouse secondary antibody (1:500) was used for nNOS immunolabeling, and Alexa Fluor 488-conjugated anti-rabbit

secondary antibody (1:500) was used for P-nNOS immunolabeling. Rabbit polyclonal anti-nNOS (#PA3-032; 1:500), mouse polyclonal anti-GAPDH (#MA-515738; 1:1000), and rabbit polyclonal anti-phospho-nNOS (Ser1417; PA1-032; 1:1 000) antibodies were purchased from Thermo Fisher Scientific. Horseradish Peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies (1:8 000) were purchased from Thermo Fisher Scientific and was used for Western blotting.

2.6. Protein extraction

The female rats were euthanized by decapitation following treatment. After rapid removal of the brain, the meninges and optic chiasm were carefully removed. The POA and ME-ARC regions of the hypothalamus were dissected using a dissecting microscope and micro-dissecting scissors. After dissection, tissue fragments from each region were placed into microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80 °C. Protein extracts were prepared by successive homogenization of the tissue fragments using 22- and 26-gauge needles in tissue extraction lysis buffer containing protease and phosphatase inhibitors (#MSSAFE). Following homogenization, tissue lysates were clarified by centrifugation at 12000×g for 15 min at 4 °C. Protein concentrations in the lysates were determined using Bradford assay. Protein samples for Western blotting were prepared by combining them with a 2X sample loading buffer, boiled for 5 min, and then stored at -80 °C until further use.

2.7. Immunofluorescence

The animals were deeply anesthetized with an overdose of ketamine (200 mg/kg) and transcardially perfused with phosphate buffer saline (PBS), followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and immersed in the same fixative overnight at 4 °C. The brain was immersed in 20% and 30% sucrose until it sank into the solution and then stored in optimal cutting temperature (OCT) embedding medium at -80 °C until cryosection. Free-floating coronal sections of the brain (25 μ m thick) containing the POA and ME-ARC regions were cut using a cryo-microtome (CM-1520; Leica). Sections were then collected in PBS (ice-cold) and incubated for 1 h in blocking buffer [PBS containing 5% normal goat serum[27] and 0.3% Triton X-100] at room temperature. After blocking, the sections were incubated for 72 h with mouse monoclonal anti-nNOS and rabbit polyclonal anti-phosphorylated nNOS (Ser-1417) antibodies prepared in blocking solution at 4 °C. Prior exposure to secondary antibodies, Alexa Fluor 488 and 568 conjugated anti-rabbit and anti-mouse antibodies in a blocking solution for 1 h at room temperature, the sections were thoroughly rinsed four to five times in PBST and PBS at room temperature. After secondary antibodies incubation and washing with PBST and PBS, the sections

were mounted on glass slides and coverslipped with mowiol (81381; Sigma Aldrich). Double immunofluorescence images were acquired using an Olympus confocal microscope (FV1200). Fluorescent images were prepared using Fluoview software (FV-10; 4.2) using digitalized images acquired with a 20× objective (NA, 0.5).

2.8. Western blotting

The protein samples were thawed, reboiled for 5 min, and electrophoresed at 100 V in 4%-10% SDS-polyacrylamide gels using a mini tetra cell system (Bio-Rad). After size separation, proteins were transferred to polyvinylidene difluoride membranes (PVDF) with 0.45 µm pore-size (#1620177) at 30V overnight. Following protein transfer, the membranes were blocked in blocking buffer [TBS with 0.1% Tween 20 (TBST) and 5% fat-free milk] for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with the appropriate primary antibody diluted in the blocking buffer. Before exposure to horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocking buffer for 1 h at room temperature, the membranes were washed with 0.1% TBST and TBS. Proteins were visualized using an ECL detection kit (#1705061; Bio-Rad). For stripping and reprobing, the membranes were in a stripping solution (62.5 mM Tris-HCl, 2% SDS, pH 6.7, and 100 mM β-mercaptoethanol) with gentle rocking at 65 °C for 30 min. An HRP-conjugated secondary antibody was used to detect all former immunoreactivities. Immunoblots were visualized using the Bio-Rad ChemiDoc system, and the intensities of the bands were measured using ImageJ software.

2.9. Treatments

2.9.1. Senktide (NK3R agonist)

Diestrus ($n=4$), proestrus ($n=4$), and ovariectomized (OVX, $n=3$) female rats received a single intraperitoneal injection (100 µg/kg) of senktide or PBS at 14:00 h. After 90 min, the rats were decapitated and the POA and ME-ARC regions were dissected and processed for Western blotting. Rats underwent deep anesthesia prior to transcardial perfusion with 4% paraformaldehyde (PFA) solution, allowing brain fixation. Subsequently, fixed brains were used for immunofluorescence analysis. The optimal dose of senktide was determined based on the findings of a preceding study[29].

2.9.2. SB222200 (NK3R antagonist) treatment

Diestrus ($n=4$) and proestrus ($n=4$) rats received a single intraperitoneal injection (*i.p.*) of SB222200 (10 mg/kg⁻¹) at 14:00 h. After 30 min, the rats were decapitated and the POA and ME-ARC regions were dissected and processed for Western blotting. In the co-administered group, SB222200 was initially administered,

followed by a subsequent injection of senktide after 30 min. After an additional 90 min, the rats were decapitated for isolation of the POA and ME-ARC regions. The optimal dose of SB222200 was determined based on findings from a previous pharmacokinetic and pharmacological study[28].

2.9.3. Ovariectomy and hormone treatments

Female rats were bilaterally ovariectomized (day 0) under anesthesia by injection of ketamine (60 mg/kg⁻¹) and xylazine (10 mg/kg⁻¹). The animals were divided into four groups. The first group ($n=3$) of animals was killed at 14:00 h on day 17, without receiving any treatment. The second group ($n=3$) received a single subcutaneous injection of 17 β-Estradiol 3-benzoate (E2) (3 µg/rat) at 10:00 h, 15 days after ovariectomy, and were killed at 14:00 h, two days later. Group 3 ($n=3$) was injected with E2+Progesterone (P4, 30 µg/rat) and sacrificed at 14:00 h on day 17. Trunk blood samples were collected for the LH assay. The ME-ARC and POA regions were isolated and subjected to Western blotting. This protocol was standardized in a previous study[30] to assess the role of estrogen in the phosphorylation of nNOS in the hypothalamic areas of adult cycling female rats.

2.10. Hormone assay

For serum isolation, blood was collected from each group of animals, allowed to clot at room temperature for 20 min, and centrifuged at 1500×*g* for 15 min. Serum was collected and stored at -80 °C. The serum concentration of LH was analyzed using LH ELISA KIT (# E-EL-R0026; ELAB SCIENCE) with a sensitivity of 0.94 mIU/mL and <10% coefficient of variation.

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data were compared using one-way analysis of variance (ANOVA) and *post-hoc* Tukey and Dunnett tests for multiple comparisons against the control group. Data between the two groups were compared using Student's *t*-test. All data are represented as mean±standard deviation (mean±SD). All significant differences in the data are represented as $P<0.05$.

2.12. Ethics statement

All experiments were performed in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) of the CUPB and MRSPTU university (approval No. MRSPTU/IAEC/2021/14) on date 22-3-2021 regarding mammalian research.

3. Results

3.1. Effect of senktide on the phosphorylation of nNOS in the ME-ARC region during the diestrus and proestrus phases

During diestrus phase, using immunofluorescence techniques, we observed an increase in nNOS phosphorylation within the ME-ARC after senktide treatment [Figure 1A(V)] when compared to the control group [Figure 1A(II)]. Upon analyzing the phosphorylation of nNOS during diestrus phase using immunoblot techniques, we observed increased phosphorylation at the active site Ser-1417 of nNOS in the senktide-treated group compared to the control group in the ME-ARC region, as shown in Figure 2A. Interestingly, female rats treated with SB222200 during the diestrus phase did not show significant changes in nNOS phosphorylation in the ME-ARC region of the hypothalamus compared to the control group (Figure 2A). The SB222200 and senktide co-administered group did not show significant changes in nNOS phosphorylation compared to the control group, highlighting the role of NKB-mediated nNOS phosphorylation during diestrus phase in the ME-ARC region.

During proestrus phase, using immunofluorescence techniques, we observed an increase in nNOS phosphorylation within the ME-ARC after senktide treatment [Figure 1B(V)] when compared to the control group [Figure 1B(II)]. Immunoblot analysis revealed increased phosphorylation of nNOS in the senktide-treated group compared to that in the control group (Figure 2B). Interestingly, SB222200 treatment also resulted in increased phosphorylation in the ME-ARC region compared to that in the control group during proestrus phase, as shown in Figure 2B. Rats co-administered SB222200 and senktide during proestrus phase did not show any significant change in nNOS phosphorylation compared with the control, as shown in Figure 2B. These findings indicate that senktide facilitates nNOS phosphorylation in the hypothalamic ME-ARC region during the diestrus and proestrus phases.

3.2. Effect of senktide on the phosphorylation of nNOS in the POA region during the diestrus and proestrus phases

In our immunofluorescence study, increased phosphorylation of nNOS was observed during diestrus phase after senktide treatment

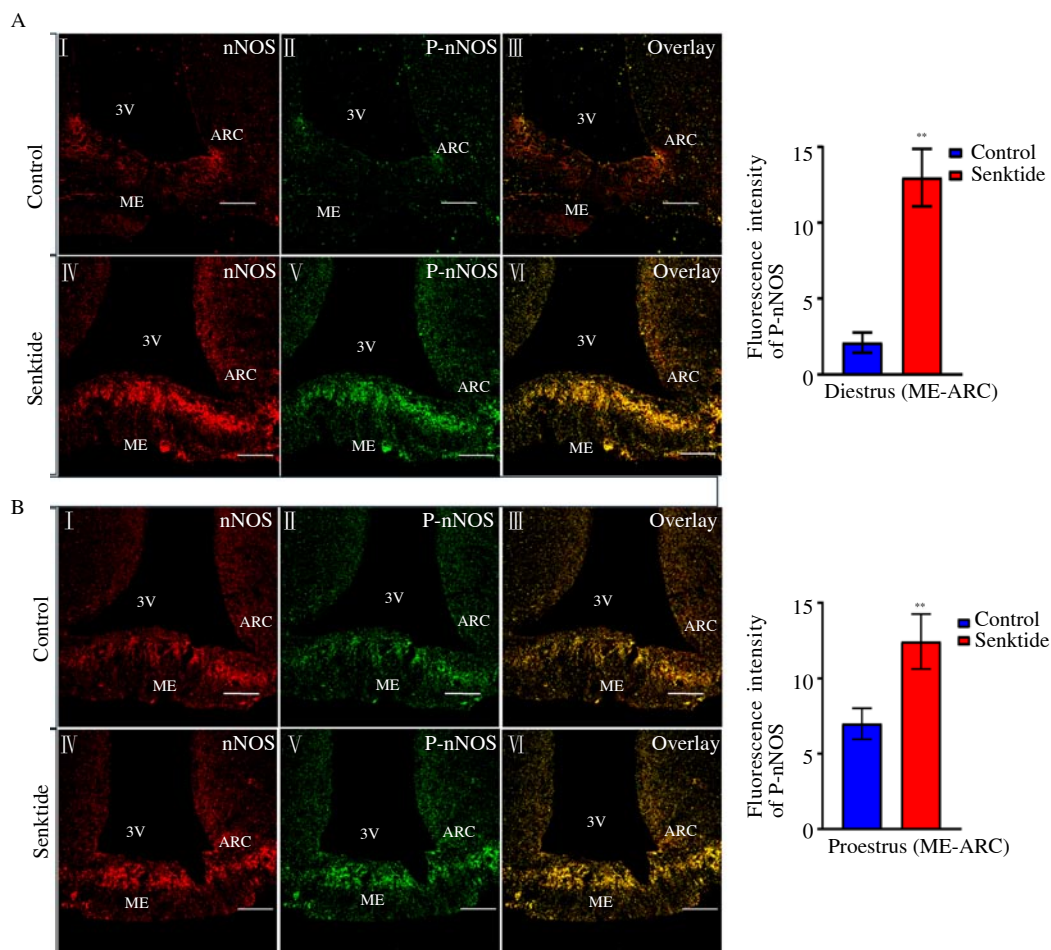


Figure 1. Immunofluorescence images and bar graphs show the effect of senktide on the phosphorylation of nNOS (P-nNOS) in the median eminence-arcuate nucleus (ME-ARC) region of the hypothalamus during the diestrus (A) and proestrus (B) phases in adult cycling female rats. ARC: arcuate nucleus of the hypothalamus; ME: median eminence; 3V; third ventricle; nNOS: neuronal nitric oxide synthase. Scale bar=100 μ m. ** P <0.01: compared to the control group.

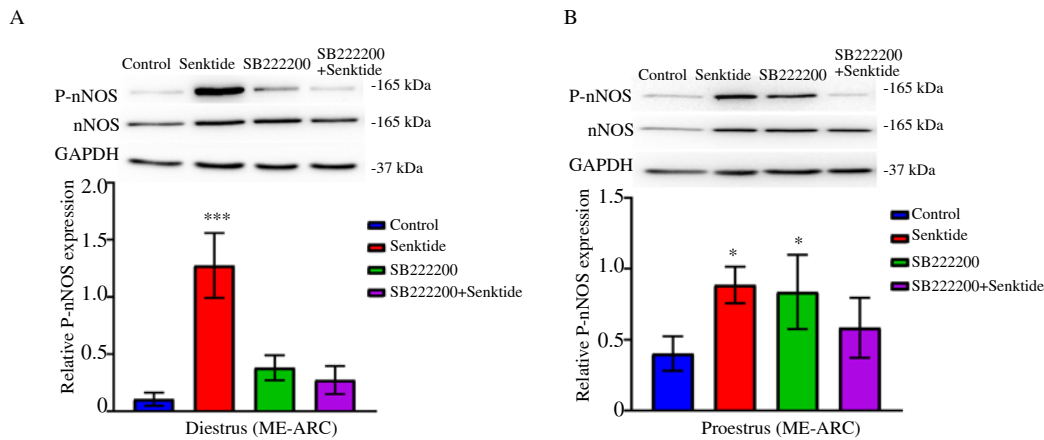


Figure 2. Immunoblots show the effect of senktide on the phosphorylation of nNOS (P-nNOS) in the median eminence-arcuate nucleus (ME-ARC) region of the hypothalamus during diestrus (A) and proestrus (B) phases of female rats. * $P < 0.05$, *** $P < 0.001$: compared to the control group. nNOS: neuronal nitric oxide synthase. P-nNOS is normalized against GAPDH.

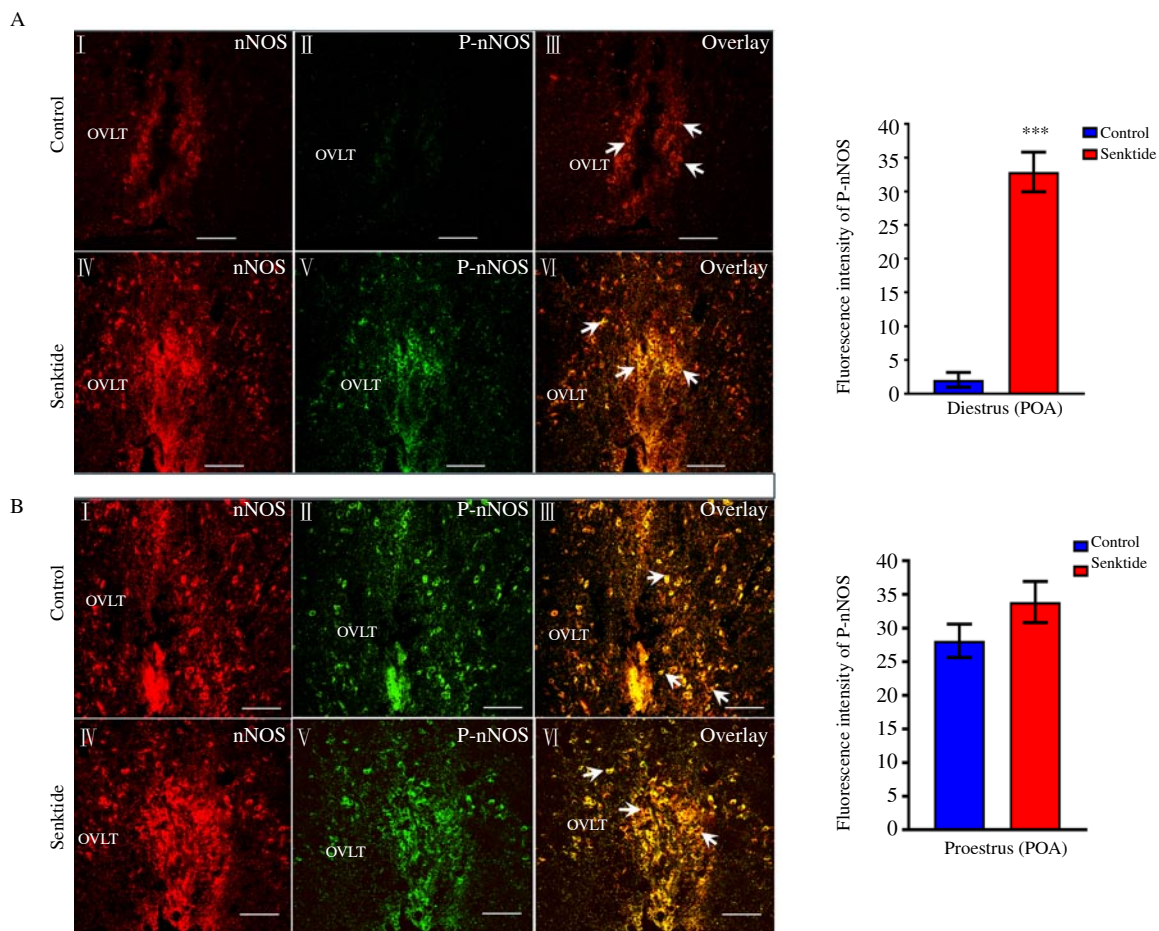


Figure 3. Immunofluorescence images and bar graphs show effect of senktide on the phosphorylation of nNOS (P-nNOS) in the OVLT region of the hypothalamus during the diestrus (A) and proestrus (B) phases in adult cycling female rats. OVLT: organum vasculosum of the lamina terminalis; POA: preoptic area; nNOS: neuronal nitric oxide synthase; Scale bar=100 μm . *** $P < 0.001$: compared to the control group.

[Figure 3A(V)] compared to the control group [Figure 3A(II)]. in the POA region. Subsequent Western blotting revealed that senktide administration resulted in a significant increase in the phosphorylation of nNOS during diestrus phase compared with the control group, as shown in Figure 4A. No significant change in

nNOS phosphorylation was observed between the SB222200-treated group and the control group. Furthermore, the group receiving both SB222200 and senktide did not show significant variance in nNOS phosphorylation compared to the control group during the diestrus phase in the POA region, as shown in Figure 4A.

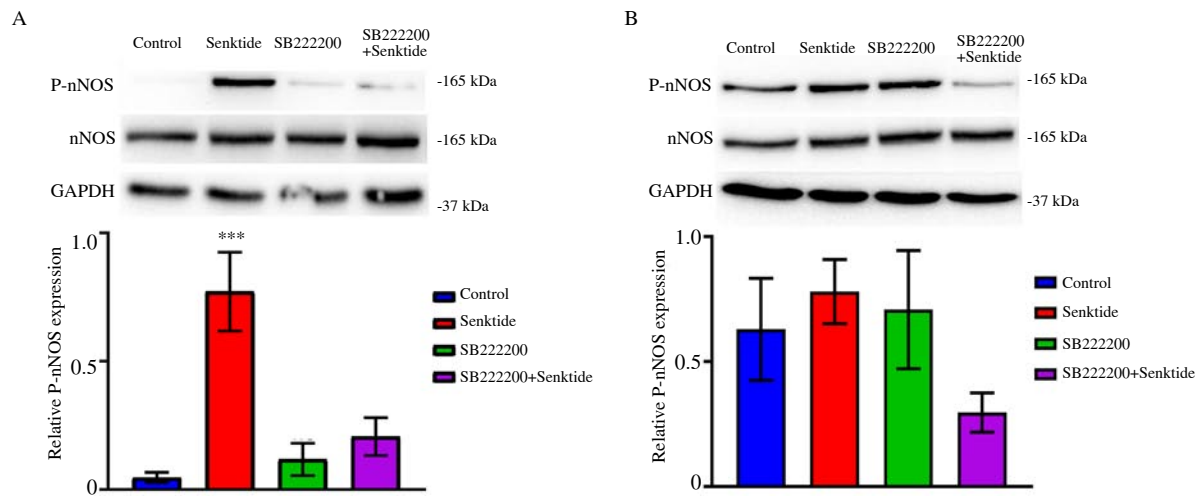


Figure 4. Immunoblots show the effect of senktide on the phosphorylation nNOS (P-nNOS) in the preoptic area (POA) region of the hypothalamus during the diestrus (A) and proestrus (B) phase of female rats. *** $P < 0.001$: compared to the control group. nNOS: neuronal nitric oxide synthase. P-nNOS is normalized against GAPDH.

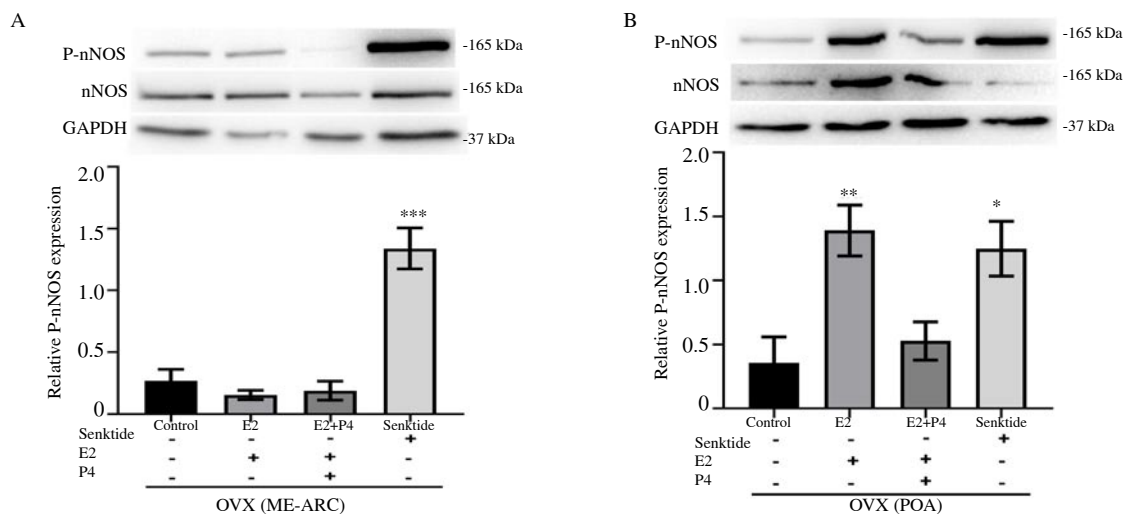


Figure 5. Immunoblots show the effect of senktide in ovariectomized (OVX) female rats on phosphorylation nNOS (P-nNOS) in the ME-ARC (A) and POA (B) region of the hypothalamus, respectively. ME-ARC: median eminence-arcuate nucleus; POA: preoptic area. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: compared to the control group. nNOS: neuronal nitric oxide synthase; E2: 17 β -Estradiol 3-benzoate; P4: progesterone. P-nNOS is normalized against GAPDH.

Similarly, during proestrus phase our immunofluorescence study found no change in nNOS phosphorylation between the control group [Figure 3B(II)] and the senktide treated group [Figure 3B(V)] in POA region. In subsequent investigation using Western blotting during proestrus phase, we observed no changes in nNOS phosphorylation between the control group and the senktide-treated group, as shown in Figure 4B. No significant variation in the nNOS phosphorylation was found in the SB222200 and senktide co-administered group compared to the control group (Figure 4B).

3.3. Effect of senktide on the phosphorylation of nNOS in the ME-ARC and POA regions in OVX female rats

The role of steroid signaling in senktide-induced phosphorylation at

the active site Ser-1417 of nNOS in the POA and ME-ARC regions of the hypothalamus was investigated. Senktide increased nNOS phosphorylation compared to the control in the ME-ARC region of OVX female rats. No changes in nNOS phosphorylation were observed after treatment with E2, E2 and P4 co-administered groups compared to the control group in the ME-ARC region (Figure 5A).

In the POA area, immunoblot analysis showed increased nNOS phosphorylation after treatment with senktide and E2 compared to that in the control group. Co-administration of E2 and P4 showed no variance in nNOS phosphorylation compared to the control group (Figure 5B). Our results indicated that senktide in the POA and ME-ARC, independent of sex steroid hormones, activates nNOS.

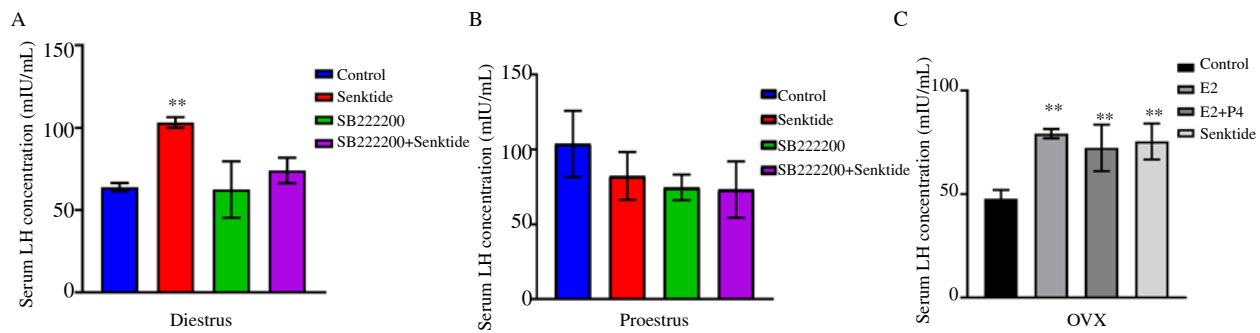


Figure 6. Bar graphs in (A, B) show the effects of senktide treatment on serum luteinizing hormone (LH) concentration in female rats during the diestrus (A) and proestrus (B) phases. The bar graphs in (C) represent serum LH concentration after the treatment of senktide in ovariectomized (OVX) female rats. E2: 17 β -Estradiol 3-benzoate; P4: progesterone.

3.4. Effect of senktide on serum LH concentration in diestrus, proestrus, and OVX female rats

We conducted serum sample analysis using ELISA to examine the impact of senktide-induced nNOS phosphorylation on LH secretion. An increase in serum LH concentration was found after senktide treatment during diestrus phase compared to that in the control. After treatment with SB222200 and co-administration of SB222200 and senktide, there was no significant change in serum LH concentration compared to that in the control group during diestrus phase, as shown in Figure 6A. During proestrus phase, there were no significant changes in serum LH concentrations between the control and treated groups in adult female rats, as shown in Figure 6B.

Furthermore, we investigated whether NKB-induced phosphorylation depended on the sex steroid hormone milieu. Bilaterally, OVX female rats were administered E2, P4, and senktide. LH concentration increased in the senktide, E2, E2 and P4 co-administered groups in OVX female rats compared to the control group, as shown in Figure 6C.

4. Discussion

Previous studies have mainly focused on the involvement of neuropeptides, particularly kisspeptin, and their interactions with other regulators on the HPG axis[2,6,31–33]. Furthermore, we investigated the significance of NKB (using senktide), which serves as a crucial regulator of the pulsatile secretion of GnRH/LH. Our study investigated the role of NKB in nNOS activation in the ME-ARC and POA regions of the hypothalamus during the proestrus and diestrus phases of the estrous cycle. Using immunohistochemistry and Western blotting, we showed that senktide during the diestrus and proestrus phases is involved in promoting phosphorylation at the active site Ser-1417 leading to the activation of nNOS in the POA and ME-ARC regions of the hypothalamus. During the diestrus phase, under the influence of sex steroid hormones NKB released from the KNDy neurons, acts *via* NK3R within the KNDy-

KNDy circuits in an autocrine and paracrine manner, releasing kisspeptin from the KNDy neurons[34]. NKB plays a modulatory role on KNDy neurons by adjusting their resting potential and increasing the latter's firing rate[35]. The NKB-NK3R interaction makes these changes possible in the intrinsic cation channels of the KNDy neuron, a form of modulation that has been established[10]. As a result, this change causes calcium (Ca^{2+}) oscillations to be initiated within these neurons[10]. These oscillations are particularly important in orchestrating the concerted activity of KNDy neurons and subsequent release of neuropeptides during diestrus phases, including NKB, kisspeptin, and dynorphin. Kisspeptin operates *via* its Kiss1R present in the GnRH neuron's terminals region in the ME-ARC region of the hypothalamus and results in the pulsatile release of GnRH into the fenestrated portal blood capillaries[36]. Fragments extracted from the ME of female rats demonstrated an episodic release of nitric oxide[37]. An earlier study provided evidence that during diestrus, the level of nitric oxide is low and increases to a maximum during proestrus in the afternoon[37]. nNOS neurons in the ME-ARC region are closely associated with kisspeptin fibers, where KNDy neurons are present, raising the possibility of NKB-mediated regulation of nNOS in the ME-ARC region[38]. This cascade of events plays a pivotal role in the control of the pulsatile release of the GnRH. The role of NKB and its interactions with nNOS in the POA and ME-ARC regions during proestrus and diestrus is not well known in context to the GnRH/LH secretion.

During diestrus phase both in the ME-ARC and POA regions treatment with senktide results in the activation of the nNOS. Senktide treatment significantly increases the phosphorylation of the nNOS compared to the control group during diestrus phase in ME-ARC and POA regions. No significant changes in the phosphorylation of nNOS compared to the control after treatment with SB222200 further confirmed the stimulatory action of senktide on nNOS phosphorylation. In the co-administered group, SB222200 blocked the stimulatory effect of senktide on nNOS phosphorylation, providing further evidence of senktide-mediated activation of nNOS during diestrus phase in the ME-ARC and POA regions.

No changes in the nNOS phosphorylation after the SB222200 and co-administered group (SB222200 and senktide) compared to the control groups during diestrus provide strong evidence for the involvement of the NKB/NK3R signaling promoting nNOS activation in the POA and ME-ARC region during diestrus phase. Interestingly, increased phosphorylation of the nNOS after the senktide in the POA highlights the role of the NKB not only confined to ME-ARC but also in the POA that can regulate LH secretion during diestrus phase. Increased LH concentration after the treatment of the senktide compared to the control group during diestrus confirms the role of the NKB-mediated nNOS activation in LH secretion.

During proestrus, earlier study showed that AVPV kisspeptin and nNOS neurons regulate GnRH in POA region of the hypothalamus[2]. During proestrus phase, kisspeptin released from AVPV Kiss1 neurons leads to activation of the nNOS neurons *via* Kiss1R present in nNOS neurons[2,18]. In the POA area, the role of the NKB and nNOS interaction is unexplored during the proestrus phase. During proestrus, our results showed that senktide promotes the phosphorylation of nNOS in the ME-ARC region compared to the control. In POA during proestrus treatment of senktide has no significant effect on the nNOS phosphorylation compared to the control group. Our results showed that during the proestrus phase, SB222200 treatment does not decrease nNOS phosphorylation compared to the control group in the POA and ME-ARC suggesting the involvement of the other pathways. Similarly, the co-administration does not have significant effect on the phosphorylation of nNOS in the POA and ME-ARC region compared to the control group during proestrus. It is well known that kisspeptin-mediated phosphorylation of nNOS during proestrus phase is highest prior to ovulation. No significant changes in serum LH concentration were found after the treatment of the NK3R agonist and antagonist further suggesting no role of NKB-mediated nNOS activation in GnRH/LH secretion during proestrus in female rats.

Sex steroids provide continuous feedback to the Kiss1 neurons and the nNOS neuronal population during the reproductive cycle[2]. Impaired negative feedback mechanisms and fertility were observed after ablation of the estrogen receptor alpha (ER) ablation, mainly in the ARC region of the hypothalamus[39]. The kisspeptin-specific ER knockout mice (KERKO) do not exhibit LH surge in mice[40]. Our results have shown that in OVX rats senktide independent of the sex steroid milieu can activate the nNOS in the ME-ARC and POA regions. Treatment with senktide in OVX rats suggests that NKB-induced phosphorylation of nNOS increases LH secretion independent of sex steroid levels.

There are some limitations. Our study is limited to female animals and given the potential for sex-specific differences in NKB-nNOS signaling and its downstream effects; the absence of male data represents a gap in our understanding that warrants further

exploration. Our analysis was restricted to selected hypothalamic regions, specifically, the ME-ARC and POA regions. It remains unclear whether other brain regions involved in reproductive neuroendocrine regulation are affected similarly. Although associations among NKB signaling, nNOS activation, and LH secretion have been identified, causal relationships remain to be fully established. Future investigations employing various pharmacological and molecular techniques will be crucial in exploring these mechanisms in greater depth, potentially leading to the development of novel therapeutic interventions for neuroendocrine disorders and a more comprehensive understanding of GnRH surge and pulse dynamics.

In conclusion, this study provides valuable insights into the complex interplay between NKB, nNOS, and regulation of GnRH/LH secretion during different phases of the estrous cycle. Our findings demonstrate that senktide, an NK3R agonist, activates nNOS in the POA and ME-ARC regions of the hypothalamus in a phase-dependent manner. The activation of nNOS by senktide during diestrus suggests a potential mechanism by which KNDy neurons, *via* NKB, could trigger nNOS activation in the ARC and POA regions, thereby regulating GnRH/LH secretion. The observed increase in serum LH concentration following senktide treatment further supports NKB's involvement of NKB in LH release during the diestrus phase.

Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

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Data availability

The data of the current study is available from the corresponding author on a reasonable request.

Authors' contributions

Jyoti Parkash and Vikash Prashar contributed to the conception and design. Vikash Prashar conducted material preparation, experiments, data collection, and analysis. The original manuscript draft was written by Vikash Prashar and reviewed by all authors. Tania Arora and Randeep Singh assisted with sample collection and draft writing. Arti Sharma assisted in data analysis, writing, supervision, and review of the manuscript. Jyoti Parkash contributed resources, provided supervision, and reviewed and approved the final manuscript.

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