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Experimental Research

Immunofluorescent Imaging of Kisspeptin in Some Brain Regions in Different Stages of Sexual Cycles of Female Mice

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ABSTRACT

Objective: The aim of the study was to determine kisspeptin intensity by immunofluorescence method in hypothalamus arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) and ventromedial nucleus (VMN) in the diestrus, proestrus and estrus phases of the sexual stages of female mice.

Material and Method: In the study, 40 female Balb/c mice aged 5-6 months with an average weight of 35-40 grams were used. All female mice were cycled by vaginal smear with an average of 3 oestrus cycles. Total of 21 animals with regular cycles were selected and divided into 3 groups according to estrus phases (diestrus, proestrus, estrus and groups, n =7). Animals were decapitated without administering any anesthetic and then brain tissues were frozen on dry ice. Sections were taken from the brain tissue and fluorescent staining steps were applied. At the end of the study, kisspeptin density in ARC, PVN, DMN and VMN nuclei of the hypothalamus was calculated by immunofluorescence method.

Results: It was found that the intensity of kisspeptin higher significantly in the oestrus group compared to the diestrus group in ARC, DMN and VMN. When the intensity of kisspeptin in the PVN was statistically evaluated, insignificant difference was found among the groups. In the DMN and VMN regions, when the estrus and proestrus groups were compared to the diestrus group, the intensity of kisspeptin higher significantly.

Conclusion: It was determined that kisspeptin intensity was different in hypothalamic ARC, DMN, VMN and PVN regions at estrous, diestrus and proestrus stages of female mice.

Keywords: *Hypothalamus, Immunofluorescent, Kisspeptin, Sexual Cycles.*

ÖZ

Dişi Farelerin Cinsel Döngülerinin Farklı Aşamalarında Bazı Beyin Bölgelerinde Kisspeptinin İmmünofloresan Görüntülemesi

Amaç: Bu çalışma ile dişi farelerin cinsel dönemlerinin östrus, diöstrus ve proöstrus fazlarında hipotalamusun arkuat nükleus (ARC), paraventriküler nükleus (PVN), dorsomedial nükleus (DMN) ve ventromedial nükleus (VMN) bölgelerinde kisspeptin ekspresyonunun immünofloresan yöntemi ile belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmada, 40 adet ortalama ağırlığı 35-40 gram olan 5-6 aylık Balb/c ırkı dişi fare kullanılmıştır. Tüm dişi farelere, ortalama 3 östrus döngüsü olacak şekilde vajinal smear ile siklus takibi yapılmıştır. Düzenli siklus gösteren toplam 21 adet hayvan seçilmiş ve östrus fazlarına göre 3 gruba ayrılmıştır (diöstrus proöstrus, östrus ve grupları olmak üzere, n =7). Hayvanlara, herhangi bir anestezik madde uygulamadan dekapite edilmiştir ve ardından beyin dokuları kuru buzda dondurulmuştur. Çalışmanın sonunda, hipotalamusun ARC, PVN, DMN ve VMN çekirdeklerinde kisspeptin yoğunluğu immunofloresan yöntemle hesaplanmıştır.

Bulgular: ARC'de östrus grubunun diöstrus grubuna göre kisspeptin yoğunluğunun istatistiksel olarak anlamlı derecede arttığı bulunmuştur. PVN'de kisspeptin yoğunluğu, istatistiksel olarak değerlendirildiğinde gruplara göre anlamlı bir farklılık bulunmamıştır. DMN ve VMN bölgelerinde, östrus ve proöstrus grupları diöstrus grubuyla kıyaslandığında kisspeptin yoğunluğunun istatistiksel olarak anlamlı derecede arttığı bulunmuştur.

Sonuç: Dişi farelerin östrus, diöstrus ve proöstrus dönemlerinde hipotalamik ARC, PVN, DMN ve VMN bölgelerinde kisspeptin yoğunluğunun farklı olduğu tespit edilmiştir.

Anahtar Sözcükler: *Hipotalamus, İmmünflorasan, Kisspeptin, Cinsel Siklus.*

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Kisspeptin was defined as a high affinity RF-amide (Arg-Phe-NH2) peptide ligand for the G-protein coupled receptor 54 (GPR54) encoded by the kiss-1 gene in 2001 (1). Kisspeptin - kisspeptin receptor signaling is key to reproduction in mammals. Kisspeptin consists of four peptide families (Kisspeptin-54, -14, -13 and -10) formed by proteolytic cleavage from a common precursor protein. All kisspeptins specifically bind to the kisspeptin receptor (Kiss1r) known as G-protein-coupled receptor-54 (GPR54) (2), activating it (1, 3), and mutations of the receptor cause hypogonadotropic hypogonadism (4, 5). Kisspeptin stimulates GnRH secretion via Kiss1r in GnRH neurons in the hypothalamus. With the secretion of GnRH, kisspeptin controls the activity of the hypothalamus-pituitary-gonadal axis (HHG) and as a result, it regulates reproductive system functions in many ways, including the onset of puberty, estrus cycle and spermatogenesis (6-8).

The GPR54 receptor was first discovered in rats in

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1999. While this receptor is highly expressed in the hypothalamus, preoptic area (POA), hippocampus, midbrain, amygdala and medulla, limbic system and basal ganglia in the brain (1, 9, 10), it is expressed in placenta, pituitary, pancreas, heart, muscle, kidney, liver, lung, thymus, intestine and testicles (11). Hypothalamus located in the ventral part of the forebrain plays an important role in the regulation of energy homeostasis, fluid balance, stress, growth, reproductive behavior, emotion, and circadian rhythms (12). In the hypothalamus, there are several important regions with well-known anatomical and physiological features such as paraventricular nucleus (PVN), dorsomedial nucleus (DMN), arcuate nucleus (ARC), ventromedial nucleus (VMN) (13). These nuclei have important roles in the regulation of food intake and appetite, hunger-satiety states, stress, body's energy metabolism and homeostatic regulation of body weight (13).

The neuroanatomical distribution of the kisspeptin-expressing cell populations is conserved among mammalian species. Kisspeptin-expressing neurons are mainly located in two different hypothalamic regions in the brain, namely the rostral periventricular region (RP3V) of the third ventricle and the ARC (14-16). Expression of kisspeptin in ARC has also been demonstrated in different species such as mouse (16-18), rat (19), hamster (20) and human (21).

The second largest kisspeptin-positive cell population is localized in RP3V (RP3V, anterior ventral periventricular region (AVPV) and periventricular nucleus in mice (16, 18, 22), rats (15, 19), hamsters (20), and humans (21). (PeN). These two hypothalamic regions are regulated differently by testosterone and estradiol, both during the growth period and in adulthood (17, 22).

Kisspeptin immunoreactive fibers are also found in DMN, ARC and PVN and send projections to preoptic areas (23, 24). It has been shown that GPR54 is synthesized in the medial preoptic area (MPOA), DMN, ARC and lateral hypothalamic region by the in situ hybridization method (9, 24, 25). However, changes in kisspeptin expression in female mice at different stages of the sexual cycle are not well known. For this purpose, in this study, it was aimed to investigate the expression intensity of kisspeptin in the brain regions that have important roles in the regulation of reproduction, energy balance and metabolism in different stages of estrus cycles of female mice by immunofluorescence method.

MATERIAL AND METHOD

Experimental Animals

In the study, 21 adult Balb / c breed female mice with an average weight of 35-40 grams were used. Experimental studies were carried out in the University Experimental Research Center and the University Faculty of Medicine, Department of Physiology.

The mice were housed in standard cages in rooms that were kept constant at temperature (22-25 ° C) and relative humidity (40-55%) in Firat University Experimental Research Center, ventilated and a 12-hour light/dark cycle was applied. The photoperiod change was made with an automatic time adjuster at 07:00 in the morning in a bright phase. The mice were fed with special rat feeds in pellet form (Korkutelim Yem Gıda San. Tic. AŞ., Korkuteli/ANTALYA). The water requirement of the animals was met with tap water in bottles placed in special sections in the cages with droppers at the ends.

The cycles of the female mice were followed by vaginal smear method for 15 days (average 3 estrus cycles) between 08:00 and 12:00 every day. Cycle follow-up was started from the day the vaginal opening was seen. The mice that showed regularity periodically during the cycles were divided into 3 groups with 7 animals in each group:

Diestrus (n = 7)
Proestrus (n =7)
Estrus (n =7)

The metaestrus group was not included in the study group because it was defined as a transition period in the first period of the first day of diestrus (diestrus 1).

Immunfluorecent Analysis

Taking Brain Sections

Mice in all three groups were decapitated during the specified sexual periods without any chemical agent application. Brain tissues were quickly removed and frozen in dry ice. 18 micrometer sections were taken from the frozen brains in the cyrostat device (Leica). Sections were taken using the mouse brain atlas (26) to cover the areas of interest. The sections taken were glued to the slides and the slides were dried on a heating plate at 40 ± 5ºC for 30 minutes.

Fluorescent Imaging

Brain sections were fixed in ethanol at -20°C for 15 minutes. The brains whose fixation was completed were shaken for 3x5 minutes in Phosphate Buffer Solution (PBS) at 80 RPM on a shaker. Blocking Solution prepared with 5% Bovine Serum Albumin (BSA) (Sigma) and PBS-T was added to each brain at 30 microliters (µl) and shaken at 80 RPM for 1 hour at room temperature. Then primary antibody (Proteintech) was added and incubated at 80 RPM overnight (approximately 12-16 hours) at 4°C.

The next day, the sections were washed in PBS for 3x5 min at 80 RPM on the shaker. Secondary antibody (Jackson Immuno Research) was added and incubated at 80 RPM for 1 hour at room temperature. After secondary antibody, sections were washed 3x5 min with PBS on a shaker. Core dye (DAPI, 4',6 Diamidine-2-Phenylindole Dihydrochloride) was added and treated in the dark for 5 minutes. After core staining, sections were washed with PBS for 5 min at 80 RPM. After the sections were dried on the slide in the dark, they were covered with Gel / Mount fluid preservative and covered with coverslip. Images were examined and photographs taken at 10X magnification under the ZEISS

fluorescence microscope. Density analysis of the relevant regions was made.

Statistical Analysis

Statistical analysis of the results of the study was performed using the SPSS 23.0 for Windows program. Data are given as Mean ± Standard error (Mean ± SEM). Kruskal-Wallis test was used to evaluate the data. In all analyses, p-value was less than 0.05 were considered statistically significant.

RESULTS

Female mice whose cycle periods were determined by vaginal smear were divided into groups in the phases of diestrus, proestrus and estrus. Among the determined groups, the mean ± SEM values of kisspeptin concentrations in the ARC, PVN, DMN and VMN regions of the hypothalamus, respectively, are shown in table 1.

Table 1. *Mean ± SEM values of kisspeptin concentration in hypothalamic nuclei in diestrus, proestrus and estrus groups.*

	ARC	PVN	DMN	VMN
Grups	**Mean±SEM**	**Mean ±SEM**	**Mean ±SEM**	**Mean ±SEM**
Diestrus	155,70±8,06	279,78±38,64	162,12±8,66	167,67±8,56
Proestrus	194,97±10,19	247,47±25,19	228,87±7,85*	217,95±10,22*
Estrus	230,83±25,82**	291,00±21,32	271,30±32,49***	222,25±26,00*

** p <0.05, ** p <0.01 and *** p <0.001 compared to the diestrus group.*

Arcuate Nucleus

The mean ± SEM values of kisspeptin intensity between groups in the arcuate nucleus are shown in table 1. It is observed that kisspeptin intensity higher significantly in estrus group compared to diestrus group (p <0.01). Although there is no statistically significant difference between diestrus and proestrus groups, it is seen that the intensity of kisspeptin in proestrus is high (Figure 1, 2). Similarly, although there is no statistically significant difference between estrus and proestrus groups, it is seen that the intensity of kisspeptin in estrus is higher than that of prooestrus (Figure 1, 2).

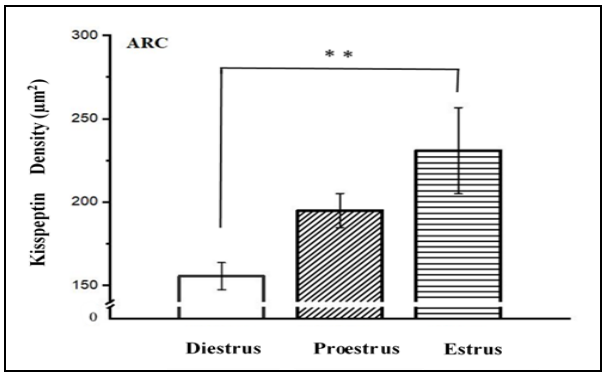


Figure 1. *Kisspeptin density in the arcuate nucleus in the diestrus, proestrus and estrus groups.*

*** p <0.01 compared to the diestrus group.*

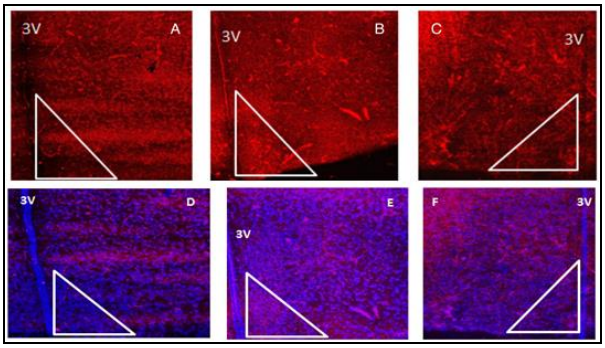


Figure 2. *A, D-Diestrus; Kisspeptin immunoreactivity in Arcuate nucleus in B, E-proestrus and C, F-estrus groups. Magnification A-E; 10X. Kisspeptin immunoreactivity (Red fluorescent, Cy3), DAPI: Blue Fluorescent, 3V: Third Ventricle.*

Paraventricular Nucleus

The mean ± SEM values of kisspeptin density between groups in the paraventricular nucleus are shown in table 1. Although there is no statistically significant difference in kisspeptin density in PVN compared to the groups, it is seen that it is the lowest in the proestrus group, and it is similar in the diestrus and estrus groups (Figure 3, 4).

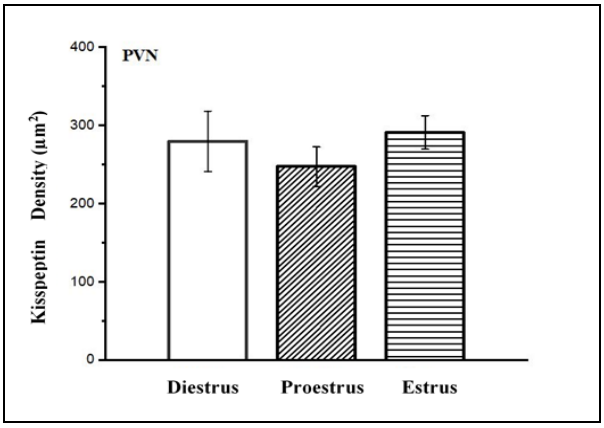
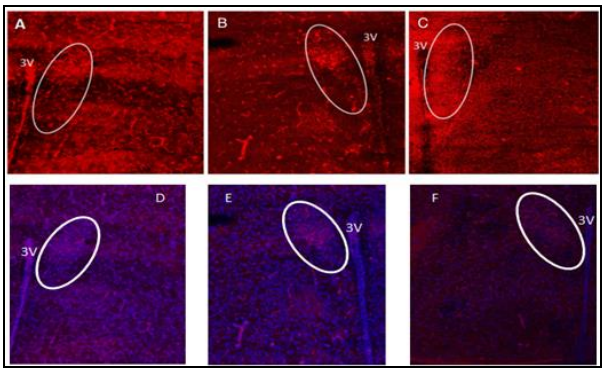


Figure 3. *Kisspeptin density in the diestrus, proestrus and estrus groups in the paraventricular nucleus.*



Fgure 4. *A, D-Diestrus; Immunoreactivity of kisspeptin in paraventricular nucleus in B, E-proestrus and C, F-estrus groups. Magnification A-E; 10X. Kisspeptin immunoreactivity (Red fluorescent, Cy3), DAPI: Blue Fluorescent, 3V: Third Ventricle.*

Dorsomedial Nucleus

The mean ± SEM values of kisspeptin intensity between groups in the dorsomedial nucleus are shown in

table 1. Kisspeptin density higher significantly in the estrus group compared to diestrus ($p < 0.001$), similarly, kisspeptin intensity higher significantly in the proestrus group compared to the diestrus group ($p < 0.05$). There was no statistically significant difference between the proestus and estrus groups (Figure 5, 6).

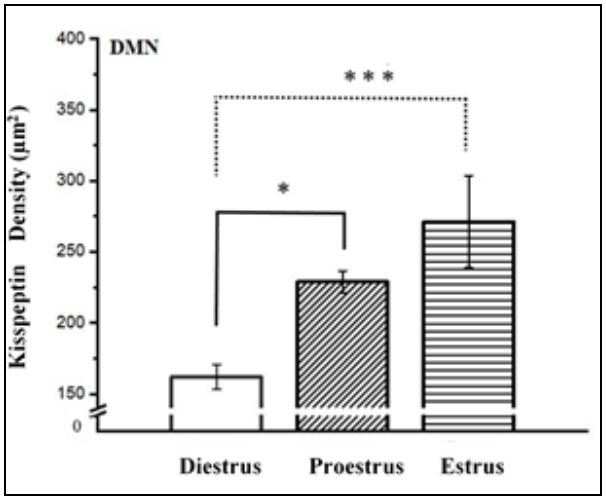


Figure 5. *Kisspeptin density in the diestrus, proestrus and estrus groups in the dorsomedial nucleus. (DMN; Dorsomedial nucleus).*
** p <0.05 ve *** p <0.001, compared to the diestrus group.*

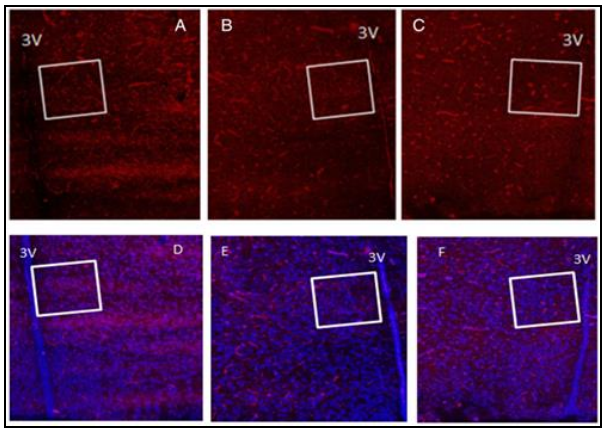


Figure 6. *A, D-Diestrus; Immunoreactivity of kisspeptin in dorsomedial nucleus in B, E-proestrus and C, F-estrus groups. Magnification A-E; 10X. Kisspeptin immunoreactivity (Red fluorescent, Cy3), DAPI: Blue Fluorescent, 3V: Third Ventricle.*

Ventromedial Nucleus

The mean ± SEM values of kisspeptin intensity between groups in the ventromedial nucleus are shown in table 1. Kisspeptin intensity higher significantly in both proestrus and estrus groups compared to diestrus group ($p < 0.05$) (Figure 7, 8).

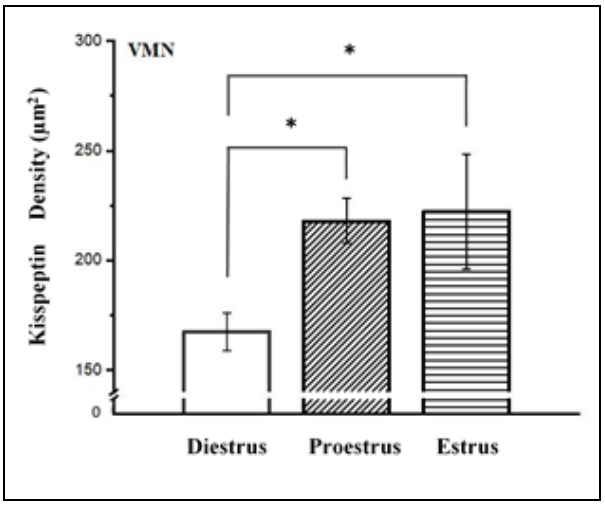


Figure 7. *Kisspeptin density in the diestrus, proestrus, estrus groups in the ventromedial nucleus.*
** p <0.05, compared to the diestrus group.*

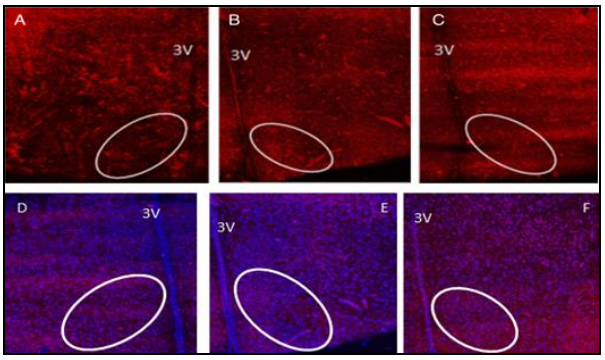


Figure 8. *A, D-Diestrus; Immunoreactivity of kisspeptin in ventromedial nucleus in B, E-proestrus and C, F-estrus groups. Magnification A-E; 10X. Kisspeptin immunoreactivity (Red fluorescent, Cy3), DAPI: Blue Fluorescent, 3V: Third Ventricle.*

DISCUSSION

Since kisspeptin is an important regulator of the hypothalamus-pituitary-gonadal axis, it is of great importance to examine the modulation of this system in different sexual periods. Kisspeptin expressions in four important brain regions in female mice in different estrus cycles were investigated by immunofluorescence method.

The arcuate nucleus is an important brain region in the basomedial hypothalamus that regulates food intake and energy consumption, where kisspeptin expression is most common (27). It has been shown that kisspeptin expression in ARC is necessary for pulsatile release of GnRH, and it has been observed that the application of kisspeptin antagonist to ARC decreases the LH pulse frequency (28) and LH release is stimulated when kisspeptin is applied to ARC (29). Studies show that kisspeptin neurons in ARC can be a neuronal pacemaker source that provides pulsatile release of GnRH (16, 30). In this study, it was observed that the intensity of kisspeptin was significantly higher in the estrus group in female mice compared to the proestrus and diestrus groups in ARC. The high estrogen levels dur-

ing the proestrus and oestrus periods of the female mice and low estrogen levels explain the changes in the intensity of kisspeptin in this study. It can be said that estrogen higher gradually starting from estrus to proestrus and estrus causes an higher in kisspeptin density in ARC in parallel. In addition, the higher in kisspeptin may have triggered the formation of the LH peak, leading to ovulation. In parallel with this study, it was observed that maximal responses to kisspeptin occurred in estrus (31).

In the study conducted on Spraque Dawley female rats, it has been shown that the expression of Kiss1 in ARC is regulated in the opposite way in AVPV. In ARC, Kiss1 mRNA peaks towards the end of the diestrus, while it is lowest in the proestrus and estrus stages (14). These studies yielded results contrary to our findings. The reason for this may be that the study is done in different types and with different methods. As a matter of fact, when looking at the literature, kisspeptin expression also varies according to species. For example, the demonstration of less peptide storage in cell bodies in rats compared to mice may reflect the different dynamics between both species in kisspeptin release (32). Another difference between mice and rats has been demonstrated in the kisspeptinergic cell population in the DMN. Unlike mice, no kisspeptin immunoreactive cells could be detected in the DMN region in rats in proestrus (32, 33). Whether this reflects a true species difference, a particular physiological arrangement or methodological differences between studies need to be investigated.

As shown in this study in ARC, which is an important brain region where food intake and energy balance are regulated, the change in the density of kisspeptin at different cycle stages suggests that kisspeptin may have a role in the change of food intake and energy consumption at these stages. However, food intake and energy regulation have not been investigated since it is not one of the main objectives of this study. However, in our study, it can be said that the changes that occur in the brain regions related to food intake may change in different estrus cycles, and this may be through leptin, kisspeptin and other orexigenic and anorexigenic hormones.

Kisspeptin fibers extend from cell bodies in RP3V and ARC to major GnRH neurons and different hypothalamic areas. Among them, PVN is one of the main targets of this system (34, 35). In different studies, it has been shown that PVN is intensely innervated with kisspeptin fibers in rodents (23, 33, 35). Within the hypothalamus, PVN is a region with different neuronal populations that play important roles in neuroendocrine / autonomic regulation and control of energy balance, and is a critical regulatory center for energy homeostasis (36, 37). Although PVN is not a very important region in the regulation of the HHG axis, it is thought that following the application of kisspeptin-54 to PVN, the stimulation of the HHG axis is mediated by interneurons extending to preoptic GnRH neurons. It has been reported that this region shows much less neuronal activation in response to peripheral kisspeptin compared to other regions of the hypothalamus, especially the preoptic nucleus (38). Further studies are needed to identify kisspeptin-sensitive neurons in PVN and investigate their relationship with GnRH neurons in other areas of the hypothalamus. Although GPR54 expression is not specifically reported in PVN, it is known that kisspeptin immunoreactive fibers and cell bodies and GnRH immunoreactive neurons are present in this region (23, 39). In the present study, no significant difference was observed in the intensity of kisspeptin in the different phases of the estrus cycle in the PVN region. The fact that the difference is not seen in accordance with the literature supports that it does not have an important role in the HHG axis. However, the presence of kisspeptin positive fibers spread throughout the PVN suggests that kisspeptin may play a role in the regulation of many physiological activities other than reproduction controlled by PVN. However, the presence of kisspeptin positive fibers spread throughout the PVN suggests that kisspeptin may play a role in the regulation of many physiological activities other than reproduction controlled by PVN.

It has been shown in an in situ hybridization study that Kiss1r is co-expressed in subpopulations of oxytocin (OT) neurons in the medial part of the PVN in female rats in diestrus (36). Oxytocin neurons in PVN have been shown to be projected from kisspeptin neurons in RP3V where kisspeptin expression is high in proestrus (2, 36, 40, 41) and oxytocin higher lordosis behavior in rats (42) and there is a significant higher in PVN Kiss1r expression in proestrus. These effects can higher female receptive behavior. Oxytocin higher lordosis behavior in rats (42) and the presence of kisspeptin fibers in PVN in proestrus (40) suggest that kisspeptin may be associated with receptive behavior in female rats through modulation of oxytocin secretion (36). Although there is no significant difference in the intensity of kisspeptin in the PVN region in this study, more studies are needed to reveal whether kisspeptin release and kisspeptin amplify the effects of oxytocin in the oxytocin-producing regions of PVN.

In a study conducted in female rats, higher expression of kisspeptin in estrus was observed in PVN (14). Similar to this study, higher kisspeptin concentration in estrus was observed in PVN in this study. The higher concentration of kisspeptin in estrus suggests that it may lead to receptive behavior in the female associated with oxytocin neurons in this area.

Another hypothalamic core that plays an important role in the regulation of energy homeostasis and stress is DMN. Although direct application of kisspeptin-10 to DMN does not cause any change in circulating LH and testosterone, this indicates that kisspeptin, its input to this core, does not play a role in the activity of the HHG axis (29).

Kisspeptin immunoreactivity was found in some cells in DMN in a study conducted on female mice in diestrus (33, 43). In this study, significant differences were observed in the intensity of kisspeptin in different

estrus stages in the DMN region, especially between prooestrus and diestrus stages and between diestrus and estrus stages. Seeing these differences suggests that DMN may be related to different functions. Especially in estrus, higher kisspeptin concentration compared to other stages may be related to stress and energy metabolism in animals. Unlike mice, no kisspeptin immunoreactive reaction was observed in DMN in a study in female rats in proestrus. The relatively high level of GPR54 mRNA in this region indicates that kisspeptin may have other roles independent of reproduction (43).
A subgroup of neurons expressing neuronal nitric oxide synthase (nNOS) in the ventrolateral part of the ventromedial hypothalamus and communicating with kisspeptin neurons is shown. In accordance with the knowledge that nitric oxide (NO) is an important neurotransmitter in the effects of kisspeptin neurons, a significant decrease in lordosis behavior has been detected in female mice with impairment in nNOS. The results obtained in the study show that kisspeptin manages both mate preference and sexual motivation in female mice, and sexual behavior and ovulation are coordinated by the same neuropeptide (44). In the current study, kisspeptin density in the ventromedial nucleus, proestrus and estrus groups was found to be higher than in the diestrus group.
Consistent with the study mentioned in the literature, the higher in the intensity of kisspeptin in VMN, especially in the estrus phase of the cycle, suggests that kisspeptin neurons may have a central function that regulates sexual behavior in the female mouse brain, as it is known to affect nitric oxide-synthesizing neurons in the ventromedial hypothalamus.
Consequently, in this study, the intensity of kisspeptin expression in the brain regions that have important roles in the regulation of reproduction, energy balance and metabolism in different stages of the estrus cycles of female mice was demonstrated by immunofluorescence method. Based on the data obtained from the study, the expression of kisspeptin in important nuclei in the hypothalamus shows that it may affect many physiological mechanisms apart from the reproductive axis.

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