UNIVERSITY OF CALIFORNIA SAN DIEGO

Sexual Dimorphism in Kisspeptin and Kiss1R in the Ventrolateral Periaqueductal Gray

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in

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by

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The Thesis of Jacqueline Hernandez is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I'd like to dedicate my thesis to:

My parents, for their sacrifices, endless love, and support.

My siblings, for their joyous energy and comfort.

My friends, for empowering and believing in me.

To the underrepresented communities who have endured multiple adversities to pave the path and opportunity for us to fulfill our dreams.

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LIST OF ABBREVIATIONS

ARC	Arcuate Nucleus
CFA	Complete Freund's Adjuvant
GABA	Gamma Aminobutyric Acid
GIRK	G Protein-Coupled Inwardly-Rectifying Potassium Channel
GnRH	Gonadotropin Releasing Hormone
HA	Hemagglutinin
Kiss1	Kisspeptin
Kiss1R	Kisspeptin Receptor
PAG	Periaqueductal Gray
TRPC	Canonical Transient Receptor Potential Channel
vlPAG	Ventrolateral Periaqueductal Gray
WT	Wildtype

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Pamela L. Mellon, Chair
Professor Deborah Yelon, Co-chair

Chronic pain manifests as a debilitating pathological state that affects up to 10% of the global population. Previous investigations revealed a disproportionate distribution of pain experience between sexes with females reporting a higher prevalence of chronic pain compared to males. The physiological mechanisms underlying the sex difference in pain remains unclear.

The ventrolateral periaqueductal gray is a midbrain region notably involved in modulating opioid activity and nociception, a measurable alternative of pain. Recently, the neuropeptide

kisspeptin and its receptor, Kiss1R, known for their role in the reproductive system, have been generally identified in the periaqueductal gray. Hypothalamic kisspeptin expression is sexually dimorphic and directly controlled by sex steroids. Kisspeptin and Kiss1R have been recently attributed to mediate nociceptive experience. Limited research has investigated their role in the ventrolateral periaqueductal gray and contribution to sex differences in nociception.

This study aimed to explore the relationship of sex steroids, kisspeptin, and Kiss1R in the periaqueductal gray to identify the physiological components underlying the sex difference in nociception. We identified the presence of kisspeptin neurons in the ventrolateral periaqueductal gray with no sex difference in quantity and further revealed the presence of Kiss1R on GABAergic neurons in the ventrolateral periaqueductal gray. Additionally, we discovered a sexual dimorphic pattern of *Kiss1* and *Kiss1r* mRNA expression in the ventrolateral periaqueductal gray (vlPAG), with higher levels in female mice that are not regulated by sex steroids. Our current results suggest that known inducers of vlPAG neuroplasticity, chronic pain and chronic opioid treatment, upregulate and downregulate PAG *Kiss1* and *Kiss1r*. Kisspeptin levels in female mice remains higher than males regardless of these conditions.

Our findings establish the presence, sexual dimorphism, and modulation of kisspeptin and Kiss1R in the ventrolateral periaqueductal gray. We hypothesize that these populations may be contributing to sex differences seen in nociception. Our work further characterizes the regulation and profile of kisspeptin and Kiss1R in the ventrolateral periaqueductal gray that will aid in conducting further studies examining the sex differences of transcriptional mechanisms and neuronal pathways involved in nociception.

INTRODUCTION

Pain has significantly detrimental effects on lifestyle quality and impairs the ability to perform daily activities. Two types of pain include acute and chronic pain [1]. Acute pain is characterized by its short duration and is associated with tissue damage, whereas chronic pain is characterized by its prolonged duration past the healing state and can even occur in the absence of tissue damage [1]. The debilitating condition of chronic pain has led it to be considered a disease state and a health burden to the global population [2]. It is estimated that at least 10% of the world's population experiences chronic pain, where one in ten people develop it every year [3]. Studies reveal a disproportionate distribution of pain experience between genders [4]. A cross-national study reported women to have a higher prevalence of chronic pain compared to men in both developed and developing countries [4,5]. Although the discrepancy of sex difference in pain is not unique to one country, there are still questions remaining regarding the physiological mechanisms underlying the phenomena.

Circulating sex steroids (estrogen and testosterone) vary between the sexes and have been considered a driving source of the sex differences in pain [6]. A study conducted on rodents revealed that females have lower acute nociception thresholds compared to males, causing females to be more sensitive to noxious stimuli [7]. The same study conducted by Bradshaw et al. (2000) associated the presence of high estradiol levels with the lower acute threshold seen in female subjects. This finding illuminates the possible role sex steroids have in eliciting sex differences in pain experience.

To locate potential areas where sex steroids might be altering or influencing pain experience, it is important to draw attention to how and where pain stimuli is processed. Pain

experience is subjective to each individual and is impossible to measure in animal models because it requires the ability to self-report. Instead, nociception is the sensation of noxious stimuli, such as chemical, mechanical, and thermal stimuli, and can be measured in rodents [8]. The brain can regulate incoming afferent nociceptive signals through the descending modulation pathway [9]. This pathway is composed of midbrain neurons in the ventrolateral periaqueductal gray (vlPAG) which project to the brainstem's rostral ventromedial medulla (RVM), which then transmits output to the dorsal horn lamina within the spinal cord [10]. The dorsal horn lamina is the site where incoming nociceptive signals can be reduced or amplified by the RVM [10]. This pathway, and particularly the vlPAG, is also an important site of opioid analgesia [11]. Despite the well documented role of the regions along the descending modulation pathway in nociception, limited research has investigated sex differences or the role of sex steroids in this pathway.

Recently, a peptide known for its role in reproduction has been identified in nociceptive processing. Kisspeptin neurons express a gene called *Kiss1*, which produces a neuropeptide called kisspeptin [12]. Expression of kisspeptin is sexually dimorphic in the hypothalamus where its transcription is directly controlled by the sex steroids of interest, estradiol and testosterone [13]. The kisspeptin neuropeptide is known to stimulate the kisspeptin receptor, Kiss1R [14]. One study has identified kisspeptin in the PAG, and others have shown it is expressed in the spinal cord within the descending modulation pathway in mice [15,16]. Additionally, Kiss1R has been identified in the PAG and spinal cord regions within the descending modulation pathway in rodents as well [16,17]. The presence of kisspeptin and Kiss1R in the descending modulation pathway makes it a candidate for a modulator of nociceptive experience.

Recent evidence identified kisspeptin and Kiss1R to influence nociception. In mice, separate injections of kisspeptin into the paws and spinal cord resulted in pronociception, the

decrease of the nociception threshold resulting in increased sensitivity to noxious stimuli [18]. Central administration of kisspeptin into the brain also elicits pronociception [19]. The study conducted by Spampinato et al. (2011) peripherally delivered a Kiss1R antagonist that resulted in antinociception, the increase of nociception threshold resulting in decreased sensitivity to noxious stimuli, suggesting a modulatory role of Kiss1R in basal nociception. Additional mouse studies centrally administered kisspeptin along with a Kiss1R antagonist resulting in antinociception, therefore blocking kisspeptin's pronociceptive effect and confirming Kiss1R to be the receptor mediating kisspeptin's role in nociception [19]. Although studies have shown the peripheral and central contribution of kisspeptin and Kiss1R in nociception, the central effects of kisspeptin and Kiss1R in the brain are still in need of further investigation. It is also still unclear if kisspeptin and Kiss1R contribute to sex differences in nociception.

Although kisspeptin and Kiss1R have been reported in the PAG, they have never been localized to the vlPAG [15,17]. Additionally, it is still unknown what cell types express Kiss1R in the PAG. It is known that in the vlPAG, GABAergic neurotransmission is pronociceptive whereas glutamatergic neurotransmission is antinociception [20]. It is possible that Kiss1R resides in GABAergic or glutamatergic neurons. Investigations have not been conducted to determine if Kiss1R profiles in the PAG differ between sexes.

The presence of kisspeptin and Kiss1R in the PAG makes the PAG, the most upstream region of the descending modulation pathway, a prime candidate region to investigate whether kisspeptin and Kiss1R expression is sexually dimorphic in the descending modulation pathway.

Sex influences chronic pain experience, but the physiological mechanism remains unclear. This project will conduct experiments aimed towards understanding the relationship between sex, sex steroids, kisspeptin, and Kiss1R in the PAG to understand why there is a sex difference in

nociception. The first aim is to quantify kisspeptin neurons in the vlPAG and determine if the amount of kisspeptin neurons differs between males and females. Transgenic kisspeptin reporter mice will be generated to fluoresce cells that express kisspeptin. The quantification of these cells will be compared between male and female mice. The second aim is to determine if Kiss1R is expressed in GABAergic neurons in the PAG and determine if this profile differs between sexes. Immunohistochemistry will be used to visualized and detect colocalization of Kiss1R with GABAergic neurons in both male and female mice PAGs. The third aim will examine the regulation of *Kiss1* and *Kiss1r* in the whole PAG in both sexes and determine if testosterone and estradiol regulate *Kiss1* and *Kiss1r* expression. The mRNA expression levels of kisspeptin and Kiss1R in the PAG will be measured in adult male and female mice in the absence and presence of sex steroids. The fourth aim will explore how two states associated with vlPAG plasticity, chronic pain and opioid treatment, alter *Kiss1* and *Kiss1r* regulation. The mRNA expression levels of kisspeptin and Kiss1R in the PAG will be compared between a mouse group that is given a chronic inflammation treatment and a control group. For chronic opioid treatment, *Kiss1* and *Kiss1r* mRNA levels will be compared between opioid naïve mice and mice that have been treated with morphine for one week. The overall goal is to bridge the gaps in knowledge between reproductive and nociceptive components to understand the physiological processes underlying sex differences seen in nociception.

MATERIALS AND METHODS

Mice

All animal experiments were performed in accordance with the regulations set by the Institution Animal Care and Use Committee at the University of California, San Diego. All mice were group-housed with a 12-hour light/dark cycle in a temperature-controlled environment and given *ad libitum* access to chow and water. Wildtype (WT) mice for micropunch studies were acquired from various colony lines. Kisspeptin reporter mice were created by crossing Kiss-Cre mice from the Elias Lab [21] with ROSA-td-Tomato reporter mice #007914 from Jackson Laboratory [22]. Tissue from Kiss1-2XHA mice was kindly provided by Dr. Andy Babwah (unpublished). Kiss1-2XHA mice were generated by inserting a 2X HA tag after the start codon of the *Kiss1r* gene with CRISPR technology at Rutgers Genome Editing Shared Resource.

Sex Steroid Treatments

WT female and male mice were gonadectomized (GDX) by ovariectomy in females and castration in males. A subset of GDX female mice were given two injections of estrogen (GDX+E) five and six days after ovariectomy, a paradigm that has been demonstrated to induce a luteinizing hormone surge in females [23]. A subset of GDX male mice received testosterone pellets (GDX+T) implanted subcutaneously with a SILASTIC brand (Dow Corning Corp; internal diameter 1.02 mm; external diameter 2.16 mm) capsule packed with testosterone (Sigma; T1500; 6 mm). These implants have been demonstrated to produce physiological levels of T [24].

Chronic CFA and Morphine treatment

A WT cohort of male and female mice were injected with a 50 µL 1:1 dilution of Complete Freund's Adjuvant (CFA) and sterile saline in the hindpaw. Successful inflammation of the hindpaw was determined by observation. These mice were sacrificed 5 days post injection.

A separate WT cohort of male and female mice were injected intraperitoneally twice a day for 7 days with a 5 mg/kg dose of morphine. Injections occurred 12 hours apart each day. On day 7, mice were sacrificed 1 hour after the second morphine injection.

Brain processing

All brain tissues were collected on dry ice at the time of sacrifice and immediately stored at -80°C. Frozen brains were sectioned into 40 µm thick slices on a cryostat. Brain sections for immunohistochemistry experiments were immediately stored in PBS at -4°C. Brain sections for quantitative PCR (qPCR) were sectioned at 200 µm. 2 mm micropunches were taken from the arcuate nucleus (ARC) [25] and periaqueductal gray (PAG) regions and stored at -80°C.

Immunohistochemistry

Brain sections were processed in a 2-day protocol and light protected during both days. On day 1 of the protocol, brain sections of the periaqueductal gray were washed 4 times with 1X PBS and placed in a warm citra antigen retrieval solution for 10 minutes. Brain sections were then washed 2 times with 1X PBS and placed in 3% H_2O_2 block solution for 10 minutes. Slices were washed twice with 1X PBS and placed in an Avidin and protein block solution for 30 minutes. Brain sections were washed again two times with 1X PBS and placed in a Biotin block solution for 30 minutes. The brain sections were placed in a primary antibody solution with primary antibodies (1:500 rabbit anti-HA, 1:500 chicken anti-GAD67) and stored overnight at 4°C.

On day 2 of the protocol, brain sections were washed four times with 1X PBS and placed in a secondary antibody solution for 1 hour containing 1:300 biotin anti-rabbit. Brain sections were then washed four times with 1X PBS and placed in an ABC kit solution for 1 hour. Sections then underwent tyramide signal amplification. Brain sections were washed four times in 1X PBS and placed in a fluorescent-conjugated streptavidin secondary solution for 30 minutes. Brain sections were washed 4 times with 1X PBS and placed in a streptavidin-conjugated 488 solution for 30 minutes. Slices were washed and then process for 60 minutes in 1:100 goat anti-chicken 647. Brain sections were washed again four times in 1X PBS. All brain sections were mounted and air dried. Sections were then coverslipped with Prolong Gold reagent.

All imaging was performed at the UCSD Nikon Imaging Center on a Ti2E microscope equipped with a laser-scanning confocal. Images were processed using FIJI.

RNA Isolation and cDNA Synthesis

RNA from all brain micropunches was isolated with RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. RNA concentrations were measured using a nanodrop. 500 ng of purified RNA was then converted to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). All cDNA samples were diluted to 1:5 for qPCR analysis.

Quantitative PCR

Every qPCR reaction was made of 8 µL of master mix and 2 µL of 1:5 diluted cDNA. The 8µL of master mix in each reaction consisted of 2 µL of distilled water, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 5 µL of iQ SYBR Green Supermix (Bio-Rad Laboratories). All

cDNA products were detected on a Bio-Rad CFX Connect quantitative real-time PCR system (Bio-Rad Laboratories). The primer sequences used are listed in **Table 1**.

qPCR Analysis

All qPCR reactions were run in triplicates. The Cq values for all three samples in a triplicate were averaged. The standard deviation for each triplicate was calculated and analyzed for outliers if deviation exceeded a value of 0.5. Outliers were identified as any Ct value more than two standard deviations of the mean of the closes two samples, and removed.

The housekeeping genes *H2afz* and *Ppia* were used for Cq normalization of ARC samples, and *Gapdh* was used for Cq normalization of PAG samples. The average Cq value for H2afz and Ppia were averaged together, and the average Cq of the genes of interest were subtracted by the average housekeeping gene Cq previously described. This calculation resulted in a deltaCt. The deltaCt for control animals were averaged. The deltaCt for the genes of interest were subtracted by the average deltaCt from control animals. This resulted in deltadeltaCt values. The average fold change for gene expression was calculated as $2^{-\text{deltadeltaCt}}$. All primer sequences used are listed in **Table 1**.

Statistical Analysis

Two-way ANOVA were used to determine differences between groups. Sidak's multiple comparison test was used for post hoc analysis. Statistics were run in Prism 9 (Graphpad) with $p<0.05$ indicating significance. All data are expressed as the mean +/- SEM.

Table 1: Primer sequences used for qPCR. Forward and reverse primers were used in qPCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Gapdh	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
Ppia	AAGTTCCAAAGACAGCAGAAAAC	CTCAAATTTCTCTCCGTAGATGG
H2afz	TCACCGCAGAGGTACTTGAG	GATGTGTGGGATGACACCA
Kiss	TGCTGCTTCTCCTCTGT	ACCGCGATTCCTTTTCC
Kiss1r	GCACATGCAGACAGTTACC	GCAGCACAGTAGGAAAGTGAC

RESULTS

To quantify and identify potential differences in kisspeptin neuron quantity in the ventrolateral periaqueductal gray between both sexes, Kiss-Cre tdTomato female and male brains were collected and sectioned to visualize fluorescence of kisspeptin neurons in the rostral, middle, and caudal sections of the ventrolateral periaqueductal gray.

Our results reveal no substantial difference in the quantities of kisspeptin neurons in the rostral, middle, and caudal sections of the ventrolateral periaqueductal gray **(Figure 1)**. There was also no observable difference in kisspeptin neuron quantity between females and males **(Figure 1)**. Statistical analysis was not conducted due to low sample size. The current findings suggest that sex differences in nociception may not be attributed to difference in kisspeptin neuron quantity in the ventrolateral PAG.

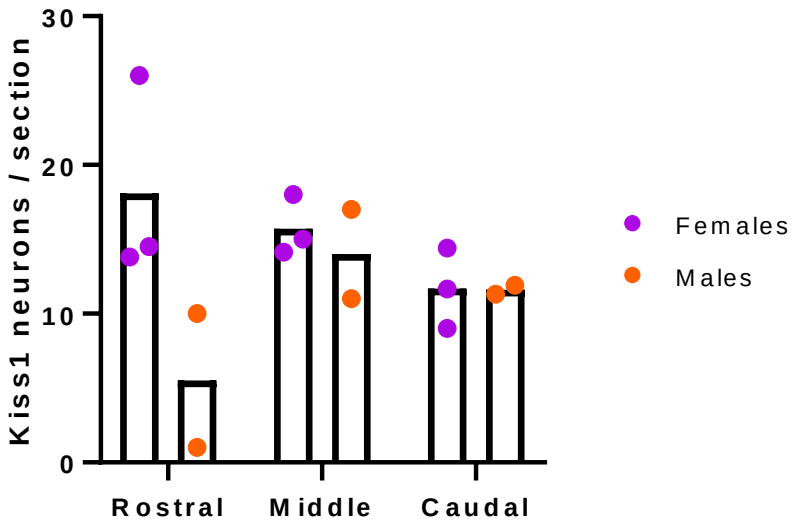

Figure 1. Quantification of kisspeptin neurons in the rostral, middle, and caudal sections of the ventrolateral periaqueductal gray in both females and males. Fluorescence of kisspeptin neurons in Kiss-Cre tdTomato females (purple dots; n=3) and males (orange dots; n=2) were counted. Means are expressed. No statistical test was conducted due to low sample size.

To determine if Kiss1R is present on GABAergic neurons in the ventrolateral periaqueductal gray, Kiss1-2XHA female and male mice brains were coronally sectioned to perform immunohistochemistry staining to localize GABAergic neurons and the kisspeptin receptor (Kiss1R) that possess HA-tags.

Our findings reveal a colocalization of GABAergic neurons and HA in the ventrolateral periaqueductal gray **(Figure 2)**. This colocalization was seen in both male and female mice. This finding establishes that the marker of Kiss1R, HA, is present on GABAergic neurons in the ventrolateral periaqueductal gray.

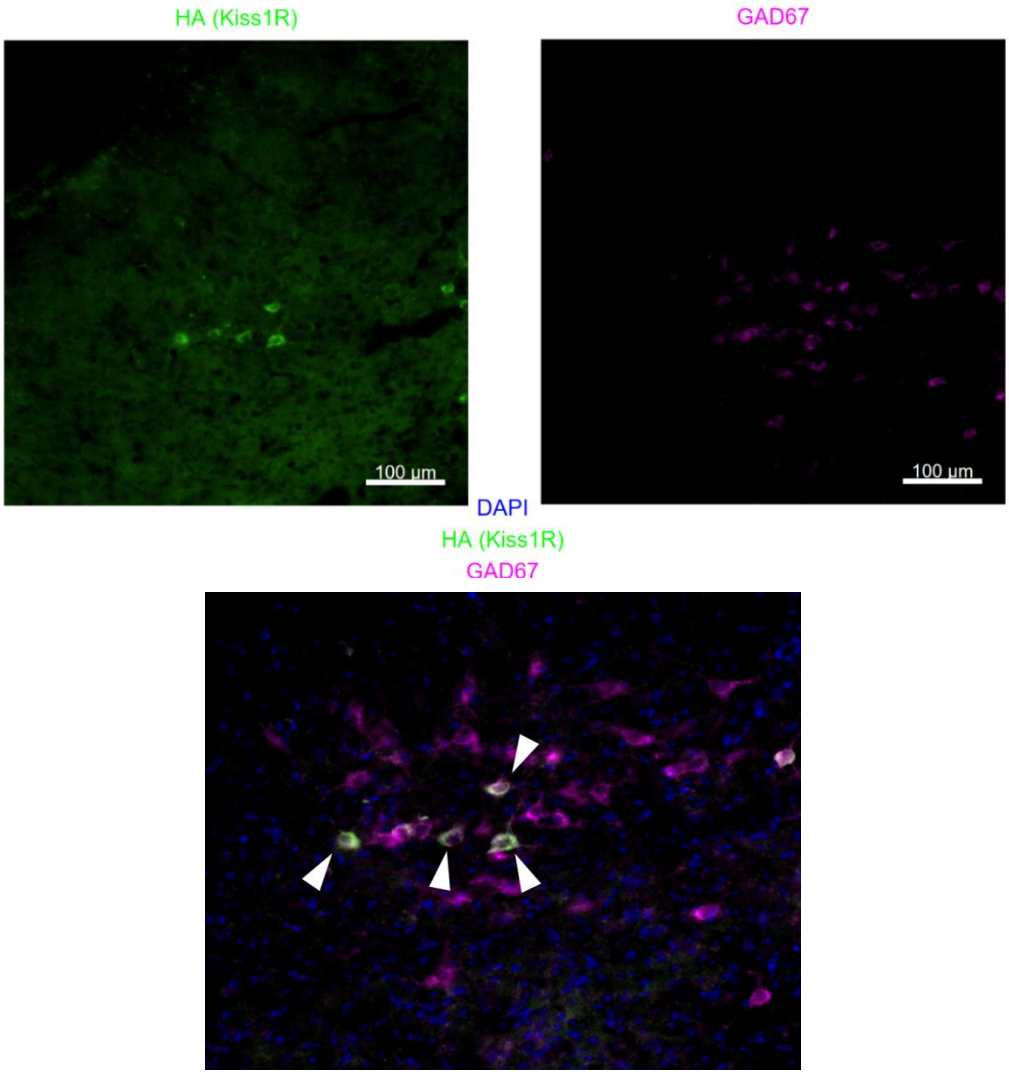

Figure 2. Identification of neuron populations containing Kiss1R in the ventrolateral periaqueductal gray (vlPAG). Fluorescent imaging depicting HA (green) and GABAergic neurons (purple) in Kiss1-2XHA mice. Arrows show colocalization of GABAergic neurons with HA-tagged Kiss1R. GABAergic neurons were identified by using a GAD67 antibody and HA was identified by using a HA-tag antibody.

To examine the influence of testosterone and estrogen on kisspeptin (*Kiss1*) and *Kiss1r* mRNA expression in the periaqueductal gray, wildtype male and female mice were gonadectomized (GDX) to remove the influence of sex steroids. Separate GDX male and GDX female cohorts were administered a testosterone pellet or two estradiol injections, respectively (GDX+T and GDX+E). The levels of administered sex steroids were selected to mimic the

presence of physiological levels of sex steroids. Brain tissues were then collected and sectioned to detect *Kiss1* and *Kiss1r* mRNA expression levels in the arcuate nucleus (ARC) and periaqueductal gray by qPCR **(Figure 3)**.

The ARC data were used as a control to verify the validity of our experimental parameters. As expected, there was a statistically significant effect of sex steroid treatment on *Kiss1* mRNA expression in both males and females in the ARC [$F(1,17) = 26.34$, $p < 0.0001$] **(Figure 3A)**. Our ability to generate expected results supports the validity of *Kiss1* and *Kiss1r* mRNA expression results for the periaqueductal gray.

There was a statistically significant effect of sex identity on *Kiss1* mRNA expression in the PAG [$F(1,18) = 15.33$, $p = 0.0010$] **(Figure 3B)**. Female GDX cohort had higher levels of kisspeptin mRNA expression compared to male GDX cohort ($p = 0.0061$) and female GDX+E cohort had higher levels of kisspeptin mRNA expression compared to GDX+T males ($p = 0.0061$) **(Figure 3B)**. These data indicate that kisspeptin mRNA levels are higher in females compared to males regardless of sex steroids. There was no statistically significant effect of sex steroid treatment [$F(1,18) = 0.2719$, $p = 0.6084$] on *Kiss1* mRNA expression in the PAG **(Figure 3B)**. These findings suggest that sex steroids do not have an influence on Kiss1 mRNA expression differences seen between males and females.

The analysis revealed a statistically significant effect of sex on *Kiss1r* mRNA expression [$F(1,20) = 7.691$, $p = 0.0117$] but no statistically significant effect of sex steroids [$F(1,20) = 7.459e\text{-}005$, $p = 0.9932$] **(Figure 3C)**. These findings suggest that *Kiss1r* mRNA levels in both female GDX and GDX+E cohorts are higher compared to both male cohorts regardless of sex steroids, similarly to *Kiss1* mRNA.

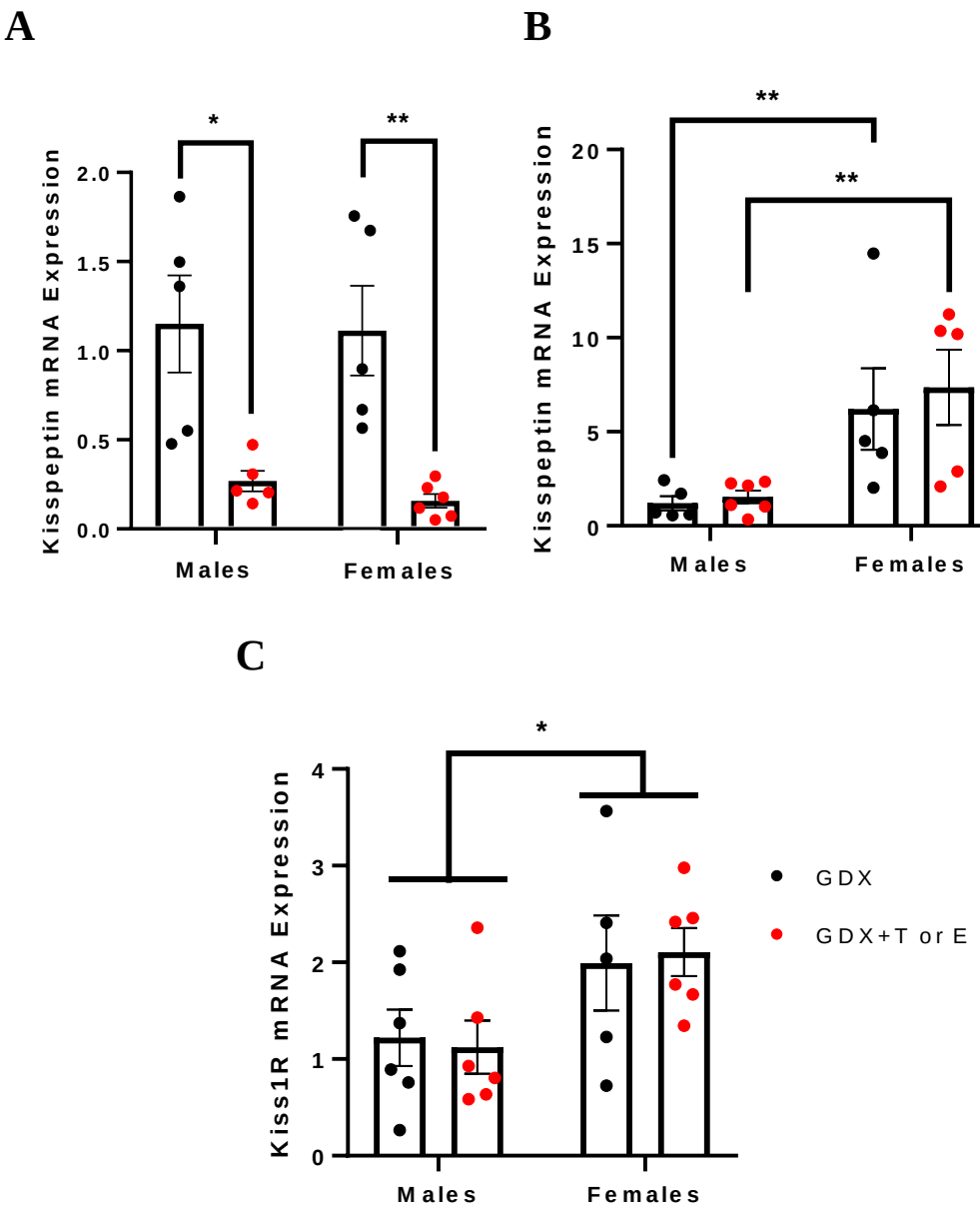

Figure 3. The effect of sex and sex steroids on (A) kisspeptin (*Kiss1*) mRNA expression in the arcuate nucleus, (B) kisspeptin (*Kiss1*) mRNA expression in the periaqueductal gray and (C) *Kiss1r* mRNA expression levels in the periaqueductal gray within gonadectomized (GDX) male and GDX female mice. One cohort each of GDX males (n=6) and GDX females (n=5) were not given sex steroid administration (black circles), whereas a separate cohort of GDX males (n=6) and GDX females (n=6) were given a testosterone (T) or estradiol (E) treatment, respectively (red circles). Brain sections of the arcuate nucleus or periaqueductal gray from each mouse were collected and mRNA levels of kisspeptin and Kiss1R were analyzed with qPCR. Expression levels were normalized with *H2afz* and *Ppia* housekeeping genes for arcuate nucleus samples and *Gapdh* housekeeping gene was used to normalized periaqueductal gray samples. Each point represents a measurement for an individual mouse. Mean ± SEM are expressed. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparison's test. * $P < 0.05$ and ** $P < 0.01$.

To examine the influence of Complete Freund's Adjuvant (CFA), a model of chronic pain, and chronic morphine exposure on kisspeptin (*Kiss1*) and *Kiss1r* mRNA expression in the periaqueductal gray, separate wildtype cohorts of male and female mice were given a control saline treatment, CFA treatment, or morphine treatment. Brain tissues were collected and sectioned to detect *Kiss1* and *Kiss1R* mRNA expression levels in the periaqueductal gray by qPCR **(Figure 4)**.

There was a statistically significant effect of sex on *Kiss1* mRNA expression [$F(1,24) = 4.644$, $p = 0.0414$] **(Figure 4A)**. *Kiss1* mRNA levels in females treated with saline, CFA, and morphine are all significantly higher compared to their corresponding male cohorts. The results support that *Kiss1* mRNA expression is higher in non-gonadectomized female mice than non-gonadectomized male mice, similar to **Figure 1**. There was a statistically significant effect of treatment on *Kiss1* mRNA expression by two-way ANOVA [$F(2,24) = 5.981$, $p = 0.0078$] **(Figure 4A)**. A post-hoc test did not reveal significant findings. The significant effect of treatment is likely driven by the upregulation of *Kiss1* by CFA treatment and downregulation of *Kiss1* by morphine treatment in both sexes, as no difference was detected by a two-way ANOVA comparing either CFA and saline or morphine and saline alone.

We did not find a significant effect of sex identity on *Kiss1r* mRNA expression [$F(1,26) = 2.548$, $p = 0.1225$] **(Figure 4B)**. The analysis revealed a statistically significant effect of treatment on *Kiss1r* mRNA expression [$F(2,26) = 5.810$, $p = 0.0082$] **(Figure 4B)**. A post-hoc test revealed that *Kiss1r* mRNA is significantly upregulated in CFA-treated females compared to control females.

The results indicate that the effects of either CFA or morphine treatment on *Kiss1* and *Kiss1r* expression warrant further investigation due to high variance and low sample size in the control group.

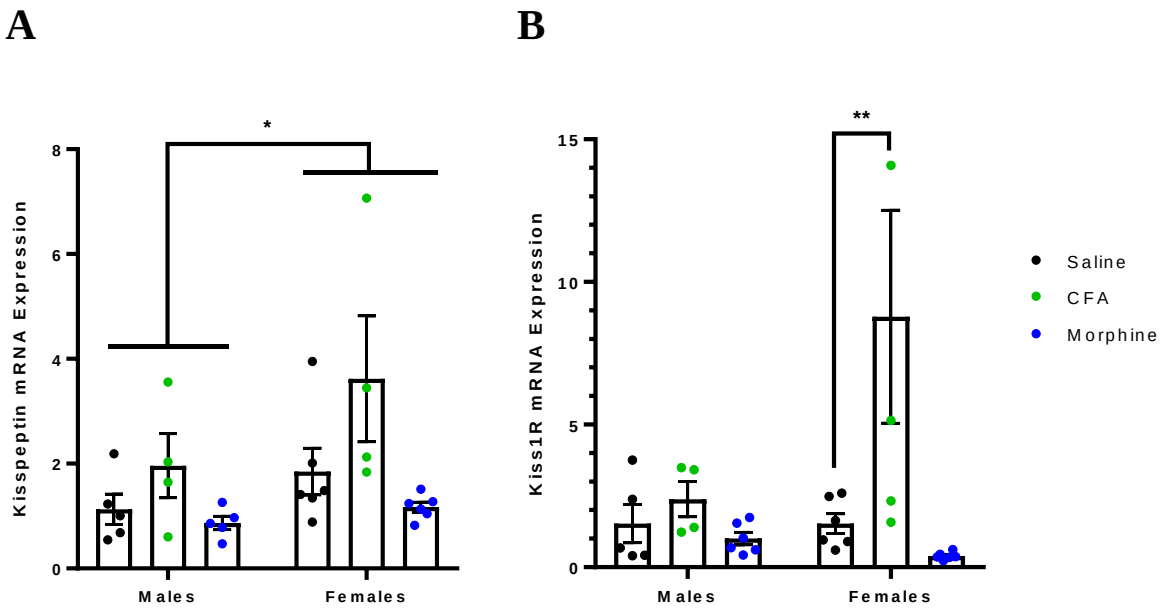

Figure 4. The effect of sex, CFA, and morphine on (A) kisspeptin (*Kiss1*) mRNA expression and (B) *Kiss1r* mRNA expression levels in the periaqueductal gray. Three separate wildtype cohorts of male and female mice were given a saline control treatment (n=5 for males and n=6 for females), Complete Freund's Adjuvant (CFA) treatment (n=4 for males and females) or morphine treatment (n=6 for males and females) for a period of 5 to 7 days. Brain sections of the periaqueductal gray were collected and analyzed with qPCR. Expression levels were normalized with *Gapdh* housekeeping gene. Each point represents a measurement for an individual mouse. Mean ± SEM are expressed. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparison's test. * $P < 0.05$ and ** $P < 0.01$.

DISCUSSION

The ventrolateral periaqueductal gray region of the descending pain modulation pathway serves an important modulatory role in nociception and opioid activity [10]. Delving into the profile and regulation of kisspeptin and Kiss1R in the ventrolateral periaqueductal is essential to begin understanding the physiological mechanisms that underly the sex differences seen in nociception. Apart from its reproductive role, kisspeptin has been identified to be pronociceptive and Kiss1R has been identified as the receptor mediating kisspeptin's role in nociception [19]. Limited research has been dedicated to analyzing the influence of sex, sex steroids, chronic nociceptive and chronic antinociceptive treatment on kisspeptin and Kiss1R in the ventrolateral region of the periaqueductal gray.

Our first set of experiments localized kisspeptin neurons in the ventrolateral periaqueductal gray and revealed no difference in kisspeptin neuron quantity between males and females in this region **(Figure 1)**. The visualization of kisspeptin neurons has allowed us to establish a profile and localize kisspeptin neurons in the ventrolateral periaqueductal gray. Our findings support the literature that identified kisspeptin neurons in the periaqueductal gray as a whole [15]. Our current findings suggest no sexual dimorphism for kisspeptin neuron quantity in the ventrolateral periaqueductal gray, but further experiments are needed to increase the sample size to finalize this finding.

We discovered that GABAergic neurons in the ventrolateral periaqueductal gray possess Kiss1R **(Figure 2)**. This finding is insightful for the nociceptive mechanism at the neuronal level. It is known that ventrolateral periaqueductal gray GABAergic neurotransmission leads to pronociceptive effects in the descending modulation pathway [20]. Kiss1R is a Gq-coupled protein

receptor. Signaling through Kiss1R has only been well-characterized in GnRH neurons, where it profoundly stimulates firing [26]. Kiss1R in GnRH neurons is phospholipase-C coupled and activates canonical transient receptor potential (TRPC) channels as well as inhibits inwardly-rectifying (GIRK) and potassium channels [26, 27, 28, 29]. If Kiss1R has a similar effect on GABAergic neurons in the vlPAG, this would increase the GABA tone within the vlPAG, making opioid-inducing disinhibition (and thus, analgesia) harder to achieve. Additional experiments investigating the activation of ventrolateral periaqueductal gray GABAergic neurons as a result of kisspeptin administration is needed to understand if kisspeptin's antinociceptive effects are mediated through increasing vlPAG GABA.

Our results indicate a sexually dimorphic pattern of kisspeptin (*Kiss1*) and *Kiss1r* mRNA expression in the periaqueductal gray **(Figure 3)**. Female mice had statistically significant higher levels of *Kiss1* and *Kiss1r* mRNA expression in the periaqueductal gray regardless of the absence or presence of sex steroids compared to male mice **(Figure 3B, 3C)**. The lack of statistical significance of sex steroid influence on *Kiss1* and *Kiss1R* mRNA expression suggests that sex steroids may not have a direct role in regulating its expression in the vlPAG. This finding differs from the sex steroid regulation of *Kiss1* expression in the hypothalamus, where estrogen and testosterone downregulate *Kiss1* in the arcuate nucleus [13]. This suggests that the sex difference seen in *Kiss1* and *Kiss1r* mRNA expression may arise from an organizational role of either sex steroid in development. A potential contributor to the sex difference seen in Kiss1 and Kiss1R expression is the amount of active kisspeptin neurons expressing Kiss1 in the periaqueductal gray. Our quantification of kisspeptin neurons in the ventrolateral periaqueductal gray utilized Kiss-Cre tdTomato mice in which fluorescent cells are those that express *Kiss1* at any point during development **(Figure 1)**. This is a limitation due to the lack of strict specificity of *Kiss-Cre*

expression. Although we observed no substantial difference of kisspeptin neuron quantity between males and females, it is possible that not all of these neurons actively express *Kiss1* during adulthood and may contribute to the differences in *Kiss1* expression seen in **Figure 3**. To rule out this possibility, active expression of *Kiss1* could be observed by utilizing Kiss-Cre GFP mice. Ultimately, further investigation is required to determine the factors influencing sex differences in *Kiss1* and *Kiss1r* expression.

The final set of findings reveal a potential capability of a chronic pain state and chronic opioid treatment to alter kisspeptin and Kiss1R levels in the ventrolateral periaqueductal gray. We found that female mice, regardless of pain condition, had higher levels of kisspeptin compared to males, supporting the findings in **Figure 3B**. Kiss1R is significantly upregulated in a pain state, but its levels are not significantly different between the male and female groups **(Figure 4B)**. A chronic pain condition tends to increase kisspeptin and Kiss1R expression levels, whereas opioid treatment does the opposite **(Figure 4A, 4B)**. The presence of high variance and low sample size warrants further investigation to draw a firm conclusion. The current results further support the literature that suggests the role of the ventrolateral periaqueductal gray in nociception and opioid analgesia [11]. Our results further characterize kisspeptin and Kiss1R to be the components that are altered in these two processes.

Kisspeptin and Kiss1R influence nociception, and this work further characterizes their regulation and profile in the ventrolateral region of the periaqueductal gray involved in nociception. The sexually dimorphic expression of kisspeptin and Kiss1R in the ventrolateral periaqueductal gray is a potential contributor to the sex differences seen in nociception. Our findings enhance the growing knowledge of kisspeptin's role in nociceptive processing. Further nociception

investigations are needed to verify kisspeptin and Kiss1R as potential analgesic targets to control the debilitating global impact of chronic pain.

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