Development of a Murine Model for Hot Flashes

Ashley Angell Krull

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2017

Reading Committee:

Robert A. Steiner, Chair

Richard D. Palmiter

G. Stanley McKnight

Program Authorized to Offer Degree:

Neuroscience

University of Washington

Abstract

Development of a Murine Model for Hot Flashes

Ashley Angell Krull

Chair of the Supervisory Committee:
Professor Robert A. Steiner
Physiology & Biophysics, Obstetrics & Gynecology

Hot flashes plague millions of reproductive age men and women who have natural or iatrogenic loss of sex steroid production. Many affected individuals are left without treatment options because of contraindications to hormone replacement therapy and the lack of equally effective non-hormonal alternatives. Moreover, development of safer, more effective therapies has been stymied by the lack of an animal model that recapitulates the hot flash phenomenon and enables direct testing of hypotheses regarding the pathophysiology underlying hot flashes. To address these problems, we developed a murine model for hot flashes and a method for determining thermal preference in mice. We designed and constructed a novel instrument, a thermocline, that produces a thermal gradient that allows mice to behaviorally adapt to a thermal challenge to their core body temperature set point and permits visual tracking and recording of their thermal preference over time. We tested and validated this murine model for “hot flashes”

by administration of a TRPV1 agonist and a NK3R agonist, capsaicin and senktide respectively, to unrestrained mice and observed their autonomic and behavioral responses. Following both treatments, the mice exhibited a hot flash-like response characterized by a drop in core body temperature and cold-seeking behavior on the thermocline. Attempts were made to use this positive control to guide experiments testing the hypothesis that neurons of the reproductive axis – so-called "KNDy neurons" – drive hot flashes. Unfortunately, efforts to use chemogenetic and optogenetic stimulation to stimulate KNDy neurons and induce thermoregulatory disturbances have been unsuccessful. However, we have gained insight into the limitations of these tools and conducted proof-of-concept experiments that support the use of the thermocline in studying thermoregulatory dysfunction following neuronal stimulation. This apparatus may be used to fully explore the cellular and molecular basis for hot flashes and to develop and test new therapeutic options in the future.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I sincerely thank my advisors, Robert Steiner and Don Clifton, for their mentorship, guidance, and support. I am a better scientist, writer, and philosopher because of their tutelage. I thank past members of the Corps of Discovery. Drs. Amy Oakley and Simina Popa provided early experimental and emotional assistance. Jessica Lancaster brought chocolate and logistics I needed to survive stressful times. I am grateful for enthusiastic, talented students: Lee Organick, Paige Haas, Jaden Blazier, Jasmine Correa, and Alejandra Cabrera. Above all, I want to thank Sarah Larsen, a passionate scientist who brought the expertise and engineering needed for the project to succeed. Without her know-how, determination, and excitement, this dissertation would not be here today.

I am grateful to my thesis committee and their lab members for valuable support, questioning, and insight. Richard Palmiter designed the mouse lines, and Stephanie Padilla and Chris Johnson supplied surgical expertise and counsel. Charles Chavkin provided the Ethovision tracking software, and Ben Land shared advice and antibodies. Stan McKnight advised experimental plans and provided helpful feedback on results. Drs. de la Iglesia and Raible served as my Graduate Student Representative, ensuring that I progressed through my degree program with support and confidence.

I am thankful for the constant encouragement and advocacy that I received from Drs. David Perkel and Jane Sullivan of the Neuroscience Graduate Program. Thank you to Ann Wilkinson, Lucia Wisdom, and Margie Trenam for tying up administrative loose ends. Thank you to fellow students who have turned into best friends. To my family and friends in faith, your love and support carried me through. To Jordan, my collaborator in science and life, I love you and thank you for all that you are for me.

DEDICATION

This work is dedicated to all the women who strive to advance scientific knowledge, improve lives, and leave this world a better place than we found it.

Chapter 1. INTRODUCTION

1.1 HOT FLASHES

Hot flashes (or flushes) are characterized by a constellation of vasomotor symptoms (VMS) that arise following a loss of circulating sex steroid hormones. VMS are characterized by intermittent periods of flushing, perspiration, and tachycardia. Moderate to severe VMS adversely affect quality of life and economic productivity. In the United States, the vast majority of women who undergo natural menopause and up to more than 90% of women who undergo surgical menopause will be plagued by hot flashes for several years and potentially indefinitely (1-3). Men also suffer from hot flashes following treatment with inhibitors of testosterone production (*e.g.,* for prostate cancer)(4, 5). Many of these people are left without recourse for treatment because of contraindications to hormone therapy and the lack of effective non-hormonal alternatives. Several non-hormonal remedies, including black cohosh, are barely more effective than placebo at diminishing hot flashes(6). To complicate matters further, the underlying physiological mechanisms that drive VMS remain poorly understood. Notwithstanding, the occurrence of VMS in both men and women suggests that the phenomenon is produced by a conserved pathophysiological mechanism that somehow links the reproductive and thermoregulatory control systems in the brain.

1.2 EARLY IDEAS ABOUT WHAT DRIVES HOT FLASHES

When scientists first began giving serious consideration to solving the VMS conundrum, early hypotheses lacked a rational basis in physiology. As early as the 1940's, physicians knew that hormone replacement therapy successfully treated hot flashes, but no one ventured to figure

out why. In the 1970's, researchers improved methods for objectively measuring hot flashes in patients, but the black box still remained. In 1981, Voda claimed that no pattern could be found to shed light on how and why women experience hot flashes(7). The first hypotheses regarding the origin of hot flashes came in the 1980's, when researchers concluded that hot flashes arise due to a change in the person's thermoregulatory set point and an effective narrowing of the "thermal neutral zone" (8). One can see how this conclusion would have been reached. VMS are notoriously triggered by otherwise innocuous rises in temperature as well as spicy foods, caffeine, and alcohol, which induce a sensation of warmth. Based on the observation that those affected often exhibit symptoms of hyperthermia, when others nearby remain thermo-neutral, researchers reasoned that the lack of sex steroids resets the threshold for perception of hyperthermia to a lower temperature(9). Likewise, it was observed that immediately following a hot flash, people would often experience chills, suggesting that the threshold for sensing hypothermia was raised. Thus, the thermal neutral zone of those affected seems to have shrunk. However, in the 32 years since this hypothesis was proposed, no sound physiological mechanism has been proposed to explain the putative shift in thermoregulatory set points. With a lack of understanding of the physiological basis of VMS, research in this area has been stymied for more than 3 decades.

1.2.1 *The hypothalamic-pituitary-gonadal axis*

Thanks to curious, creative researchers in the latter half of the 20th century, a flood of knowledge regarding how the reproductive axis is dysregulated following the decline in circulating sex steroids reignited interest in unraveling the mystery of VMS. The hypothalamic-pituitary-gonadal (HPG) axis governs mammalian reproductive function. The hypothalamus contains the arcuate nucleus (ARC; called the infundibular nucleus in humans), in

which resides a specialized population of neurons called kisspeptin (Kiss1) neurons. These neurons release a neuropeptide called kisspeptin onto the terminals of neurons in the median eminence to stimulate the release of gonadotropin-releasing hormone (GnRH)(10-13). GnRH then enters the portal circulation, prompting the release of luteinizing hormone (LH) from gonadotropes in the anterior pituitary(14). LH travels through the circulatory system to the gonads, where it stimulates production and secretion of sex steroids (*e.g.*, estrogen or testosterone). Steroids then act in a negative-feedback fashion in the ARC to suppress Kiss1 neuronal activity and thereby reduce GnRH release(10)(15-19). Thus, circulating levels of sex steroids are tonically regulated through this negative feedback loop involving sex steroid-dependent inhibition of Kiss1 secretion(20, 21)(17).

As a woman ages, her oocyte reserves are depleted and the ovaries' ability to produce estrogen becomes greatly diminished(22, 23). Over the span of 2-5 years, a perimenopausal woman's circulating estradiol levels drop from 40-400 pg/mL to 10-20 pg/mL(24-26). The drop in circulating levels of estradiol is even more abrupt in women undergoing a surgical oophorectomy, those being treated with hormone blockers for breast cancer, and in children treated with hormone suppressants for gender identity disorder. In all of these instances, the HPG axis attempts to correct the drastic change in sex hormone status by activating the Kiss1-GnRH neuronal circuit, which triggers intermittent episodes of VMS. This constellation of VMS – flushing, perspiring, skin vasodilation, and subsequent chills – comprises what is commonly known as a hot flash(9, 27)

1.2.2 *Early leads on hunt for hot flash generator*

Casper et al. (1979) and Tataryn et al. (1979) observed that episodes of VMS often occur coincidentally with pulses of LH in the blood(28,29). Scientists reasoned that LH may be the

driving signal behind VMS; however, it was soon shown that even women with pituitary deficiencies experience hot flashes(30-32). In addition, Casper and Yen showed that GnRH signaling was not needed to stimulate hot flashes in menopausal women(33). Based on these observations it was deduced that neither the pituitary nor LH was responsible for the occurrence of VMS, leaving the mystery of VMS etiology unresolved. It was not until more was learned about Kiss1 neurons in the ARC (*i.e.*, KNDy neurons) that interest in the neurobiology of VMS became reignited.

1.3 EMERGENCE OF THE HYPOTHESIS THAT KNDY NEURONS DRIVE HOT FLASHES

1.3.1 *Kiss neurons as drivers of reproduction*

Kisspeptin (Kiss1)-expressing neurons in the arcuate nucleus (ARC) play a key role in the tonic regulation of GnRH secretion and, therefore, reproductive function and fertility(10-12, 34). Kisspeptin secretion occurs coincidently with GnRH and LH pulses, and the absence of kisspeptin signaling results in the complete loss of fertility in humans and other mammals(12, 35, 36). Thus, it has become widely accepted that Kiss1 neurons are the primary drivers of pulsatile LH secretion— and by inference, the source generator for VMS.

1.3.2 *KNDy neuron characteristics*

Kiss1 neurons in the ARC express glutamate and two other neuropeptides – neurokinin B (NKB) and dynorphin (Dyn) – inviting the moniker of "KNDy" (Candy) neurons to describe this unique population of ARC neurons(37). NKB (encoded by *Tac2* in the mouse) stimulates KNDy neurons by binding to NK3 receptors (NK3R), which stimulates GnRH and LH release(38-41). Conversely, Dyn (encoded by *Pdyn*) acts through binding of kappa-opioid receptors (KOR) to

inhibit KNDy neuronal activity(19). Together, the actions of NKB and Dyn are thought to regulate the release of kisspeptin. Work from the Rance lab has shown that KNDy neurons project to other KNDy neurons in the opposite hemisphere, causing widespread neuronal population recruitment, even when one hemisphere is stimulated(42). Thus, this system is set up for rapid recruitment and then silencing of all KNDy neurons, resulting in strong pulsatile signaling to post-synaptic targets, such as GnRH neurons.

When estradiol levels fall (as in menopause), the negative-feedback control system compensates. With the brake of estradiol removed, KNDy neurons become activated, the neurons themselves become hypertrophied, and increase their expression of *Kiss1*, *Tac2*, and *Pdyn* mRNA, which are normally kept in check by estradiol in females (or testosterone in males) (40, 43-48). The activity of KNDy neurons becomes even more dramatically pulsatile, which in turn amplifies pulses of LH (38). Looking closer, Rometo *et al.* (2007) found that infundibular Kiss1 neurons were hypertrophied and had increased *KISS1* gene expression in menopausal women. Indeed, many of these hypertrophied kisspeptin neurons also increase their expression of NKB(44).

1.3.3 *Working Hypothesis: KNDy neurons drive VMS*

A rising consensus of scientific opinion now argues that KNDy neurons drive VMS. The basic argument contends that following a decline in circulating levels of sex steroids, KNDy neurons become hyper-activated, which has two parallel effects. First, episodic Kiss1 secretion from the KNDy neurons triggers pulses of GnRH and LH secretion. Second, episodic NKB secretion activates warm-sensing neurons (WSN) in thermoregulatory nuclei to cause an aberrant perception of excess heat. Thus, the pulsatile neuropeptide release from the excited KNDy neurons is linked to the intermittent pulsatile secretion of LH and the concomitant occurrence of

VMS (Fig. 1). Two relatively recent reports bolster the argument that KNDy neurons trigger both LH pulses and VMS. First, Yeo and Herbison showed that KNDy neurons project to a critical thermoregulatory nucleus, the median preoptic area (MnPOA)(49). Second, Rance and her colleagues revealed connections between NK3R-expressing neurons in the ARC and the MnPOA(42). This same group also demonstrated that the MnPOA contains WSN that express NK3R, the receptor for NKB, which is one of the neuropeptides produced by KNDy neurons(50). Indeed, when NK3R-expressing neurons in rodents are activated by the NK3R agonist senktide, they induce vasodilation of the tail skin, which also reduces the core body temperature (CBT) of the mouse. These studies led directly to the supposition that hyperactive KNDy neurons could be triggering thermoregulatory dysfunction via NKB signaling in the MnPOA(51). Finally, studies in women have shown that NKB administered systemically triggers VMS, lending further credence to the notion KNDy neurons drive VMS through NKB signaling.

1.4 Dissertation Objectives

Only circumstantial evidence supports the idea that KNDy neurons drive VMS, and there has not been a good way to directly test the hypothesis— because 1) there is not a good animal model for VMS and 2) there hasn't been a way to selectively activate KNDy neurons *in vivo* and examine its effect on thermoregulation. I had two aims for my dissertation project. The first was to engineer a method to assess the perception of feeling hot or cold in a mouse, measure and record it accurately, and use it to identify the occurrence of a pharmacologically-induced VMS. The second was to directly stimulate KNDy neurons within the ARC of transgenic mice and examine its effect on thermoregulatory behavior.

1.4.1 *Aim 1 Experimental Strategies*

My first aim was to develop an improved animal model of VMS. Over the years, several animal models have been used to interrogate the physiological basis of the phenomena, following either gonadectomy or pharmacologically–induced sex steroid suppression(52-58). However, the results of these inquiries have been complicated and confounded by inconsistencies in animal age, sex, time-of-day, and ambient temperature.

In humans, VMS are characterized by a perception of having an elevated core body temperature and by subsequent adaptive measures taken in response to this perception (*e.g.,* sweating, flushing, disrobing, fanning, or moving to a cooler area). Mice and other small mammals also thermoregulate using a variety of adaptive measures— but *behavioral adaptation* is their dominant strategy(59-61). In other words, they will move to correct a perceived temperature imbalance, and their settled location indicates a newly preferred comfort zone. I sought to exploit this thermoregulatory preference in our development of a murine model for VMS.

First, in collaboration with my colleague, Sarah Larsen, I designed a temperature-gradient platform (a thermocline) and validated its ability to reveal an animal's changing thermal preference in response to a thermal challenge. To validate the instrument's performance, I used capsaicin to activate the transient receptor potential cation channel subfamily V member 1 (TRPV1), which opens in response to a thermal challenge or certain chemicals. This would theoretically produce a sensation of warmth, and indeed, I was able to establish that it induced an appropriate behavioral response on the thermocline (*i.e.,* moving to a cooler region of the thermal gradient). Second, I explored the putative role of NKB signaling in triggering a "murine hot flash" by challenging mice with an NKB agonist, senktide. I assessed their thermoregulatory

responses, including temperature preference on the thermocline, CBT, TST, and Fos induction in the MnPOA. Here, I report that the response to senktide recapitulates many of the autonomic and behavioral adaptations that are characteristic of a hot flash. Thus, by combining senktide and the thermocline, we have developed a method to generate and measure VMS, which we can use to perform experiments focused on revealing the etiology of VMS and identify effective new therapies.

1.4.2 *Aim 2 Experimental Strategies*

To address the second aim, I used two different approaches to activate KNDy neuronal activity. First, I tried chemogenetic stimulation, by using designer receptors exclusively activated by designer drugs (DREADD). DREADDs are essentially customized G-protein-coupled receptors, derived from the muscarinic receptor for acetylcholine (ACh)(62). Point mutations in the third intracellular loop portion of the receptor diminish binding of ACh and exclusively permit binding of the otherwise inert ligand, clozapine-N-oxide (CNO). When CNO binds to the receptor, the alpha subunit of the trimeric G-protein triggers a canonical intracellular signaling cascade. For instance, if the DREADD is coupled to a G-α-q subunit, CNO binding will promote an increase in intracellular calcium that triggers release of neurotransmitters. There are also inhibitory DREADDs that will silence neuronal activity with CNO binding. I chose to utilize an excitatory DREADD (hM3Dq). This receptor promotes neuronal activation via the same small molecule-signaling cascade triggered by NKB. Also, the ability to inject CNO systemically into the animals and then leave them unrestrained and undisturbed presented experimental advantages from a logistics perspective.

Second, I used optogenetic stimulation to provoke neuronal stimulation. In optogenetics, depolarization (or hyperpolarization) can be triggered by light activating an ion channel. I used

an algae-derived ion channel, channelrhodopsion (ChR2), which opens in response to blue light to permit passage of cations, depolarizes cell membranes and triggers action potentials. Optogenetics offers some advantages over chemogenetics as it doesn't require handling of the mice to activate neurons and affords tighter temporal-timing of neuronal stimulation, which has a slow fade-in and fade-out process.

In both techniques, we would, in theory, directly stimulate KNDy neurons in transgenic mice wherein the receptor or channel is exclusively expressed in Kiss1 neurons. In my case, with the generosity of Dr. Richard Palmiter, I used a line of transgenic mice (KissCre2) that express Cre recombinase exclusively in *Kiss1*-expressing neurons in the adult brain. When these neurons encounter the gene encoding the DREADD or ChR2—delivered by a virus injected into the ARC—the Cre recombinase flips the orientation of the DREADD or ChR2 gene to permit transcription and expression of the protein on the cell surface. This gene flipping is made possible by LoxP sites flanking the DREADD or ChR2 gene as part of a double-floxed inverse open reading frame (DIO) (Fig. 2)(63). Following the action of Cre recombinase, the gene is locked into an active orientation. Neurons that do not express Cre cannot express either the receptor or channel and, thus, cannot be stimulated by either CNO or light.

I used the chemogenetic approach in an attempt to stimulate KNDy neurons in the ARC. The DREADD I used would trigger neuronal stimulation through a similar mechanism as NKB, which signals through the canonical G_q protein pathway to activate a small molecule-signaling cascade that results in the increase of cytosolic calcium. This calcium then acts on other molecules to trigger neuronal signaling and release of neuropeptides. Unlike NKB signaling via NK3R, however, the DREADD I used, hM3Dq, would be significantly overexpressed compared

to NK3R, and so stimulation of the DREADD could itself cause neuronal firing rather than simply raising intracellular calcium.

However, despite extensive efforts, I was unable to stimulate KNDy neurons with chemogenetic stimulation. Thus, I turned to optogenetic stimulation in mice that endogenously express a mutated ChR2 in their Kiss1 neurons, thereby obviating the need for stereotaxic injection of a virus to deliver the ChR2 gene. Unfortunately, my attempts to stimulate KNDy neurons using either the chemogenetic or optogenetic approach were unsuccessful.

Despite the lack of success in chemogenetically or optogenetically stimulating KNDy neurons to trigger NKB release, I was still able to test the role of NKB signaling in driving VMS via systemic administration of the NK3R agonist, senktide. Though injection of senktide lacks the specificity of genetically-driven approaches, I was able to test indirectly whether NKB signaling could "simulate" VMS in the thermocline model. Thus, even though I was not able to directly implicate KNDy neurons in driving hot flashes, I was able to show that a KNDy neuropeptide agonist – given systemically – can drive recruitment of WSN in the MnPOA to trigger VMS and behavior on the thermocline, recapitulating a hot flash-like response. This response still lent support to my hypothesis that a KNDy neuron co-transmitter can trigger a "murine hot flash" as shown on the thermocline while providing the field with a much-needed mouse model and experimental positive control.

Chapter 2. MATERIALS, METHODS, & EXPERIMENTAL DESIGN

2.1 MOUSE LINES

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Washington in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals*. Male and female mice were housed in a temperature-controlled room (21-23 °C) with a 12:12-h or 14:10-h light:dark cycle with lights on at 0600 h. Housing was under specific-pathogen-free (SPF) conditions and animals were provided with *ad libitum* access to water and chow. All mice were bred in our *Kiss1*$^{CreGFP.v2}$ transgenic colony. Most of the mice used for Aim 1 were wild-type littermates of the mice on a F8 generation C57BL/6J background, which do not express the gene encoding the Cre-GFP protein. Animals born in house were utilized for capsaicin and senktide experiments because C57BL/6J mice shipped from Jackson Labs proved to be more difficult to habituate to handling and the thermocline following the stress of shipment. I used Cre-GFP-expressing mice that were heterozygotes (*Kiss1*$^{CreGFP.v2/+}$) to examine Fos induction following senktide administration and for Aim 2 studies unless otherwise noted. Men and women experience VMS following steroid withdrawal. Therefore, we utilized both male (M) and female (F) mice to model the human condition.

Pain assessment following administration of various treatment doses in the mice was conducted using guidelines provided by our institution's veterinary staff. Per recommendation by veterinary staff, mice were monitored for the following signs: licking/biting/scratching the injection area for more than 15 min., lethargy, tucked or hunched posture, failure to groom, eye discharge, poor responsiveness to stimuli, and failure to show normal patterns of inquisitiveness

or alertness. None of these signs of pain was observed at the dosages of capsaicin or senktide evaluated.

2.1.1 *Kiss1*$^{CreGFP.v2}$ (KissCre2)

In the new KissCre2 mice, designed with the generous help of Dr. Richard Palmiter, the expression of Cre recombinase is under the control of the kisspeptin gene (*Kiss1*) promoter, meaning that Cre will only be expressed in cells expressing *Kiss1*. Cre expression is limited by the lack of a consensus initiation codon, the removal of a nuclear localization signal, and the addition of a Myc 3' untranslated region (3'UTR) and polyadenylation sequence to the gene. These changes result in the Cre mRNA being short-lived, helping to prevent ectopic expression of Cre. Finally, these animals were generated on a C57BL/6J background, meaning they will be more like the control mouse line in every way except for *Kiss1*-coupled Cre expression.

2.1.2 *Tacr3*$^{Cre/+}$ (Tacr3Cre)

This *Tacr3*$^{Cre/+}$ (Tacr3Cre) mouse line is similar in design to the KissCre2 line and was also designed by Dr. Richard Palmiter. In this line, Cre recombinase was targeted to the gene encoding the receptor for NKB, *Tacr3*.

2.1.3 *Kiss1*$^{CreGFP.v2}$/Ai32$^{+/-}$ (KC2/Ai32)

I crossed the KissCre2 heterozygotic mice with Ai32 mice that were a generous gift from the Palmiter group. The Ai32 mice were derived from mice generated by the Allen Institute in Seattle, WA, with the official designation: Ai32(RCL-ChR2(H134R)/EYFP) (https://www.jax.org/strain/012569). These animals were designed for optogenetic studies. They express a fusion protein of channelrhodopsin (ChR2) fused to EYFP to enable histological

visualization. The protein will only be expressed following exposure to Cre recombinase. Thus, when I crossed the Ai32 mice with our KissCre2 mice, only those neurons expressing *Kiss1* (and so Cre) should express the ChR2 gene and be stimulated by blue light. For my studies, I was only able to perform one round of breeding, and so I used mice that were double heterozygotes.

2.2 THERMOCLINE DESIGN

There are four components of the thermocline system (Fig. 3; patent pending): thermocline test chamber, video camera, video streaming and recording software, and position-tracking software. The thermocline test chamber is an open topped aluminum box (100 cm x 85 cm x 14 cm) divided into lengthwise sub-sections or "lanes". The base is a ½" thick piece of aluminum in which a thermal gradient is generated across its length, with one end cool and the opposite end warm, ranging from 10–45 °C. The warm end is maintained by cartridge heaters (12v 30W) implanted in the aluminum. The cool end is maintained by solid-state refrigeration components called Peltier Junctions. A Peltier Junction contains semiconductor material that maintains a voltage-controlled thermal differential between its two sides. The cool side is placed against the base of the thermocline, and the opposite side has heat removed by attached heat sinks and fans that allow the cool side to drop below room temperature. Lane temperature is monitored via thermocouples implanted into the aluminum base. The thermocouples are connected to control circuitry, which provides a user selectable temperature range and over-limit-alarm outputs. Topping the chamber is an optically clear piece of Plexiglas that creates a consistent internal ambient temperature and allows for video tracking of animals.

2.3 RECORDING AND ANALYSIS OF THERMOCLINE BEHAVIOR

Powering up the thermocline creates a thermal gradient across the aluminum base plate. Mice were placed into each lane and they freely move to the location of their choice. The position of each mouse was tracked with a USB video camera (Vimicro USB 2.0 PC Camera, Logitech HD Pro Web cam C920) mounted above the thermocline test chamber. Using VLC software (open source software available at https://www.videolan.org/vlc), I recorded (and sometimes viewed live) a video, streamed to a remote location for monitoring. After the video was recorded, positional data were analyzed with EthoVision video-tracking software. The position along the thermal gradient at which the animal is located was mapped to its preferred temperature. From the positional data, it could be determined how interventions influenced an animal's perceived temperature.

2.4 CAPSAICIN DOSE-RESPONSE STUDY & CORE BODY TEMPERATURE MONITORING

SubCue-Mini Dataloggers (Canadian Analytical Technologies Inc., Calgary, Alberta, Canada) were initialized prior to insertion and programmed for data recording during the time window of interest. Mice (n=30, 15M/15F) were anesthetized under isofluorane (3%). Dataloggers were surgically implanted via a paralumbar abdominal incision. After surgery, animals were given buprenorphine SR (0.5 mg/kg) as an analgesic and allowed to recover for one week.

Capsaicin samples (Sigma Aldrich, M2028) were prepared as a serial dilution (100mM initial stock solution in DMSO) with saline (0.9% sodium chloride) in dose amounts 1.5, 1, 0.5,

0.25, and 0 mg/kg (vehicle, saline) all maintaining a constant dosing volume of 5 uL/g animal mass.

One week post surgery, the mice were habituated to injections by receiving a flank injection (sc) of saline once per day for 4 days. On the fifth day, the mice were divided into 5 groups (n=5-6, with 2-3M and 3F), and mice in each group received one flank injection (sc) of vehicle or of one of the four different doses of capsaicin. At the conclusion of the experiment, mice were euthanized under anesthesia by cervical dislocation, and the dataloggers were extracted to retrieve CBT data.

2.5 SENKTIDE DOSE-RESPONSE STUDY & CORE BODY TEMPERATURE MONITORING

Dataloggers were inserted into thirty mice (15M/15F) as described above, and animals were allowed to recover for one week. Senktide (Tocris Bioscience) samples were prepared as a serial dilution (1mM initial senktide stock solution in 0.9% saline with 5% DMSO v/v) with vehicle (0.9% saline) in dose amounts of 0.75, 0.50, 0.25, 0.125, and 0 mg/kg senktide (vehicle, 0.9% saline with 5% DMSO), maintaining a constant dosing volume of 5 uL/g animal mass. Senktide action was always verified by the presence of tail whips before including the animal in analyses.

Similar to the capsaicin dosing protocol, one week post surgery, animals received a flank injection (sc) of saline once per day for 4 days. On the fifth day, the animals were divided into 5 groups (n=5-6, with 2-3M and 3F) and animals in each group received one flank injection (sc) of either vehicle or one of the four doses of senktide. The animals were subsequently euthanized to retrieve the dataloggers and extract CBT data.

2.6 BEHAVIOR IN RESPONSE TO CAPSAICIN AND SENKTIDE ON THE THERMOCLINE WITH THERMAL GRADIENT OFF

For these experiments, the thermal gradient of the thermocline was turned off. Mice (n=18, 9M/9F) were placed in one of twelve thermocline lanes and habituated for 4 h per day over 5 consecutive days. Half-way through habituation period, mice were picked up, momentarily touched at the injection site, and returned to the thermocline. Investigators were not present in the room, except to place the mice on in the thermocline, handle them briefly, and subsequently remove them from the thermocline. During the second week, each mouse was placed on the thermocline again for 4 h on 3 separate days. Two hours after being placed in the thermocline, mice were removed and given an injection (sc) of one of three different treatments: 1 mg/kg of capsaicin with 3% DMSO, 0.5 mg/kg senktide with 3% DMSO, or vehicle only. The mice then were returned to thermocline for another 2 h. The vehicle used for all cases was 0.9% sodium chloride with 3% DMSO v/v. Each mouse received all 3 treatments one time each over the course of the experiment. Animal position on the thermocline and movement were monitored and recorded with USB cameras and VLC software and tracked using EthoVision position tracking software. Preferred position on the thermocline as well as total movement over the course of the experiment – and exclusively post-injection – were quantified.

2.7 BEHAVIOR IN RESPONSE TO CAPSAICIN AND SENKTIDE ON THE THERMOCLINE WITH THERMAL GRADIENT ON

For these experiments, the thermal gradient of the thermocline was turned on. Mice (n=11, 5M/6F) were implanted with a thermal datalogger and allowed to recover for one week. After recovery, they were placed in one of twelve thermocline lanes and habituated for four hours per day over five consecutive days. Half-way through habituation period, mice were

picked up, momentarily touched at the injection site, and returned to the thermocline. Investigators were not present in the room, except to place the mice on in the thermocline, handle them briefly, and subsequently remove them from the thermocline. During the second week, each mouse was placed on the thermocline again for 4 h for 3 consecutive days. Two hours after being placed in the thermocline, mice were removed and given a subcutaneous injection of one of three different treatments: 1 mg/kg of capsaicin with 3% DMSO, 0.5 mg/kg senktide with 3% DMSO, or vehicle only. The mice then were returned to thermocline for another 2 h.The vehicle used for all cases was 0.9% sodium chloride with 3% DMSO v/v. Each mouse received all 3 treatments one time each over the course of the experiment. Animal position on the thermocline and movement were monitored and recorded with USB cameras and VLC software. Movement was quantified and the position translated into temperature using EthoVision position tracking software. Following all trials, the animals were euthanized, the dataloggers were removed, and the data was processed by using the SubCue software.

2.8 TAIL SKIN TEMPERATURE CHANGES IN RESPONSE TO SENKTIDE

Mice (n=12, 6M/6F) were habituated to the thermocline daily for 2 h per day for 5 days with the thermocline at ambient temperature (off). The week following habituation, mice were again placed on the thermocline on two additional days for a 2 h period each day. After 1 h, animals were imaged with an infrared (IR) camera (FLIR E5) and five minutes later given a subcutaneous injection of either senktide (0.5 mg/kg with 3% DMSO) or vehicle in a crossover design. They were each then IR imaged at 2.5 min. intervals for 20 min. at a distance of 6 in. above the mouse. Tail skin temperature (TST) was measured from the IR image by averaging the temperature of the tail along a line starting 2 cm from the base of the tail and extending for 1 cm distally.

2.9 EXAMINATION OF FOS INDUCTION IN THE MNPOA FOLLOWING SENKTIDE

Mice (n=24, 12M/12F) were divided into 2 groups of 12 animals (6M/6F) and given a subcutaneous injection of either senktide solution (0.5 mg/kg with 3% DMSO) or vehicle (0.9 % saline with 3% DMSO v/v) solution. After 2 hours, mice were administered a ketamine/xylazine cocktail and transcardially perfused using 1x PBS and 4% PFA in 1x PBS. Brains were post-fixed in 4% PFA overnight at 4 °C and then cryoprotected in 30% sucrose solution overnight. Brains were then frozen in Tissue-Tek® O.C.T. Compound and stored at -80 °C until slicing. The MnPOA of each mouse was sliced into 30 µm sections and stored in 1 x PBS at 4 °C. A total of 20-24 sections were collected, and the section closest to 0.38 mm anterior to bregma was chosen for cell quantification.

Floating sections were immune-stained for Fos with the following protocol. Sections were washed in 1 x PBS and then blocked in blocking buffer (5% normal donkey serum, 0.3% Triton-X100, in 1x PBS) at room temperature (RT) for 60-90 minutes. Sections then were left on shaker in primary antibody (1:500, Santa Cruz Biotechnology Cat# sc-52, RRID:AB_2106783, in blocking buffer) overnight at RT. Following three washes in 1 x PBS, sections were left on shaker in secondary antibody solution (1:500, Donkey anti-Rabbit IgG H&L (DyLight® 488), Abcam Cat# ab96919, RRID:AB_10679362, in blocking buffer) for three to four hours. Slide coverslips were mounted with ProLong Diamond Antifade Mountant (Life Technologies) and allowed to dry overnight. Processed slides were imaged on a Leica DMLB fluorescence microscope with a 40x objective, and Fos-expressing neurons in the MnPOA were counted by observers who were blinded to the group assignments. Images used in Figure 5 were collected using a Leica SP8X Scanning Confocal microscope with a 40x / NA 1.30 oil immersion objective.

2.10 STATISTICAL ANALYSIS

GraphPad Prism resources (http://www.graphpad.com/quickcalcs/) were utilized for statistical analyses. We compared the responses of mice to treatment and vehicle as seen in the animals' CBT, temperature/position preferences, and TST over time. Based on preliminary dose-response studies, we identified the time window of responses to capsaicin and senktide to be 60 and 80 minutes respectively. These time windows following injection were used for statistical analyses of subsequent experiments. For the studies examining CBT and behavioral responses to treatment on the thermocline, we examined each mouse's responses over time and measured the area under the response curves (AUCs). We then used a two-tailed, paired t-test to determine if the mean AUCs for each treatment group were significantly different. This same statistical strategy was applied to the data for the TST response to senktide as well as studies utilizing Tacr3Cre mice on the thermocline. To determine whether senktide induced Fos expression in the MnPOA, counts of Fos-labeled cells were compared with a two-tailed t test. We used a 2-way ANOVA (thermocline status vs treatment) to evaluate whether thermocline status (on vs. off) affected post-injection movement in both capsaicin- and senktide-treated animals. All data are plotted as means ± SEM.

2.11 DREADD EXPERIMENTAL DESIGNS

2.11.1 *hM3Dq-mCherry virus design*

AAV-hSyn-DIO-hM3D(Gq)-mCherry. This viral construct was produced by the Palmiter Lab using established protocols(64). This AAV is adenovirus-free with a biosafety level of BSL1. It contains a gene for a designer Gq-protein-coupled receptor (hM3D(Gq)) fused to a mCherry molecule. This DREADD is a cell membrane receptor activated by CNO that has no

extraneous effects or binding partners in the body and solely serves to activate or inhibit a neuron upon CNO binding's initiation of a protein-signaling cascade endogenous to the cell. In this manuscript, this construct is referred to as AAV-DIO-hM3Dq.

2.11.2 *Clozapine-N-oxide*

The clozapine-N-oxide (CNO) used for stimulation of DREADD receptors was provided by the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (Catalog no. C-929). I always administered a 1.0 mg/kg dose of CNO via an intraperitoneal injection of a 0.1 mg/mL solution of CNO (in 0.5% DMSO v/v in 0.9% sterile saline). The vehicle solution was 0.5% DMSO v/v in 0.9% sterile saline.

2.11.3 *Intracranial injections: coordinates & general details*

Intracranial injections were performed by trained lab personnel within the University of Washington vivarium or in other approved surgical facilities. In general, injections of all viruses were performed at the following coordinates: x = ± 0.3 mm, y = -1.3 mm, z = -5.8 or -6.0 mm (F versus M). Experimenters injected 500 nanoliters of virus solution (equating to 10^9 viral particles) into each hemisphere at a rate of 100 nL/min. Following injection, animals were allowed to recover for at least 3 weeks to allow the virus to express.

2.11.4 *Unilateral stimulation of Kiss1$^{CreGFP.v2}$ mice under isofluorane*

Mice (n=12, 6M/6F) were treated with acyline five days prior to being intracranially injected with AAV-DIO-hM3Dq unilaterally into the ARC. This dose of acyline aids viral expression by increasing Cre expression by Kiss1 neurons and is washed out of the body by the time of experiments. In the first round of CNO and vehicle (VEH) injections, mice were anesthetized and a baseline blood sample was collected via retro-orbital bleed. Ten min later,

mice were given 1.0 mg/kg CNO or VEH i.p. injection. After 40 min, mice were bled again and then allowed to recover from anesthesia. In the second round of CNO/VEH injections, following the post-CNO/VEH bleed, mice were given a ketamine/xylazine cocktail and transcardially perfused.

2.11.5 *Bilateral stimulation of Kiss1$^{CreGFP.v2}$ mice*

Mice (n=16, 8M/8F) were treated with acyline one week prior to being intracranially injected with AAV-DIO-hM3Dq bilaterally into the ARC. Administration of CNO/VEH and subsequent perfusion were conducted as for KissCre2 mice in section 2.9.4. However, prior to sacrifice, mice were placed on the thermocline for 2 h (1 h habituation, 1 h post-injection) to observe their thermoregulatory response to the CNO/VEH injection.

2.11.6 *Stimulation of intact Kiss1$^{CreGFP.v2}$ mice on the thermocline*

Mice (n=11, 5M/6F) were treated with acyline 4 days before being intracranially injected with AAV-DIO-hM3Dq bilaterally into the ARC. Mice were habituated to the thermocline for 5 days prior to experiments. On experimental days, mice were allowed to settle on the thermocline for at least 90 min prior to CNO/VEH injection and then permitted to freely explore for an additional 3 h. Following completion of all experimental runs on the thermocline, mice were administered CNO or VEH and then transcardially perfused in preparation for histological analysis.

2.11.7 *Stimulation of gonadectomized Kiss1$^{CreGFP.v2}$ mice on the thermocline*

Mice (n=12, 6M/6F) were treated with acyline one week prior to being intracranially injected with AAV-DIO-hM3Dq bilaterally into the ARC. One month following intracranial

injections, the mice were gonadectomized. Two weeks later, mice were habituated to the thermocline for 5 days prior to experiments. On experiment days, mice were allowed to settle on the thermocline for 2 h prior to CNO/VEH injection and then to freely behave for an additional 2 h. Following completion of all experimental runs on the thermocline, mice were administered CNO or VEH and then transcardially perfused in preparation for histological analysis.

2.11.8 *Stimulation of intact Kiss1$^{CreGFP.v2}$ mice expressing ChR2 & hM3Dq on the thermocline*

Mice (n=9, 8M/1F) were intracranially injected with AAV-DIO-hM3Dq and AAV-DIO-ChR2 bilaterally into the ARC. Following recovery, mice were habituated to the thermocline for 5 days prior to experiments. On experimental days, mice were allowed to settle on the thermocline for 2 h prior to CNO/VEH injection and then to freely behave for an additional 2 h. These experiments were conducted in collaboration with Stephanie Padilla of the Palmiter lab, who conducted further experiments with these mice before sacrificing them. Mice were excluded from analysis based on behavioral responses and expression of virus.

2.11.9 *Stimulation of intact, cycled Kiss1$^{CreGFP.v2}$ mice on the thermocline*

Mice (n=19, 3M/16F) were intracranially injected with AAV-DIO-hM3Dq bilaterally into the ARC. Following recovery, mice were habituated to the thermocline for 5 days prior to experiments. The estrous cycles of the female mice were monitored daily, and only mice in diestrus were used for experiments each day. On experiment days, mice were allowed to settle on the thermocline for 2 h prior to CNO/VEH injection and then to freely behave for an additional 2 h. Following completion of all experimental runs on the thermocline, mice were administered CNO or VEH and then transcardially perfused in preparation for histological analysis.

2.11.10 *Stimulation of intact Tacr3Cre mice on the thermocline*

Mice (n=6, 6F) were intracranially injected with AAV-DIO-hM3Dq & AAV-DIO-ChR2 or AAV-DIO-mCherry bilaterally into the MnPOA. Following recovery, mice were habituated to the thermocline for 5 days prior to experiments. On experiment days, mice were allowed to settle on the thermocline for 1 h prior to CNO/VEH injection and then to freely behave for an additional 90 min. These experiments were conducted in collaboration with Chris Johnson of the Palmiter lab, who performed further experiments with these mice before sacrificing them.

2.12 OPTOGENETIC STIMULATION OF *KISS1*$^{CREGFP.V2}$/AI32$^{+/-}$ & TACR3CRE MICE

2.12.1 *ChR2-mCherry virus design*

AAV-hSyn-DIO-ChR2-mCherry. This viral construct was produced by the Palmiter Lab using established protocols detailed in Gore *et al.*, 2014(64). The virus was injected at a titer of 10^9 viral particles per microliter following established protocols(69). This AAV is adenovirus-free with a biosafety level of BSL1. It contains a gene for channelrhodopsin (ChR2) fused to a mCherry molecule. In this manuscript, this construct is referred to as AAV-DIO-ChR2. A control version of this virus was also constructed that lacked the ChR2 gene but expressed the mCherry marker – AAV-DIO-mCherry.

2.12.2 *Optogenetic stimulation of Tacr3Cre mice*

The same female control mice (expressing DIO-mCherry) were used for this experiment as described in section 2.9.10, along with one ChR2-expressing female and two ChR2-expressing males. When optogenetically stimulated on the thermocline, only one mouse was

placed on the thermocline at a time. Each mouse was tethered to the laser in such a way so as to not be disturbed by the tether or get caught on thermocline components. On experimental days, mice were allowed to settle on the thermocline for 1 h prior to laser stimulation and for 50-60 min following. These experiments were conducted in collaboration with Chris Johnson of the Palmiter lab, who conducted further experiments with these mice before sacrificing them.

2.12.3 *Optogenetic stimulation of* Kiss1$^{CreGFP.v2}$/Ai32$^{+/-}$ *mice*

Double heterozygotic *Kiss1*$^{CreGFP.v2}$*/Ai32*$^{+/-}$ mice (n=6, 5F,1M) were stimulated in the same manner as Tacr3Cre mice on the thermocline.

Chapter 3. AIM 1 RESULTS & DISCUSSION: DEVELOPMENT OF A MURINE MODEL FOR HOT FLASHES

This chapter contains information that was submitted to the Journal of Endocrinology by the author and other members of the Steiner-Clifton-Neal-Perry lab (Krull *et al.*, 2017).

3.1 Decreases in core body temperature in response to capsaicin and senktide doses

Mice injected with capsaicin and senktide exhibited dose-dependent decreases in CBT (Fig. 4A, 5A). Vehicle-treated mice did not experience a drop in CBT. We took particular note of the response of mice given a 1.0 mg/kg dose of capsaicin or a 0.5 mg/kg dose of senktide, which provided a clear thermoregulatory response without pain. We used these treatment doses for validation of the thermocline.

3.2 Response to capsaicin and senktide treatment when thermal gradient was active (ON)

To observe the behavioral response to capsaicin and senktide, mice implanted with dataloggers were placed in the individual lanes of the thermocline. Two hours later, mice were injected subcutaneously with 1.0 mg/kg of capsaicin, 0.5 mg/kg of senktide, or with vehicle solution. The mice were then allowed to behaviorally thermoregulate undisturbed while CBT and positional data on the thermocline were recorded.

All mice showed an initial drop in thermal preference. However, the vehicle-treated mice quickly recovered and returned to their previously preferred temperature on the thermal gradient. In contrast, the capsaicin-treated mice maintained their position on the cooler end of the

thermocline for nearly an hour following treatment (Fig. 4D). The CBT of these mice dropped further than that of the mice given capsaicin at ambient temperature during the dose-response study (nadir = 32.1±0.623 °C versus 33.5±0.709 °C; Fig. 4B). Likewise, the senktide-treated mice preferred to remain at cooler temperatures for the duration of the study post-injection (Fig. 5D). As seen with capsaicin, the CBT of these mice also dropped further than that of the mice given 0.5 mg/kg senktide at ambient temperature during the dose-response study (nadir = 31.6±0.489 °C versus 32.7±0.790 °C; Fig. 5B).

3.3 POSITION PREFERENCE IN RESPONSE TO CAPSAICIN AND SENKTIDE TREATMENT WHEN THERMAL GRADIENT WAS INACTIVE (OFF)

We examined whether the apparent cold temperature preference of capsaicin- and senktide-treated mice was attributed to a "fear or stress response" to the injection— a response that induced the mice to seek a particular end of the thermocline— notwithstanding the presence of a thermal gradient. To examine this possibility, we evaluated the behavioral response of mice on the thermocline to the same treatments— but this time, with the thermocline turned off (*i.e.,* no thermal gradient present). We reasoned that if the injection itself or treatment response caused the mice to prefer a specific end of the thermocline (independent of the thermal gradient), then we would expect the same location preference pattern to occur whether the thermal gradient was on or off. However, we found that when the thermocline temperature gradient was turned off, the mice did not demonstrate a placement preference— *i.e.,* they moved randomly between ends of the thermocline (Fig. 4C, 5C).

3.4 Comparison of movement following capsaicin and senktide treatment when thermal gradient is active versus inactive (ON vs OFF)

We also examined whether the positional preferences of the capsaicin- and senktide-treated mice could be related to "impaired mobility," which compromised their ability to direct their movement elsewhere. To address this possibility, we used the Ethovision tracking software to quantify the total movement of mice on the active or inactive thermocline following treatment. (We focused on the post-injection movement to avoid pre-injection movement differences masking any potential effect of the treatments on movement.) We found that thermal gradient status (on *vs* off) had a significant effect on post-injection movement ($p<0.001$, $F=44.75$, $Df=1$, 2way ANOVA), whereas treatment had no significant effect ($p=0.0904$, $F=2.476$, $Df=2$), with no significant interaction observed ($p=0.202$, $F=1.630$, $Df=2$, Fig. 8).

3.5 Increase in tail skin temperature and Fos expression in the MnPOA in response to senktide

To ensure that senktide induced a physiologically normal thermoregulatory response in our animals, we also measured the tail skin temperature (TST) of senktide-treated mice. Senktide-treated mice experienced a significant rise in TST compared to vehicle-treated mice (Fig. 6; 5.98±0.729 °C vs. 1.93±0.701 °C). This rise in TST lasted for about 15 min before subsiding, which reflects a difference in the time-course of the response to senktide between the CBT and TST.

Finally, to determine whether senktide could activate neurons in the MnPOA, an important thermoregulatory region in the hypothalamus, we quantified and compared the number of Fos-expressing cells following either senktide or vehicle treatment (Fig. 7). We found that

senktide treatment significantly increased the number of Fos-expressing neurons within a section of the MnPOA ($p=0.0259$, two-tailed t test).

3.6 Discussion: Use of pharmacological stimulation on the thermocline to create a murine model of hot flashes

VMS (or hot flashes) represent a physiological enigma— and a clinical problem that severely reduces the quality of life. People who experience VMS perceive an elevation in their CBT— when none actually occurs; yet, they physiologically respond to the actuating event that triggers the phenomenon. Scientists have struggled to devise an animal model in which to elucidate the etiology of VMS and test potential therapeutic options to quell their occurrence or reduce their intensity. Here, I report a novel approach to induce a perception of elevated CBT in mice and then remotely measure and record the behavioral adaptation to this thermal challenge.

Small mammals, such as mice, adapt to excessive heat by seeking cooler areas that facilitate heat loss(60). I found that mice would exhibit this same behavior if they were placed on a temperature gradient, a thermocline. The thermocline gave the mice a full range of ambient temperature choices and allowed me to record their adaptive movements on a second-to-second basis. As expected, the mice chose to sit at temperatures very close to their normal CBT (37 °C). I even observed that mice choose to defecate in the cold end of the thermocline and then return to sleep in the warm end. One of the main advantages of our thermocline is that it permits performing experiments simultaneously on up to 12 individual animals, while not permitting animals to interact and influence one another. In addition, one can record behavior in response to a variety of stimuli (*e.g.*, ambient temperature, pharmacologic agents, neural stimulation) remotely. Once I was confident that I had created a reliable observational tool, I turned to address my second aim— to create a “hot flash” in mice.

I chose to induce thermoregulatory dysfunction using two compounds – capsaicin and senktide. First, I used capsaicin since previous experience and other murine studies have shown this compound capable of inducing hypothermia(65, 66). I conducted a dose-response study to determine the dose of capsaicin to which mice would exhibit a thermoregulatory response—but no pain (as evidenced by lack of behavioral changes such as immobility, abnormal posturing, vocalization, and overt aggression), and I selected a dose of 1.0 mg/kg on this basis. When capsaicin was administered to mice on the thermocline, I found that the ability to behaviorally thermoregulate allowed the animals to quickly address their perceived thermal threat. This contention is supported by the observation that following a capsaicin injection, animals quickly dropped their CBT on the thermocline—much more rapidly than happened if they were challenged in their home cage. I suggest that the initial drop in thermal preference by all the mice following injection reflects a stress response due to handling from which the vehicle-treated mice quickly recovered, whereas the capsaicin-treated mice continue to huddle in the cold end of the thermocline in response to their perceived thermal challenge. These observations gave me confidence that I could use the thermocline to detect and measure the behavioral adaptation to a thermal challenge in mice.

Second, I wanted to examine the response of mice to a more physiologically relevant stimulus. To achieve this objective I used the NKB receptor agonist, senktide. Emerging evidence suggests that NKB produced by KNDy neurons is a neurochemical signal that induces menopausal hot flashes in women(67). Recent studies have also shown that senktide, induces thermoregulatory disturbances in mice(50, 68). Senktide binds to the type 3 tachykinin receptor, NK3R, which is expressed by KNDy neurons in the arcuate nucleus as well as WSN in the MnPOA, which may represent target neurons for the VMS phenomenon(19, 50). As with

capsaicin, I conducted my own dose-response study to determine the senktide dose to which, in my hands, mice would have a thermoregulatory response. The dose of 0.5 mg/kg was determined to cause the greatest thermoregulatory response in the mice— without any signs of discomfort (according to the same aforementioned behavioral criteria). Following senktide administration on the thermocline, mice repositioned themselves to a cooler region to rectify their perceived elevation in CBT, which occurred in parallel with the changes we observed in CBT during the dose-response trials. Again, the initial drop in thermal preference by all the mice following injection likely reflects a stress response from which the vehicle-treated mice recovered. Also, as was seen following capsaicin treatment, the ability of the mice to move to a cooler area facilitated the animal's thermoregulatory response to the perceived thermal threat, allowing it to more quickly and significantly drop its CBT before regaining thermoneutrality.

Although thermoregulatory behavior in response to senktide had not been previously demonstrated, I reasoned that senktide was not evoking a completely abnormal behavioral thermoregulatory response in the mice. I was able to reproduce the hypothermia and Fos induction in the MnPOA in response to a peripherally administered senktide, previously shown when senktide is delivered directly into the MnPOA(50, 68). My results extend this observation by showing that senktide also evokes a rise in TST in mice, demonstrating its ability to promote tail vein vasodilation, a known murine heat dissipation effector. However, the fact that the TST response differed so strikingly from the behavioral response highlights the utility and importance of measuring multiple readouts to get a complete picture of thermoregulation in the mice. I would argue that the behavioral response of the mouse provides a more holistic representation of how the mouse is "feeling" in response to a perceived—or real—thermal threat. In my case, the choice of the senktide-treated mice to remain on the cooler end of the thermocline reflects their

sustained perception of thermal threat – which persists even longer than that induced by capsaicin. Overall, the effect of senktide on autonomic and behavioral thermoregulation in mice bears a remarkable similarity to a hot flash and suggests that this compound can serve as a chemical surrogate for the VMS phenomenon in an animal model.

Chapter 4. AIM 2 RESULTS & DISCUSSION: STIMULATION OF KISS1 NEURONS TO INDUCE HOT FLASHES

4.1 RESULTS: CHEMOGENETIC STIMULATION VIA DREADDS

I hypothesized that chemogenetic stimulation of Kiss1 neurons within the arcuate nucleus would trigger aberrant behavioral thermoregulation in mice, reminiscent of a hot flash response in humans. For this aim, I injected the AAV-DIO-hM3Dq-mCherry construct containing the gene for the excitatory DREADD (hM3Dq) into the arcuate of mice from the *Kiss1*$^{CreGFP.v2}$ mouse line. Given that only those neurons actively expressing *Kiss1* in the arcuate nucleus would express the hM3Dq receptor, I anticipated visualizing the viral marker (mCherry) localized to the arcuate nucleus to show that only Kiss1 neurons were amenable to stimulation through binding of CNO to the expressed hM3Dq. A similar approach was undertaken in Tacr3Cre mice in partnership with Chris Johnson of the Palmiter lab in an attempt to chemogenetically stimulate warm-sensing neurons in the MnPOA and elicit behavioral thermoregulation in mice.

4.1.1 *Virus functionality in* Kiss1$^{CreGFP.v2}$ *mice indicates localized Cre expression in the arcuate nucleus*

Serotype 1 adeno-associated virus (AAV) constructs containing the floxed, inactive hM3Dq gene were prepared following the protocol detailed in Gore *et al.*, 2014(64). The virus was injected at a titer of 10^9 viral particles per microliter following established protocols(69). Virus prepared and injected in this way has previously been shown to be functional as evidenced by neuronal transduction and behavioral outcomes(64, 69). I expected to see localized viral

expression in the arcuate nucleus following injection as Cre expression in *Kiss1*$^{CreGFP.v2}$ mice was shown to be confined to Kiss1 neurons (Fig. 9, shared by S. Padilla). Robust expression of the AAV-DIO-hM3Dq-mCherry construct used for my experiments can be seen localized to the arcuate nucleus in our KissCre2 animals, providing a means to correlate experimental animal behavior with hM3Dq receptor expression in Kiss1 neurons (Fig. 10).

4.1.2 *CNO functionality*

CNO received from the NIMH was prepared as described in the methods chapter and based on published protocols(70). The behavioral results obtained using my stock solutions of CNO were the same as those obtained when CNO stock solutions (also obtained from the NIMH) were prepared in the Palmiter lab (Fig. 20), indicating that my CNO solutions were effective. When the Palmiter lab's CNO stock was later used to stimulate the mice prior to perfusion and histological processing, Fos induction was observed in Kiss1 neurons (Fig. 20C), suggesting that my CNO could induce a similar increase in Fos expression in activated neurons.

4.1.3 *Lack of LH response to unilateral stimulation of KNDy neurons under isoflurane*

I aimed to use LH levels as evidence of successful Kiss1 neuronal stimulation. I reasoned that, similar to what occurs during a hot flash, stimulation of Kiss1 neurons would induce both a surge in LH release and thermoregulatory dysfunction. Contrary to my prediction, I observed no rise in circulating levels of LH following administration of CNO to either male or female KissCre2 mice unilaterally infected with AAV-DIO-hM3Dq (Fig. 11). Indeed, LH levels often declined following treatment (perhaps reflecting an acute stress response). Unfortunately, an

error in brain slice storage led to slices that were too damaged to be processed for Fos induction in mCherry-expressing neurons following CNO.

4.1.4 *Lack of LH or cold-seeking response to bilateral stimulation of Kiss1 neurons in intact KissCre2 mice*

I reasoned that the lack of LH response in unilaterally-stimulated animals could be due to insufficient expression of virus and, thus, insufficient recruitment of KNDy neurons. Bilaterally-infected animals should contain twice the number of receptor-expressing KNDy neurons that could be activated by CNO. Contrary to expectations, however, I observed only subtle elevations in LH levels following administration of CNO to intact male and female KissCre2 mice that were bilaterally infected with AAV-DIO-hM3Dq (Figs. 12, 13). Based on my lab's expertise, the magnitude of these increases was far less than what would be expected if *Kiss1*-expressing neurons were truly activated. Moreover, I observed no cold-seeking behavior following CNO administration to these mice on the thermocline (Fig. 14). This lack of behavior does not reveal whether Kiss1 neurons were simply not activated or they simply didn't induce the predicted behavior. Unfortunately, an error in brain slice storage led to slices that were too damaged to be processed for Fos induction in mCherry-expressing neurons following CNO.

4.1.5 *No evidence of cold-seeking behavior following bilateral stimulation of Kiss1 neurons in intact and gonadectomized KissCre2 mice*

Considering the failure to induce LH release in intact animals, I reasoned that perhaps the presence of sex steroids in these mice was keeping Kiss1 neurons suppressed enough to prevent stimulation via hM3Dq. Thus, I decided to attempt chemogenetic stimulation in intact *and* gonadectomized (GDX) animals in which a lack of sex steroids would cause Kiss1 neurons to be

stocked with neuropeptides and more easily stimulated. I decided to include intact animals again to see if perhaps they would show a behavioral thermoregulation response without a rise in LH and for direct comparison with GDX animals. However, I found no evidence of cold-seeking behavior following administration of CNO to intact male and female KissCre2 mice bilaterally infected with AAV-DIO-hM3Dq (Figs. 15, 16). (Both sexes showed an increase in temperature preference over time, a phenomenon also shown in non-stimulated mice.) I also found no evidence of cold-seeking behavior following administration of CNO to either GDX male or female KissCre2 mice that I bilaterally infected with AAV-DIO-hM3Dq (Figs. 17, 18). Subsequent histological analysis revealed that only 2 out of 11 intact mice expressed the hM3Dq gene as evidenced by mCherry immunostaining in the ARC. On the other hand, all 6 of the male GDX mice expressed mCherry, but only 2 out of 6 female GDX mice expressed mCherry. Furthermore, I observed no evidence of Fos induction in mCherry-expressing neurons, but questions remain as to whether this was due to defective antibodies, a lack of reliable positive controls for Fos expression, or a lack of Fos induction. However, knowing that the virus is reliable and strongly expressed when correctly injected, the lack of cold-seeking response in the GDX male mice as well as mice with low virus expression suggests that Kiss1 neuronal stimulation does not drive behavioral thermoregulation in mice.

4.1.6 *Cold-seeking behavior observed following bilateral stimulation of Kiss1 neurons in KissCre2 mice expressing hM3Dq and ChR2*

I partnered with Dr. Stephanie Padilla of the Palmiter lab to test if intact mice that had been intracranially injected by her could be successfully stimulated on the thermocline. Her mice had previously been intracranially injected with AAV-DIO-hM3Dq and AAV-DIO-ChR2 in the arcuate nucleus and used for optogenetic and chemogenetic experiments. Following

administration of CNO on the thermocline, 4 out of 8 male mice and 1 female mouse displayed significant periods of cold-seeking behavior (Figs. 19, 21). However, this result was obtained following exposure of the mice to repeated neuronal stimulation. Therefore, I was reluctant to accept this outcome without repeating the experiment with a larger cohort of naïve KissCre2 mice that only expressed the hM3Dq gene. These mice did demonstrate induction of Fos expression following CNO, however, lending support to the hypothesis that Kiss1 neurons could drive behavioral thermoregulation (Fig. 20C).

4.1.7 *Lack of cold-seeking behavior observed following bilateral stimulation of Kiss1 neurons in male and cycled-female KissCre2 mice*

In the repeat experiment, Dr. Padilla performed the bilateral intracranial injections of 3 male and 16 female KissCre2 mice with AAV-DIO-hM3Dq-mCherry into the arcuate nucleus. I decided to cycle the female mice based on the rationale that female mice in the diestrus state (*i.e.*, low circulating estrogen) would have uninhibited Kiss1 neurons more primed for stimulation. However, I observed no evidence of cold-seeking behavior following administration of CNO (Fig. 22). (Rather, both sexes showed an increase in temperature preference over time, a result that was shown to be the normal behavior of non-stimulated mice on the thermocline over time.) Subsequent histological analysis revealed that all of the female mice expressed the hM3Dq gene as evidenced by mCherry immunostaining in the arcuate nucleus. The males, however, showed little to no mCherry expression. Furthermore, I observed no evidence of Fos induction in mCherry-expressing neurons, but questions remain as to whether this was due to bad antibodies, a lack of a reliable positive control, or a lack of Fos induction. However, knowing that the virus is reliable and strongly expressed when correctly injected, the lack of cold-seeking response in

the female mice suggests that KNDy neuronal stimulation does not drive behavioral thermoregulation in mice – at least in females.

4.1.8 *Cold-seeking response following chemogenetic stimulation of Tacr3Cre mice on the thermocline*

Hoping to see a positive result from chemogenetic stimulation of mice on the thermocline, I partnered with Chris Johnson of the Palmiter lab to stimulate the warm-sensing neurons in the MnPOA of intact Tacr3Cre mice. This experiment would also interrogate the ability of these neurons to drive behavioral thermoregulation. His mice had previously been intracranially injected with AAV-DIO-hM3Dq and AAV-DIO-ChR2 in the MnPOA. When I stimulated the mice with CNO on the thermocline, I observed significant cold-seeking behavior (Fig. 23; $p<0.01$). Control female mice infected with a DIO-mCherry virus (not expressing the hM3Dq gene) did not exhibit cold-seeking behavior following CNO administration. However, the response to CNO was not as robust as I would have anticipated, with animals not cold-seeking until around 20 min post-injection. I believe this experiment would have to be repeated with immediate sacrifice and perfusion of the mice to make sure such a result was due to neuronal stimulation and was not an anomalous result. [Chris Johnson is currently planning to follow up on this result, including performing histological analyses to ensure robust virus expression in the MnPOA.]

4.2 RESULTS: OPTOGENETIC STIMULATION

Due to the lack of positive results from chemogenetic stimulation, I decided to use optogenetics to stimulate Kiss1 neurons within *Kiss1*$^{CreGFP.v2}$/Ai32$^{+/-}$ (KC2/Ai32) mice. These mice endogenously express ChR2 within Kiss1 neurons in the arcuate nucleus. I reasoned that

use of this mouse line would obviate the risks associated with surgical administration of the viral construct as well as variable viral uptake and expression of ChR2 by Kiss1 neurons. The only surgery performed on these mice was to implant to the fiber-optic cannula into the arcuate nucleus. Other groups had shown that Kiss1 neurons could be stimulated *ex vivo* and *in vivo* using optogenetics(71-73). Thus, I reasoned that perhaps these neurons were better stimulated via optogenetic techniques than chemogenetic means or that a more critical mass of Kiss1 neurons would be expressing ChR2 and so be able to be recruited.

For an additional optogenetic study, I partnered with Chris Johnson of the Palmiter group to stimulate the Tacr3-expressing warm-sensing neurons in the MnPOA of Tacr3Cre mice. This experiment was designed to test the putative role of Tacr3-expressing neurons in the MnPOA (the presumed downstream targets of KNDy neurons) in driving VMS in mice. We collaborated to inject AAV constructs containing the ChR2 gene into the MnPOA, implant fiber-optic cannulas, and then stimulate the animals on the thermocline.

4.2.1 *Laser and fiber-optic functionality and power*

These experiments required materials (*e.g.*, patch cords, cannulas, lasers) generously provided by the Palmiter lab(69). The lab routinely tests the output of their patch cords. In addition, my collaborators test the output power of each fiber-optic cannula, knowing that the wattage output will be diminished between the patch cord and the cannula. Knowing how much power is lost between the patch cord and the cannula, they can then adjust the power of the laser accordingly to ensure a precise wattage emission from the fiber-optic cannula into the brain. For our studies, the output wattage of the light from the fiber-optic cannula was 10 milliwatts.

4.2.2 *Lack of cold-seeking behavior in optogenetically stimulated KC2/Ai32 mice*

Stimulation of KC2/Ai32 mice on the thermocline failed to elicit cold-seeking behavior (Fig. 24). The mice showed a significant increase in temperature preference following laser stimulation; however, the mice showed the same increase in preferred thermocline temperature when they were tethered, but not stimulated with light (Fig. 25). This result led us to conclude that mice will normally seek progressively warmer locations on the thermocline. Histological examination of the KC2/Ai32 arcuate nuclei is ongoing to determine if these mice robustly express the ChR2 channel in Kiss1 neurons and exhibit Fos expression following optogenetic stimulation.

4.2.3 *Cold-seeking response following optogenetic stimulation of Tacr3Cre mice*

I used the same intact female Tacr3Cre mice bilaterally infected with AAV-DIO-hM3Dq and AAV-DIO-ChR2 (or control DIO-mCherry virus) for this study (see 4.1.8). I observed cold-seeking behavior by the ChR2-expressing, but not control, mice (Fig. 26, $p<0.01$). [Chris Johnson is currently planning to follow up on this result, including performing histological analyses to ensure robust virus expression.]

4.3 DISCUSSION: CHEMOGENETIC AND OPTOGENETIC STIMULATION

I aimed to use chemogenetic or optogenetic stimulation to test the hypothesis that KNDy neurons drive VMS through aberrant stimulation of warm-sensing neurons (WSN) in the MnPOA. I chose these two strategies to stimulate Kiss1 neurons based on observations published by the Palmiter and Herbison labs. The Palmiter group has repeatedly activated and suppressed

hypothalamic neuronal populations using the same tools and procedures utilized by our lab(74, 75). In addition, the Herbison group has published a report detailing stimulation of LH release following optogenetic stimulation of Kiss1 neurons in anesthetized male mice and anesthetized GDX female mice(71).

Preliminary studies conducted by our group suggested that it would be possible to chemogenetically and optogenetically stimulate neuronal populations while animals are freely behaving on the thermocline. By combining autonomic measures and thermocline behavior, I aimed to produce a complete picture of how stimulation of KNDy neurons impacts thermal perception in awake, undisturbed animals. However, attempts to activate Kiss1 neurons via DREADDs and ChR2 have not produced a reliable readout of neuronal activation nor a demonstrable effect on behavioral thermoregulation in mice.

In beginning this project, I knew that I could obtain robust viral expression of the hM3Dq receptor in the arcuate nucleus of my KissCre2 mice, indicating that these neurons should be susceptible to stimulation following CNO binding. To gain evidence of Kiss1 neuronal activation, I aimed to measure LH concentrations in the blood before and after administration of CNO. I hypothesized that I would observe an increase in the circulating LH concentration following CNO treatment. However, chemogenetic stimulation of several cohorts of KissCre2 mice failed to elicit a rise in LH following CNO. The null effect of CNO on LH was observed regardless of sex, gonadal state (*i.e.*, intact or GDX), or the presence of anesthesia. Because the results of the Herbison group showed that not all intact mice were sensitive to optogenetic activation, I was not overly concerned that I couldn't evoke LH secretion with DREADD activation in my intact KissCre2 mice. However, I reasoned that the KNDy neurons of GDX mice would be optimally primed for CNO stimulation and that neuropeptide release from KNDy

neurons would induce LH secretion as seen by the Herbison group. However, because I did not observe an effect on LH in GDX mice, I reasoned that chemogenetic stimulation was insufficient to stimulate KNDy neurons and cause behavioral thermoregulation in mice.

Optogenetic stimulation, however, presented notable advantages to chemogenetic stimulation. Previous *in vitro* studies in Kiss1 neurons had shown that these neurons were capable of responding to strong optogenetic stimulation(72, 73). Stimulating neurons with light also allows for greater temporal control over neuronal stimulation, circumventing concerns about CNO penetration into the parenchyma. In addition, transgenic mice were available that endogenously express ChR2, obviating the need for intracranial viral injections. In crossing these mice with mice from the KissCre2 line, my collaborators in the Palmiter lab created $Kiss1^{CreGFP.v2}/Ai32^{+/-}$ (KC2/Ai32) cross animals that endogenously express ChR2 exclusively in Kiss1 neurons. I hypothesized that optogenetic stimulation of Kiss1 neurons in the arcuate nuclei of these double-heterozygous KC2/Ai32 mice would induce behavioral thermoregulation in mice freely behaving on the thermocline.

Subsequent studies in which intact male and female KC2/Ai32 mice were optogenetically stimulated on the thermocline, however, suggested that stimulation of Kiss1 neurons in the arcuate nucleus does not elicit behavioral thermoregulation in mice. Final conclusions must await histological analysis of ChR2 and Fos expression in the arcuate nucleus following optogenetic stimulation. Genotyping of the animals indicated that these animals should show robust ChR2 expression in the arcuate nucleus. Lack of Fos expression, however, would indicate that these arcuate neurons – even with ChR2 expression – were not stimulated by the light. If the neurons showed no signs of stimulation, I would reason that there was not sufficient ChR2 expression. There is inconsistency in the literature as to whether animals must be heterozygous

or homozygous for sufficient endogenous expression levels that would permit neuronal stimulation(76-80). Future directions for our lab will include an examination of whether murine thermoregulatory behavior differs between mice that are heterozygous and homozygous for the Ai32 allele. However, breeding has been complicated by the occurrence of gene recombination early in development, leading to the expression of the transgene throughout the body. Careful genotyping will be conducted to ensure that only mice with the proper genetics are used for future studies.

4.4 Conclusions from chemogenetic and optogenetic stimulation of Kiss1 neurons

I reason that my results point to one of two conclusions. First, it is possible I never stimulated Kiss1 neurons. Despite ensuring reliable virus preparation and visualizing robust hM3Dq expression in the arcuate nucleus, I was unable to visualize co-localization of mCherry with another marker to indicate expression in Kiss1 neurons or with a Fos marker to indicate that mCherry-expressing neurons were activated. Thus, the argument remains that stimulation of Kiss1 neurons never took place.

The second possibility is that stimulation of Kiss1 neurons in the arcuate nucleus does not induce behavioral thermoregulation. However, additional experiments could provide less equivocal results to refute or support my original hypothesis. For instance, using a dual-virus strategy, it would be possible to stimulate only those KNDy neurons that project to the MnPOA. Should stimulation of these MnPOA-projecting neurons cause thermo-deregulation, then one could reason that there exists a specific population of KNDy neurons that drive hot flash production. Perhaps when the entire KNDy population is recruited – as would have occurred in my animals – this thermoregulatory signal is lost in the noise of mass KNDy neuronal activity.

By isolating only those MnPOA-projecting KNDy neurons, one could interrogate solely the role of these neurons in driving VMS in mice. The other experiment I would undertake would examine if non-KNDy, NKB-expressing neurons in the arcuate nucleus could drive hot flashes. Like KNDy neurons, these neurons express NKB but do not express the other KNDy neuropeptides. Perhaps these neurons also become hyperactive following hormone loss (due to vacant estrogen receptors or stimulation by other hormone-sensitive neurons) and signal strongly to NK3R-expressing neurons in the MnPOA. Both of these prospective studies could lend insight into hypothalamic neuronal populations and expand our understanding of circuits governing thermoregulation.

4.5 Conclusions regarding the thermoregulatory effect of stimulating Tacr3-expressing neurons in the MnPOA

In addition to the above-mentioned follow-up experiments, I anticipate sharing our resources with personnel in the Palmiter lab to follow up on our observation of cold-seeking behavior following stimulation of Tacr3-expressing neurons in the MnPOA. The positive result from the Tacr3Cre mouse line is encouraging that the underlying experimental strategy is sound – that combining neuronal stimulation and thermocline technology can be useful in interrogating the thermoregulatory system. For instance, we are curious to know to what other nuclei the warm-sensing neurons project in order to bring about recruitment of heat dissipation effectors, such as vasodilation and cold-seeking. Work by Dr. Knight at UCSF suggests that discrete populations of neurons reside in the MnPOA, each potentially mediating a downstream thermoregulatory effector mechanism or behavior(81). Thus, the discrete population of NK3R-expressing neurons to which KNDy neurons project could be playing a specific role in thermoregulation. By combining tract tracing, dual-virus protocols, and single-cell RNA-seq, I

may be able to parse out more phenotypic information on this neuronal population and continue to expand our understanding of thermoregulation and hot flash generation. Perhaps discrete NK3R-expressing populations in the MnPOA could be stimulated to evoke specific thermoregulatory responses on the thermocline. Overall, the preliminary findings in the Tacr3Cre line could foreshadow future discoveries made with the thermocline and neuronal stimulation strategies.

Chapter 5. CONCLUSIONS & FUTURE DIRECTIONS

This chapter contains information that was submitted to the Journal of Endocrinology by the author and other members of the Steiner-Clifton-Neal-Perry lab (Krull *et al.*, 2017).

5.1 UTILITY OF THERMOCLINE FOR READOUT OF MURINE BEHAVIORAL THERMOREGULATION

I report the development of a reliable murine model of hot flashes. Using our engineered temperature gradient, we can remotely observe murine behavioral thermoregulation in response to a variety of perturbations, including pharmacologic agents such as capsaicin and senktide. Most notably, mice administered senktide – which acts on the NKB receptor implicated in hot flash production – demonstrate a clear change in their thermal preference, causing them to spend significantly more time in cooler temperatures along our thermocline. Like hot flash patients, these mice only perceive a threat to their thermoneutrality, and yet respond behaviorally. We aim to use this model to elucidate the neurological basis of hot flashes. In addition, we plan to utilize the thermocline, neural stimulation, and novel therapeutics to conduct pre-clinical trials that could motivate the use of newer, safer medications to treat hot flashes in humans.

5.2 FUTURE EXPERIMENTAL DIRECTIONS AND PRE-CLINICAL TRIALS

We anticipate the thermocline being used for pre-clinical testing of potential hot flash therapeutics. Should any thermoregulatory behavior be induced on the thermocline via KNDy stimulation or other means, scientists could then attempt to block such behavior with pharmacological treatments. There are at least two new potential therapies that could be tested for efficiency with this technology. First, studies have shown that kappa opioid receptor (KOR) agonists – acting in the same manner as Dyn – could silence the KNDy neuronal activity driving VMS(38). Moreover, KOR agonists have been engineered to be peripherally-restricted (PRKAs), having limited access to the central nervous system. PRKAs would – in theory – obviate the side effects of non peripherally-restricted agonists (*e.g.,* nausea, cognitive impairment, and dysphoria), yet still block VMS. Although the ideal PRKA would not cross the blood-brain barrier (BBB), they would in all likelihood inhibit the activity of KNDy neurons in the arcuate/infundibular nucleus, which resides outside of the confines of the BBB. A clinical trial conducted by our group demonstrated that a *non-peripherally-restricted* KOR agonist can suppress the frequency of menopausal VMS(82), providing a tantalizing proof-of-concept for the idea. Second, NK3R antagonists have proven efficacy in the treatment of polycystic ovarian syndrome (PCOS) and VMS(83, 84). These compounds presumably work by suppressing the activity of either KNDy neurons or WSN in the MnPOA – or both. Our results showing that senktide induces Fos expression in the MnPOA supports a strategy of targeting the WSN in the MnPOA for VMS relief. By combining neuronal stimulation on the thermocline with such treatment compounds, we could elucidate the neurobiological basis of VMS and test the efficacy of would-be blockers that could bring relief to people affected by hot flashes.

Bibliography

1. Avis NE, SL Crawford, SM McKinlay 1997 Psychosocial, behavioral, and health factors related to menopause symptomatology. Womens Health 3:103-120
2. Feldman BM, A Voda, E Gronseth 1985 The prevalence of hot flash and associated variables among perimenopausal women. Res Nurs Health 8:261-268
3. Hunter MS, A Gentry-Maharaj, A Ryan, M Burnell, A Lanceley, L Fraser, I Jacobs, U Menon 2012 Prevalence, frequency and problem rating of hot flushes persist in older postmenopausal women: impact of age, body mass index, hysterectomy, hormone therapy use, lifestyle and mood in a cross-sectional cohort study of 10,418 British women aged 54-65. BJOG 119:40-50
4. Ahmadi H, S Daneshmand 2013 Androgen deprivation therapy: evidence-based management of side effects. BJU Int 111:543-548
5. Spetz AC, EL Zetterlund, E Varenhorst, M Hammar 2003 Incidence and management of hot flashes in prostate cancer. J Support Oncol 1:263-6, 269
6. Reed S, MPH MD, K Newton, A LaCroix, L Grothaus, V Grieco, K Ehrlich 2008 Vaginal, endometrial, and reproductive hormone findings: randomized, placebo-controlled trial of black cohosh, multibotanical herbs, and dietary soy for vasomotor symptoms: the Herbal Alternatives for Menopause (HALT) Study. Menopause 15:51-58
7. Voda AM 1981 Climacteric hot flash. Maturitas 3:73-90
8. Kronenberg F, LJ Cote, DM Linkie, I Dyrenfurth, JA Downey 1984 Menopausal hot flashes: thermoregulatory, cardiovascular, and circulating catecholamine and LH changes. Maturitas 6:31-43

9. Kronenberg F 1990 Hot flashes: epidemiology and physiology. Ann N Y Acad Sci 592:52-86; discussion 123
10. Irwig MS, GS Fraley, JT Smith, BV Acohido, SM Popa, MJ Cunningham, ML Gottsch, DK Clifton, RA Steiner 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264-272
11. Arreguin-Arevalo JA, CA Lents, TA Farmerie, TM Nett, CM Clay 2007 KiSS-1 peptide induces release of LH by a direct effect on the hypothalamus of ovariectomized ewes. Anim Reprod Sci 101:265-275
12. Keen KL, FH Wegner, SR Bloom, MA Ghatei, E Terasawa 2008 An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology 149:4151-4157
13. Goodman RL, SM Hileman, CC Nestor, KL Porter, JM Connors, SL Hardy, RP Millar, M Cernea, LM Coolen, MN Lehman 2013 Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. Endocrinology 154:4259-4269
14. Millar RP, ZL Lu, AJ Pawson, CA Flanagan, K Morgan, SR Maudsley 2004 Gonadotropin-releasing hormone receptors. Endocr Rev 25:235-275
15. Eghlidi DH, GE Haley, NC Noriega, SG Kohama, HF Urbanski 2010 Influence of age and 17beta-estradiol on kisspeptin, neurokinin B, and prodynorphin gene expression in the arcuate-median eminence of female rhesus macaques. Endocrinology 151:3783-3794

16. Tomikawa J, T Homma, S Tajima, T Shibata, Y Inamoto, K Takase, N Inoue, S Ohkura, Y Uenoyama, K Maeda, H Tsukamura 2010 Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. Biol Reprod 82:313-319
17. Mittelman-Smith MA, H Williams, SJ Krajewski-Hall, J Lai, P Ciofi, NT McMullen, NE Rance 2012 Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology 153:2800-2812
18. Pielecka-Fortuna J, Z Chu, SM Moenter 2008 Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. Endocrinology 149:1979-1986
19. Navarro VM, ML Gottsch, C Chavkin, H Okamura, DK Clifton, RA Steiner 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci 29:11859-11866
20. Smith JT, HM Dungan, EA Stoll, ML Gottsch, RE Braun, SM Eacker, DK Clifton, RA Steiner 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976-2984
21. Smith JT, MJ Cunningham, EF Rissman, DK Clifton, RA Steiner 2005 Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692
22. Santoro N 2005 The menopausal transition. Am J Med 118 Suppl 12B:8-13
23. Broekmans FJ, EA Knauff, ER te Velde, NS Macklon, BC Fauser 2007 Female reproductive ageing: current knowledge and future trends. Trends Endocrinol Metab 18:58-65

24. Judd HL, GE Judd, WE Lucas, SS Yen 1974 Endocrine function of the postmenopausal ovary: concentration of androgens and estrogens in ovarian and peripheral vein blood. J Clin Endocrinol Metab 39:1020-1024

25. Rannevik G, S Jeppsson, O Johnell, B Bjerre, Y Laurell-Borulf, L Svanberg 1995 A longitudinal study of the perimenopausal transition: altered profiles of steroid and pituitary hormones, SHBG and bone mineral density. Maturitas 21:103-113

26. Meldrum DR, BJ Davidson, IV Tataryn, HL Judd 1981 Changes in circulating steroids with aging in postmenopausal women. Obstet Gynecol 57:624-628

27. Casper RF, SS Yen 1985 Neuroendocrinology of menopausal flushes: an hypothesis of flush mechanism. Clin Endocrinol (Oxf) 22:293-312

28. Casper RF, SS Yen, MM Wilkes 1979 Menopausal flushes: a neuroendocrine link with pulsatile luteninizing hormone secreation. Science 205:823-825

29. Tataryn IV, DR Meldrum, KH Lu, AM Frumar, HL Judd 1979 LH, FSH and skin temperaure during the menopausal hot flash. J Clin Endocrinol Metab 49:152-154

30. Meldrum DR, Y Erlik, JK Lu, HL Judd 1981 Objectively recorded hot flushes in patients with pituitary insufficiency. J Clin Endocrinol Metab 52:684-688

31. DeFazio J, DR Meldrum, L Laufer, W Vale, J Rivier, JK Lu, HL Judd 1983 Induction of hot flashes in premenopausal women treated with a long-acting GnRH agonist. J Clin Endocrinol Metab 56:445-448

32. Gambone J, DR Meldrum, L Laufer, RJ Chang, JK Lu, HL Judd 1984 Further delineation of hypothalamic dysfunction responsible for menopausal hot flashes. J Clin Endocrinol Metab 59:1097-1102

33. Casper RF, SS Yen 1981 Menopausal flushes: effect of pituitary gonadotropin desensitization by a potent luteinizing hormone- releasing factor agonist. J Clin Endocrinol Metab 53:1056-1058

34. Lehman MN, LM Coolen, RL Goodman 2010 Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. Endocrinology 151:3479-3489

35. Seminara SB, S Messager, EE Chatzidaki, RR Thresher, JSJ Acierno, JK Shagoury, Y Bo-Abbas, W Kuohung, KM Schwinof, AG Hendrick, D Zahn, J Dixon, UB Kaiser, SA Slaugenhaupt, JF Gusella, S O'Rahilly, MB Carlton, WFJ Crowley, SA Aparicio, WH Colledge 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614-1627

36. Funes S, JA Hedrick, G Vassileva, L Markowitz, S Abbondanzo, A Golovko, S Yang, FJ Monsma, EL Gustafson 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312:1357-1363

37. Goodman RL, MN Lehman, JT Smith, LM Coolen, CV de Oliveira, MR Jafarzadehshirazi, A Pereira, J Iqbal, A Caraty, P Ciofi, IJ Clarke 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752-5760

38. Wakabayashi Y, T Nakada, K Murata, S Ohkura, K Mogi, VM Navarro, DK Clifton, Y Mori, H Tsukamura, K Maeda, RA Steiner, H Okamura 2010 Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci 30:3124-3132

39. Ramaswamy S, SB Seminara, B Ali, P Ciofi, NA Amin, TM Plant 2010 Neurokinin B stimulates GnRH release in the male monkey (Macaca mulatta) and is colocalized with kisspeptin in the arcuate nucleus. Endocrinology 151:4494-4503

40. Navarro VM, JM Castellano, SM McConkey, R Pineda, F Ruiz-Pino, L Pinilla, DK Clifton, M Tena-Sempere, RA Steiner 2011 Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. Am J Physiol Endocrinol Metab 300:E202-E210

41. Navarro VM, ML Gottsch, M Wu, D Garcia-Galiano, SJ Hobbs, MA Bosch, L Pinilla, DK Clifton, A Dearth, OK Ronnekleiv, RE Braun, RD Palmiter, M Tena-Sempere, M Alreja, RA Steiner 2011 Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. Endocrinology 152:4265-4275

42. Krajewski SJ, MC Burke, MJ Anderson, NT McMullen, NE Rance 2010 Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. Neuroscience 166:680-697

43. Rance NE, NT McMullen, JE Smialek, DL Price, WS Young 1990 Postmenopausal hypertrophy of neurons expressing the estrogen receptor gene in the human hypothalamus. J Clin Endocrinol Metab 71:79-85

44. Rance NE, WS Young 1991 Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. Endocrinology 128:2239-2247

45. Rance NE, TR Bruce 1994 Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. Neuroendocrinology 60:337-345

46. Rometo AM, SJ Krajewski, ML Voytko, NE Rance 2007 Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab 92:2744-2750

47. Rometo AM, NE Rance 2008 Changes in prodynorphin gene expression and neuronal morphology in the hypothalamus of postmenopausal women. J Neuroendocrinol 20:1376-1381

48. Hrabovszky E, CS Molnar, MT Sipos, B Vida, P Ciofi, BA Borsay, L Sarkadi, L Herczeg, SR Bloom, MA Ghatei, WS Dhillo, I Kallo, Z Liposits 2011 Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women. Front Endocrinol (Lausanne) 2:80

49. Yeo SH, AE Herbison 2011 Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. Endocrinology 152:2387-2399

50. Mittelman-Smith MA, SJ Krajewski-Hall, NT McMullen, NE Rance 2015 Neurokinin 3 Receptor-Expressing Neurons in the Median Preoptic Nucleus Modulate Heat-Dissipation Effectors in the Female Rat. Endocrinology 156:2552-2562

51. Rance NE, PA Dacks, MA Mittelman-Smith, AA Romanovsky, SJ Krajewski-Hall 2013 Modulation of body temperature and LH secretion by hypothalamic KNDy (kisspeptin, neurokinin B and dynorphin) neurons: a novel hypothesis on the mechanism of hot flushes. Front Neuroendocrinol 34:211-227

52. Gervais NJ, SS Viechweg, JA Mong, A Lacreuse 2016 The middle-aged ovariectomized marmoset (Callithrix jacchus) as a model of menopausal symptoms: Preliminary evidence. Neuroscience 337:1-8

53. Kobayashi T, M Tamura, M Hayashi, Y Katsuura, H Tanabe, T Ohta, K Komoriya 2000 Elevation of tail skin temperature in ovariectomized rats in relation to menopausal hot flushes. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology Am J Physiol Regul Integr Comp Physiol 278:R863
54. Albertson AJ, DC Skinner 2009 A Novel Animal Model to Study Hot Flashes: No Effect of GnRH. Menopause Menopause (New York, NY) 16:1030-1036
55. Holinka CF, M Brincat, B Coelingh, H. J. T. 2008 Preventive effect of oral estetrol in a menopausal hot flush model. Climacteric Climacteric 11:15-21
56. Shuto H, A Yamauchi, M Ikeda, Y Sohda, A Koga, K Tominaga, T Egawa, Y Kataoka 2005 Forced Exercise-Induced Flushing of Tail Skin in Ovariectomized Mice, as a New Experimental Model of Menopausal Hot Flushes. Journal of Pharmacological Sciences 98:323-326
57. Mittelman-Smith MA, H Williams, SJ Krajewski-Hall, NT McMullen, NE Rance 2012 Role for kisspeptin/neurokinin B/dynorphin (KNDy) neurons in cutaneous vasodilatation and the estrogen modulation of body temperature. Proc Natl Acad Sci U S A 109:19846-19851
58. Sanchez-Alavez M, S Alboni, B Conti 2011 Sex- and age-specific differences in core body temperature of C57Bl/6 mice. AGE 33:89-99
59. Gordon CJ 1983 Behavioral and autonomic thermoregulation in mice exposed to microwave radiation. Journal of Applied Physiology J Appl Physiol 55:1242
60. Gordon CJ 1985 Relationship between autonomic and behavioral thermoregulation in the mouse. Physiology & Behavior 34:687-690

61. Gaskill BN, CJ Gordon, EA Pajor, JR Lucas, JK Davis, JP Garner 2012 Heat or Insulation: Behavioral Titration of Mouse Preference for Warmth or Access to a Nest. PLOS ONE PLOS ONE 7:e32799

62. Armbruster BN, X Li, MH Pausch, S Herlitze, BL Roth 2007 Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A Proceedings of the National Academy of Sciences of the United States of America 104:5163-5168

63. Smedemark-Margulies N, JG Trapani 2013 Tools, methods, and applications for optophysiology in neuroscience. Front Mol Neurosci Frontiers in Molecular Neuroscience 6:18

64. Gore BB, ME Soden, LS Zweifel 2013 Manipulating gene expression in projection-specific neuronal populations using combinatorial viral approaches. Curr Protoc Neurosci Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al] 4:4.35.1-4.35.20

65. Feketa VV, Y Zhang, Z Cao, A Balasubramanian, CM Flores, MR Player, SP Marrelli 2014 Transient receptor potential melastatin 8 channel inhibition potentiates the hypothermic response to transient receptor potential vanilloid 1 activation in the conscious mouse. Crit Care Med Critical care medicine 42:e355-e363

66. Shimizu I, T Iida, N Horiuchi, MJ Caterina 2005 5-Iodoresiniferatoxin Evokes Hypothermia in Mice and Is a Partial Transient Receptor Potential Vanilloid 1 Agonist in Vitro. Journal of Pharmacology and Experimental Therapeutics J Pharmacol Exp Ther 314:1378

67. Jayasena CN, AN Comninos, E Stefanopoulou, A Buckley, S Narayanaswamy, C Izzi-Engbeaya, A Abbara, R Ratnasabapathy, J Mogford, N Ng, Z Sarang, MA Ghatei, SR Bloom, MS Hunter, WS Dhillo 2015 Neurokinin B Administration Induces Hot Flushes in Women. Scientific Reports 5:8466

68. Dacks PA, SJ Krajewski, NE Rance 2011 Activation of neurokinin 3 receptors in the median preoptic nucleus decreases core temperature in the rat. Endocrinology 152:4894-4905

69. Padilla SL, J Qiu, ME Soden, E Sanz, CC Nestor, FD Barker, A Quintana, LS Zweifel, OK R√∏nnekleiv, MJ Kelly, RD Palmiter 2016 AgRP Neural Circuits Mediate Adaptive Behaviors in the Starved State. Nat Neurosci Nature neuroscience 19:734-741

70. Padilla SL, J Qiu, CC Nestor, C Zhang, AW Smith, BB Whiddon, OK R√∏nnekleiv, MJ Kelly, RD Palmiter 2017 AgRP to Kiss1 neuron signaling links nutritional state and fertility. Proceedings of the National Academy of Sciences

71. Han SY, T McLennan, K Czieselsky, AE Herbison 2015 Selective optogenetic activation of arcuate kisspeptin neurons generates pulsatile luteinizing hormone secretion. Proc Natl Acad Sci U S A Proceedings of the National Academy of Sciences of the United States of America 112:13109-13114

72. Qiu J, CC Nestor, C Zhang, SL Padilla, RD Palmiter, MJ Kelly, OK R√∏nnekleiv 2016 High-frequency stimulation-induced peptide release synchronizes arcuate kisspeptin neurons and excites GnRH neurons. eLife eLife 5:e16246

73. Nestor CC, J Qiu, SL Padilla, C Zhang, MA Bosch, W Fan, SA Aicher, RD Palmiter, OK R√∏nnekleiv, MJ Kelly 2016 Optogenetic Stimulation of Arcuate Nucleus Kiss1 Neurons Reveals a Steroid-Dependent Glutamatergic Input to POMC and AgRP Neurons in Male Mice. Molecular Endocrinology 30:630-644

74. Carter ME, ME Soden, LS Zweifel, RD Palmiter 2013 Genetic identification of a neural circuit that suppresses appetite. Nature 503:111-114

75. Padilla SL, J Qiu, ME Soden, E Sanz, CC Nestor, FD Barker, A Quintana, LS Zweifel, OK Ronnekleiv, MJ Kelly, RD Palmiter 2016 Agouti-related peptide neural circuits mediate adaptive behaviors in the starved state. Nat Neurosci 19:734-741

76. Madisen L, T Mao, H Koch, J- Zhuo, A Berenyi, S Fujisawa, Y-WA Hsu, AJ Garcia, X Gu, S Zanella, J Kidney, H Gu, Y Mao, BM Hooks, ES Boyden, G Buzs√°ki, JM Ramirez, AR Jones, K Svoboda, X Han, EE Turner, H Zeng 2012 A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nat Neurosci Nature neuroscience 15:793-802

77. Hsu Y-WA, SD Wang, S Wang, G Morton, HA Zariwala, lI de, Horacio O, EE Turner 2014 Role of the Dorsal Medial Habenula in the Regulation of Voluntary Activity, Motor Function, Hedonic State, and Primary Reinforcement. J Neurosci The Journal of Neuroscience 34:11366-11384

78. Hsu Y-WA, G Morton, EG Guy, SD Wang, EE Turner 2016 Dorsal Medial Habenula Regulation of Mood-Related Behaviors and Primary Reinforcement by Tachykinin-Expressing Habenula Neurons. eNeuro eNeuro 3:ENEURO.0109-ENEU16.2016

79. Han VZ, G Magnus, Y Zhang, AD Wei, EE Turner 2014 Bidirectional modulation of deep cerebellar nuclear cells revealed by optogenetic manipulation of inhibitory inputs from Purkinje cells. Neuroscience 277:250-266

80. Hedrick T, B Danskin, RS Larsen, D Ollerenshaw, P Groblewski, M Valley, S Olsen, J Waters 2016 Characterization of Channelrhodopsin and Archaerhodopsin in Cholinergic Neurons of Cre-Lox Transgenic Mice. PLoS One PLoS ONE 11:e0156596

81. Tan C, E Cooke, D Leib, Y-C Lin, G Daly, C Zimmerman, Z Knight 2016 Warm-Sensitive Neurons that Control Body Temperature. Cell 167:47-59.e15

82. Oakley AE, RA Steiner, C Chavkin, DK Clifton, LK Ferrara, SD Reed 2015 kappa Agonists as a novel therapy for menopausal hot flashes. Menopause

83. George JT, R Kakkar, J Marshall, ML Scott, RD Finkelman, TW Ho, J Veldhuis, K Skorupskaite, RA Anderson, S McIntosh, L Webber 2016 Neurokinin B Receptor Antagonism in Women With Polycystic Ovary Syndrome: A Randomized, Placebo-Controlled Trial. The Journal of Clinical Endocrinology & Metabolism 101:4313-4321

84. 2016 Stream 16 ,Äì Health in mid-life and Beyond (FC16). BJOG: An International Journal of Obstetrics & Gynaecology BJOG: Int J Obstet Gy 123:78-82

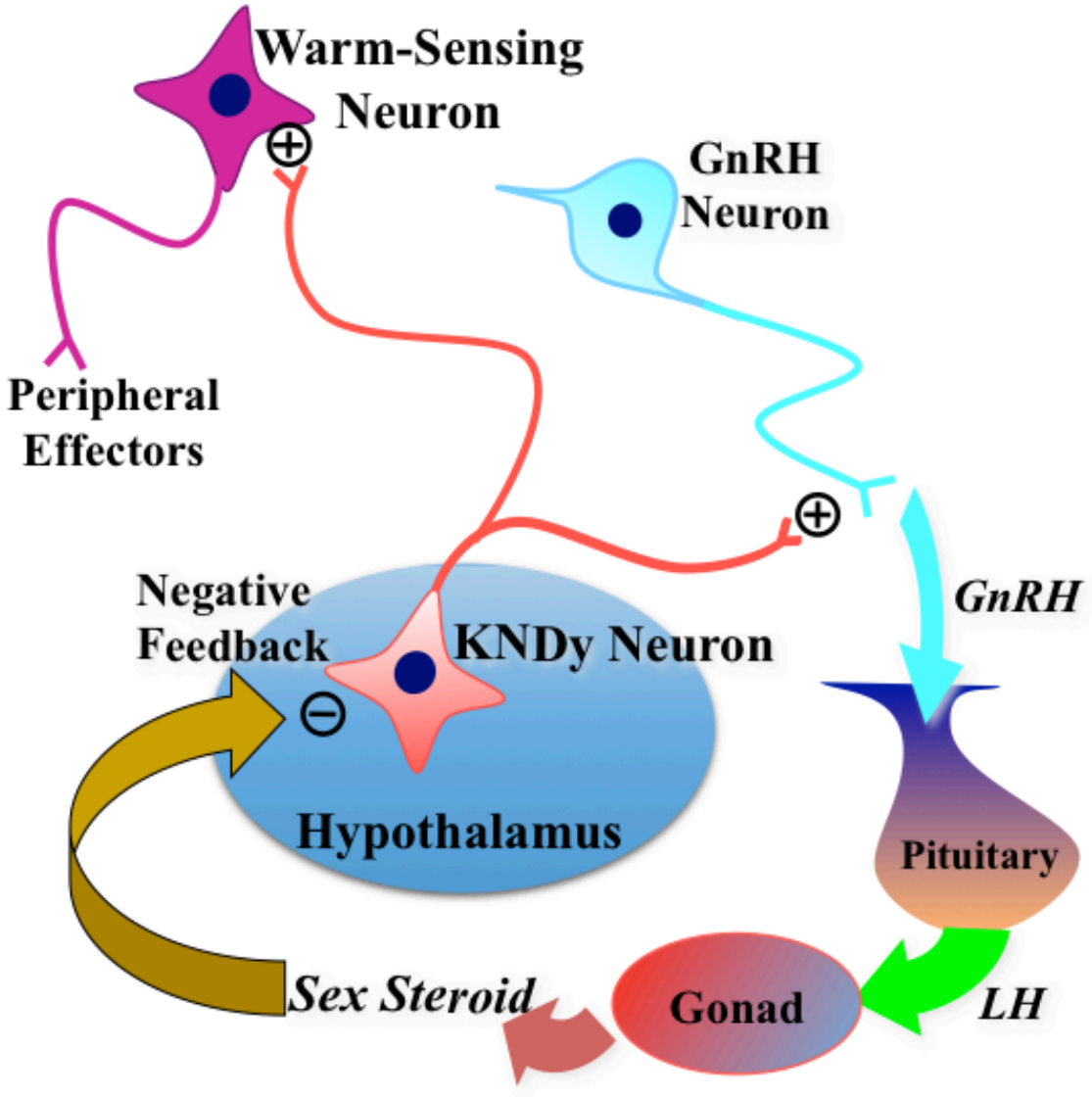

Figure 1. Hypothesized model for hot flash generation. Following the loss of sex steroids, hyperactive KNDy neurons increase their pulsatile secretion of neuropeptides onto GnRH neurons and warm-sensing neurons. This increased signaling triggers LH pulses and aberrant recruitment of heat dissipation effectors to produce the vasomotor symptoms characteristic of a hot flash.

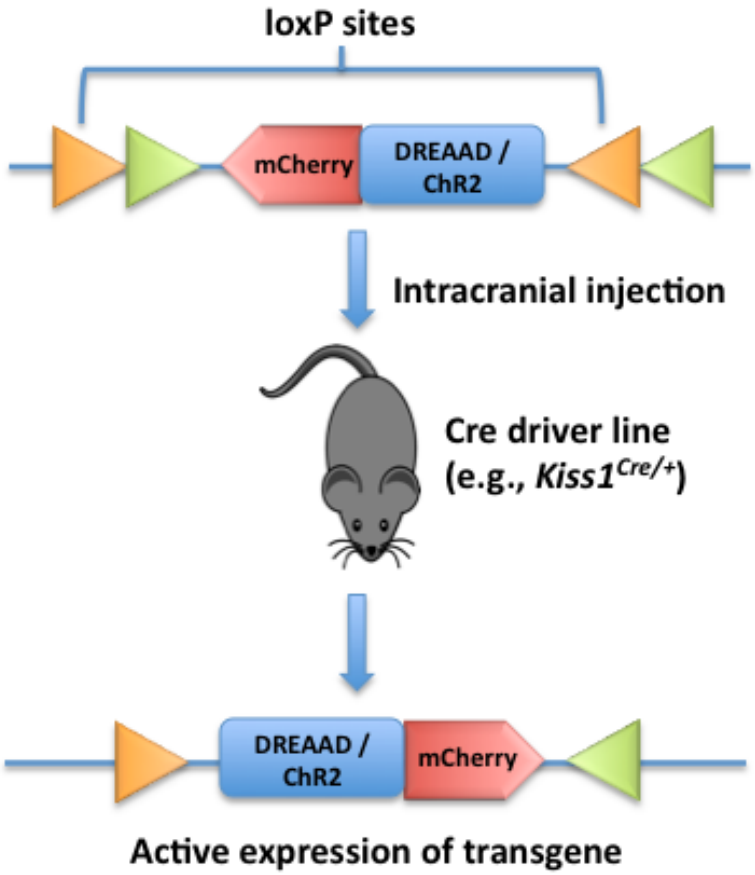

Figure 2. Explanation of double-floxed inverse open reading frame (DIO) utilization to express a transgene in a Cre driver mouse line. Figure adapted from Smedemark-Margulies et al., 2013(63).

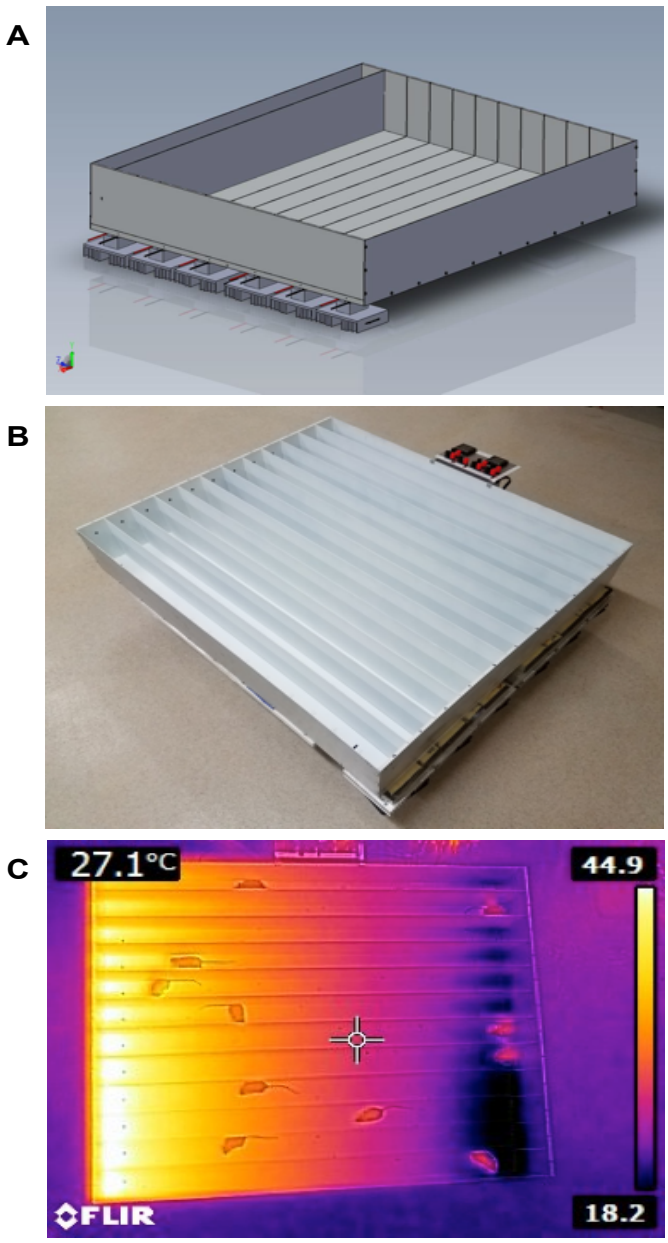

Figure 3. Thermocline design. (A) Computer-generated schematic of the thermocline showing Peltieer junctions with heat sinks and slots for lane dividers, (B) image of the constructed thermocline showing the control panel and twelve divided lanes, including markings to aid habituation of the mice to the temperature gradient, and (C) thermal camera-generated image of the functioning thermocline with freely behaving mice. The numbers on the right reflect the temperature range represented in the image. The number in the upper left corner reflects the temperature at the crosshairs.

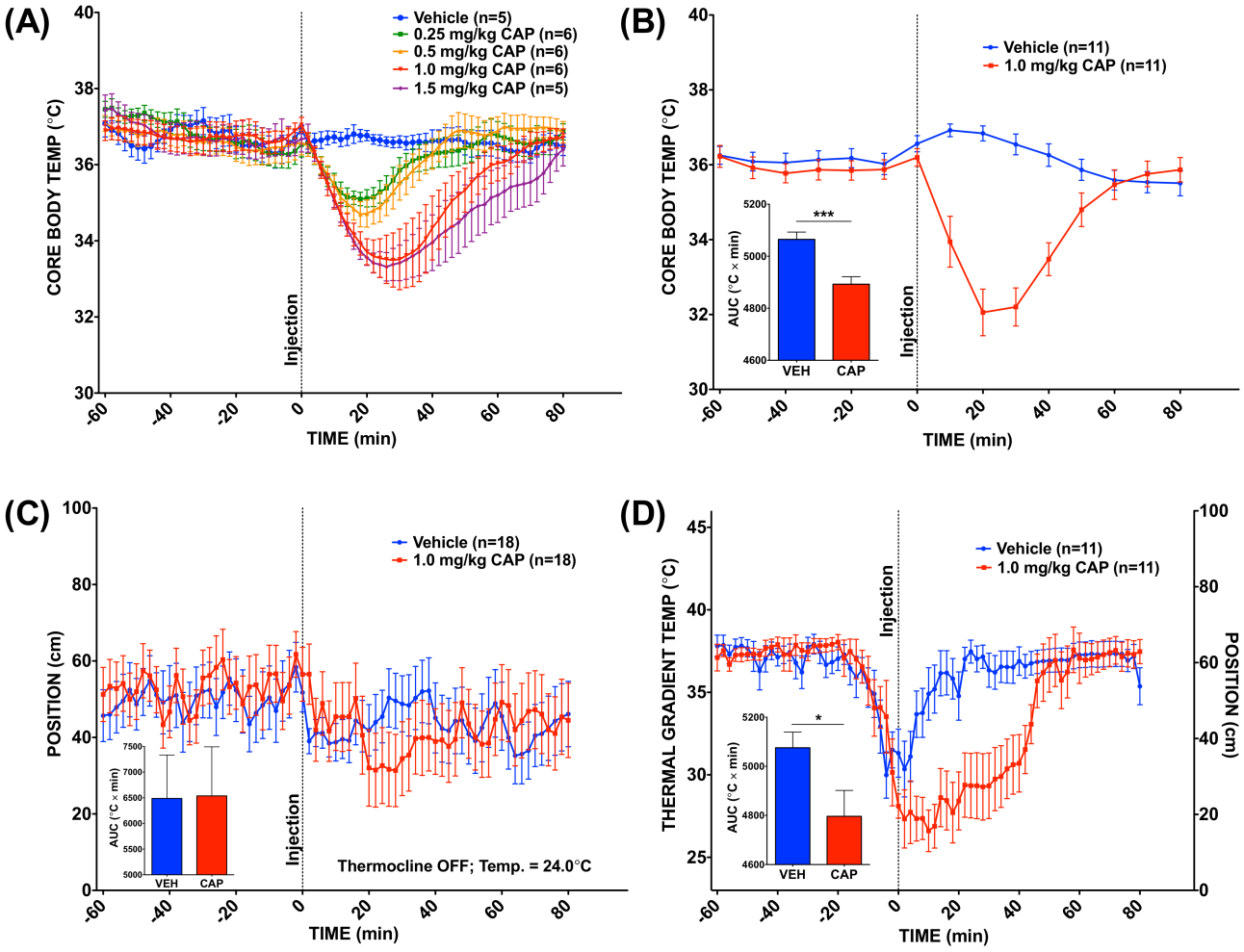

Figure 4. Core body temperature (CBT) changes and thermocline behavior in response to capsaicin. (A) CBT response to capsaicin doses in cages (n=5-6 per group), (B) CBT response in freely behaving mice administered 1.0 mg/kg capsaicin versus vehicle on the active thermal gradient (***p<0.001, paired t test of AUC, n=11 per group), (C) behavioral response to 1.0 mg/kg capsaicin versus vehicle on the inactive thermocline (p=0.959, NS, paired t test of AUC, n=18 per group), (D) behavioral response to 1.0 mg/kg capsaicin versus vehicle treatment on the active thermocline (*p=<0.05, paired t test of AUC, n=11 per group). Dotted line at time point 0 reflects the time of the injection. Values are means ± SEM. CAP = capsaicin. VEH = vehicle.

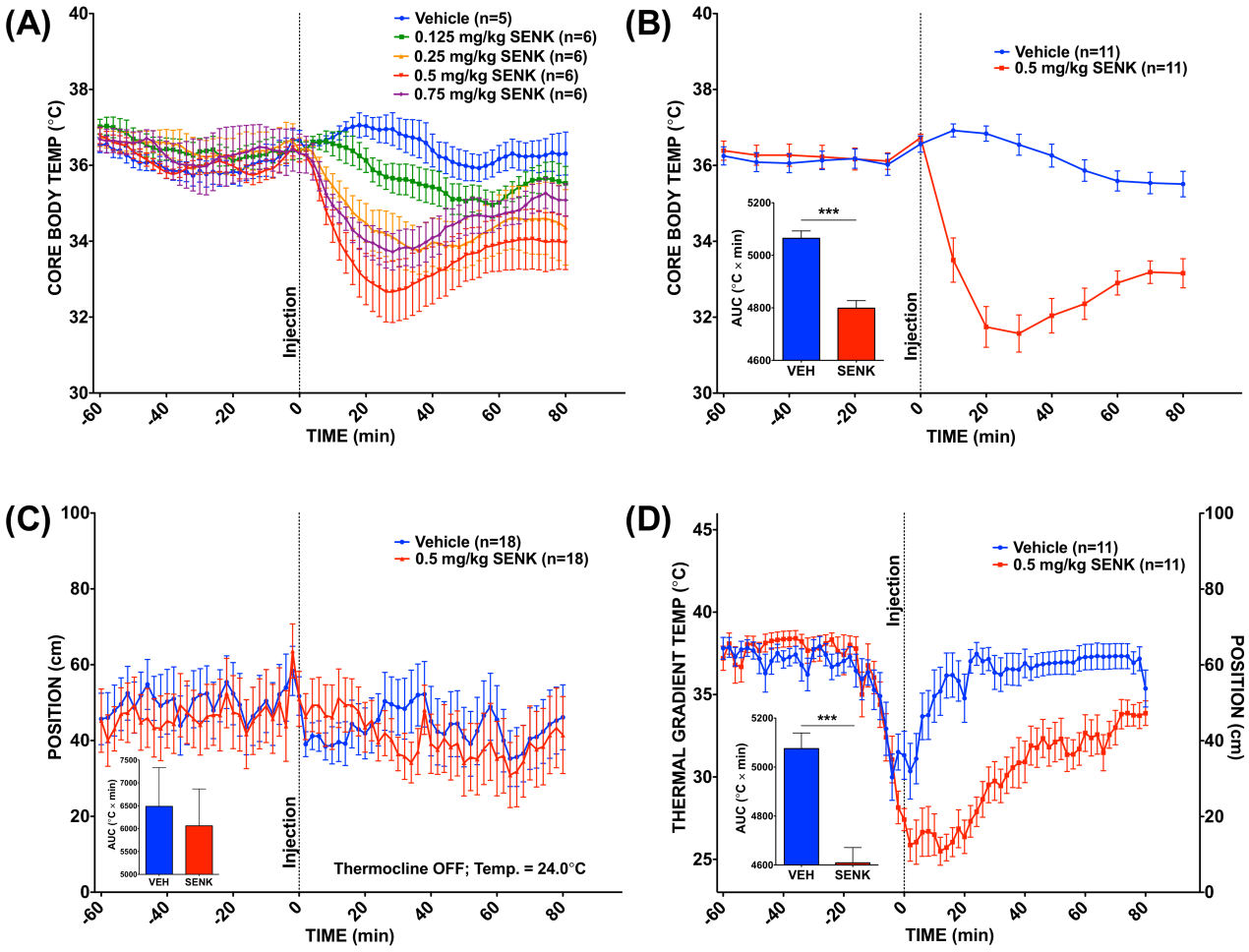

Figure 5. Core body temperature (CBT) changes and thermocline behavior in response to senktide. (A) CBT response to senktide doses in cages (n=5-6 per group), (B) CBT response in freely behaving mice administered 0.5 mg/kg senktide versus vehicle on the active thermal gradient (***p<0.001, paired t test of AUC, n=11 per group), (C) behavioral response to 0.5 mg/kg senktide versus vehicle on the inactive thermocline (p=0.698, NS, paired t test of AUC, n=18 per group), (D) behavioral response to 0.5 mg/kg senktide versus vehicle treatment on the active thermocline (***p<0.001, paired t test of AUC, n=11 per group). Dotted line at time point 0 reflects the time of the injection. Values are means ± SEM. SENK = senktide. VEH = vehicle.

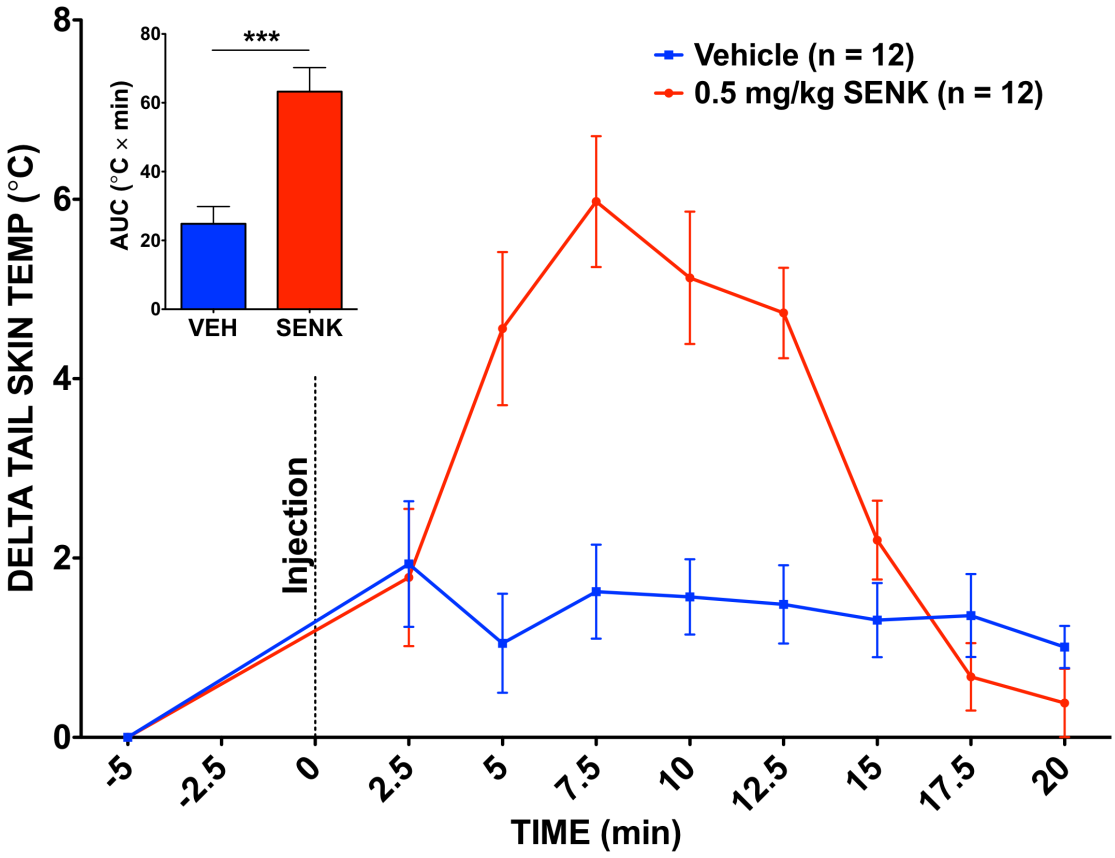

Figure 6. Tail skin temperature (TST) in response to senktide. Significant TST response to 0.5 mg/kg senktide versus vehicle (***$p<0.001$, paired t test of AUC, n=12 per group). Values are means ± SEM. SENK = senktide.

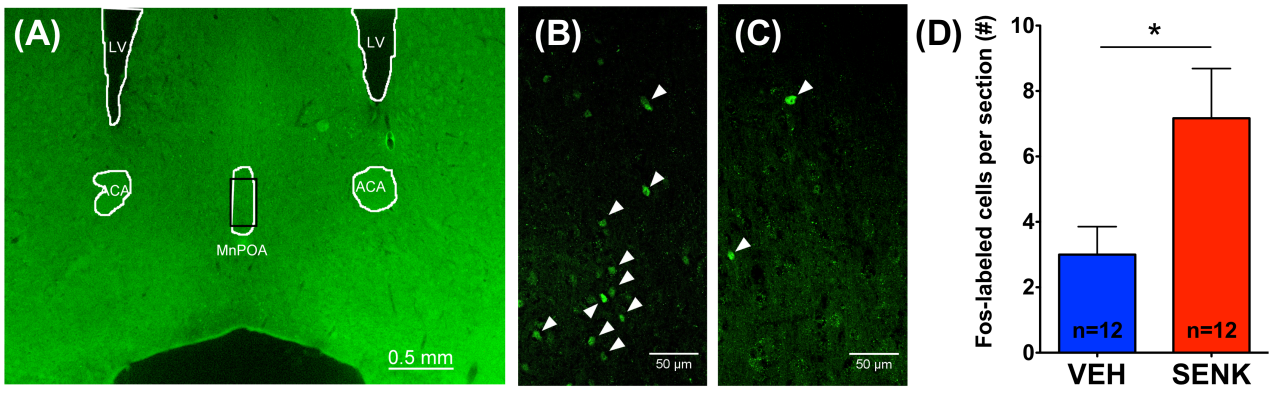

Figure 7. Fos induction in the MnPOA in response to senktide. Senktide treatment significantly induces Fos expression in the MnPOA. (A) Representative image of the MnPOA section used for quantification with landmarks noted for guidance (LV=lateral ventricle; ACA=anterior part of anterior commissure), (B) 40x magnification image from a senktide-treated male mouse, representative of the images used for quantification, (C) 40x magnification image from a vehicle-treated male mouse, (D) Quantification of Fos induction by senktide treatment (*$p<0.05$, two-tailed t test, n=12 per group). Values are means ± SEM. VEH = vehicle. SENK = senktide. White arrowheads indicate positively-labeled nuclei.

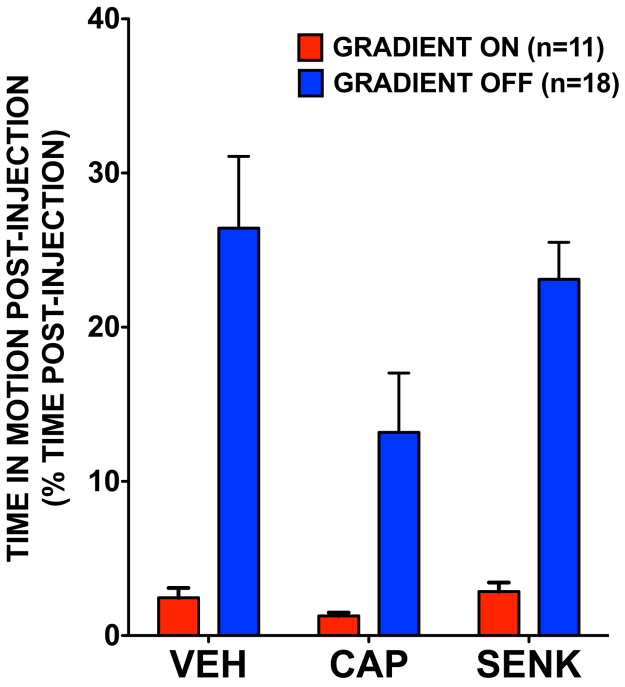

Figure 8. Movement observed following treatment with the thermal gradient ON versus OFF. Thermal gradient status (active versus inactive) significantly affects the amount of movement by mice treated with vehicle, capsaicin, and senktide (***$p<0.001$, 2way ANOVA, n=11-18 per group). Treatment had no significant effect on movement, regardless of thermocline status ($p=0.0904$, NS) and there was no significant interaction ($p=0.202$, NS) .

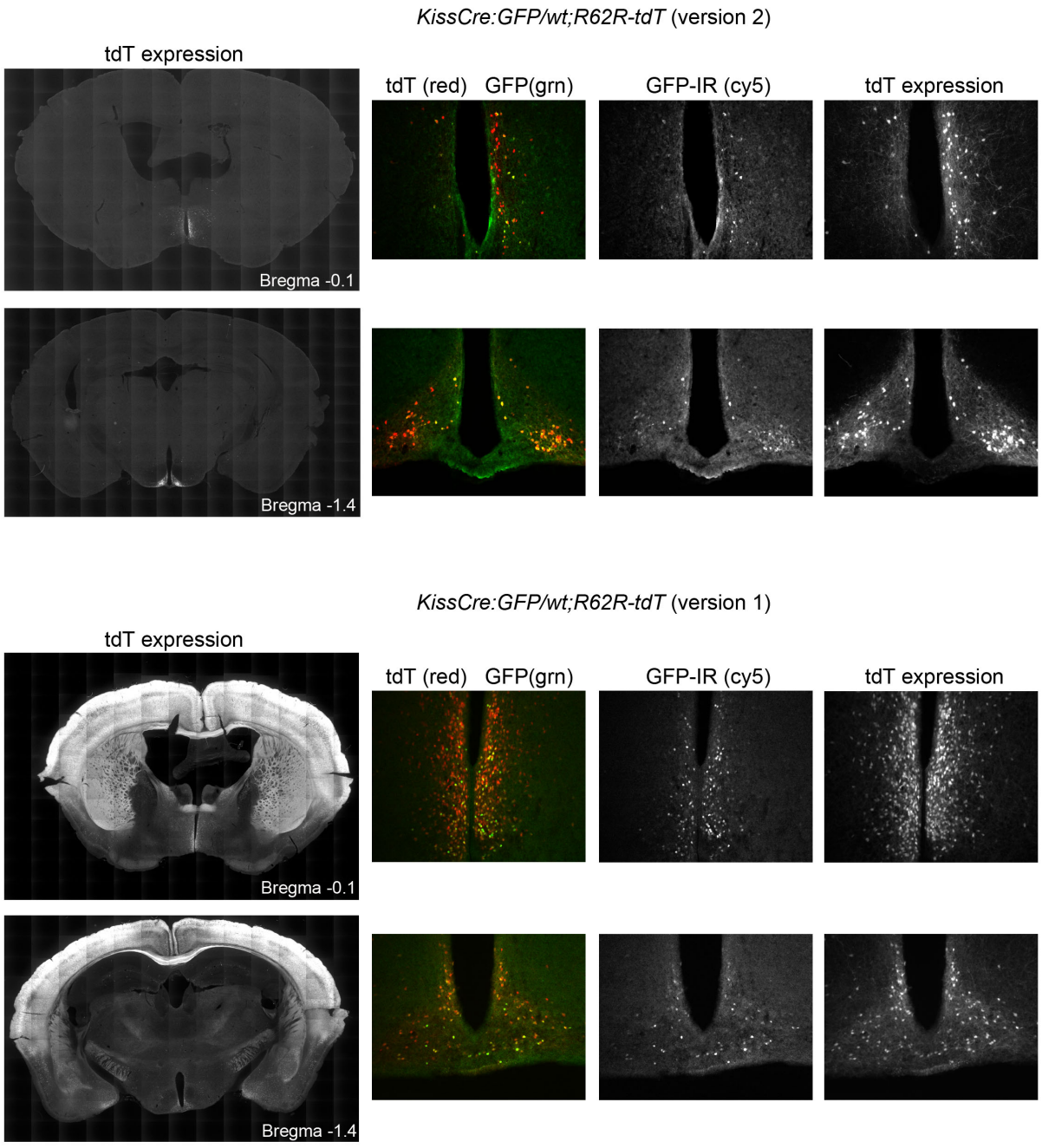

Figure 9. Cre expression localized to Kiss1 neurons in *Kiss1*$^{CreGFP.v2}$ mice. Figure courtesy of Dr. Stephanie Padilla.

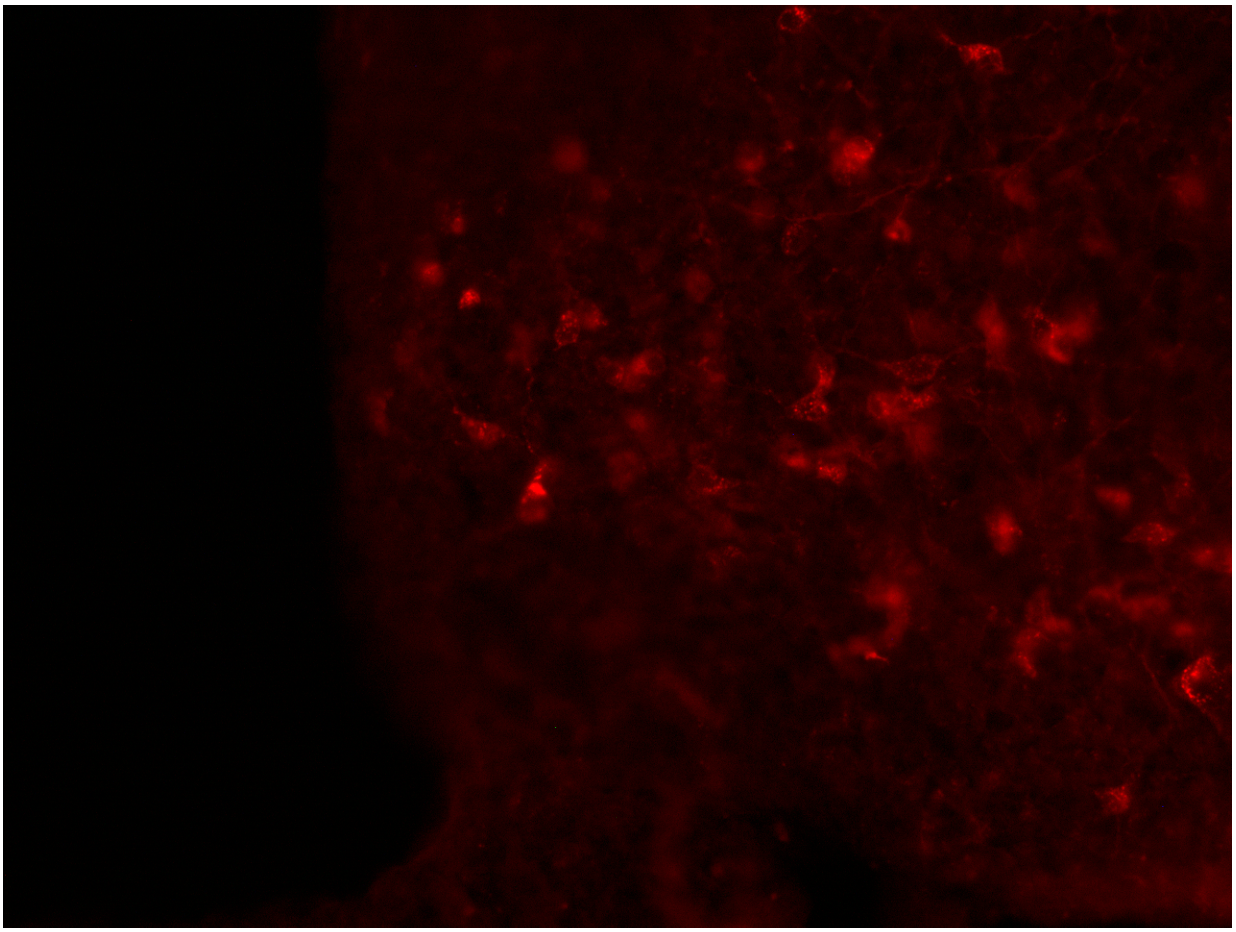

Figure 10. Representative image of AAV-DIO-hM3Dq-mCherry expression in the arcuate nucleus of an intact *Kiss1*$^{CreGFP.v2}$ mouse.

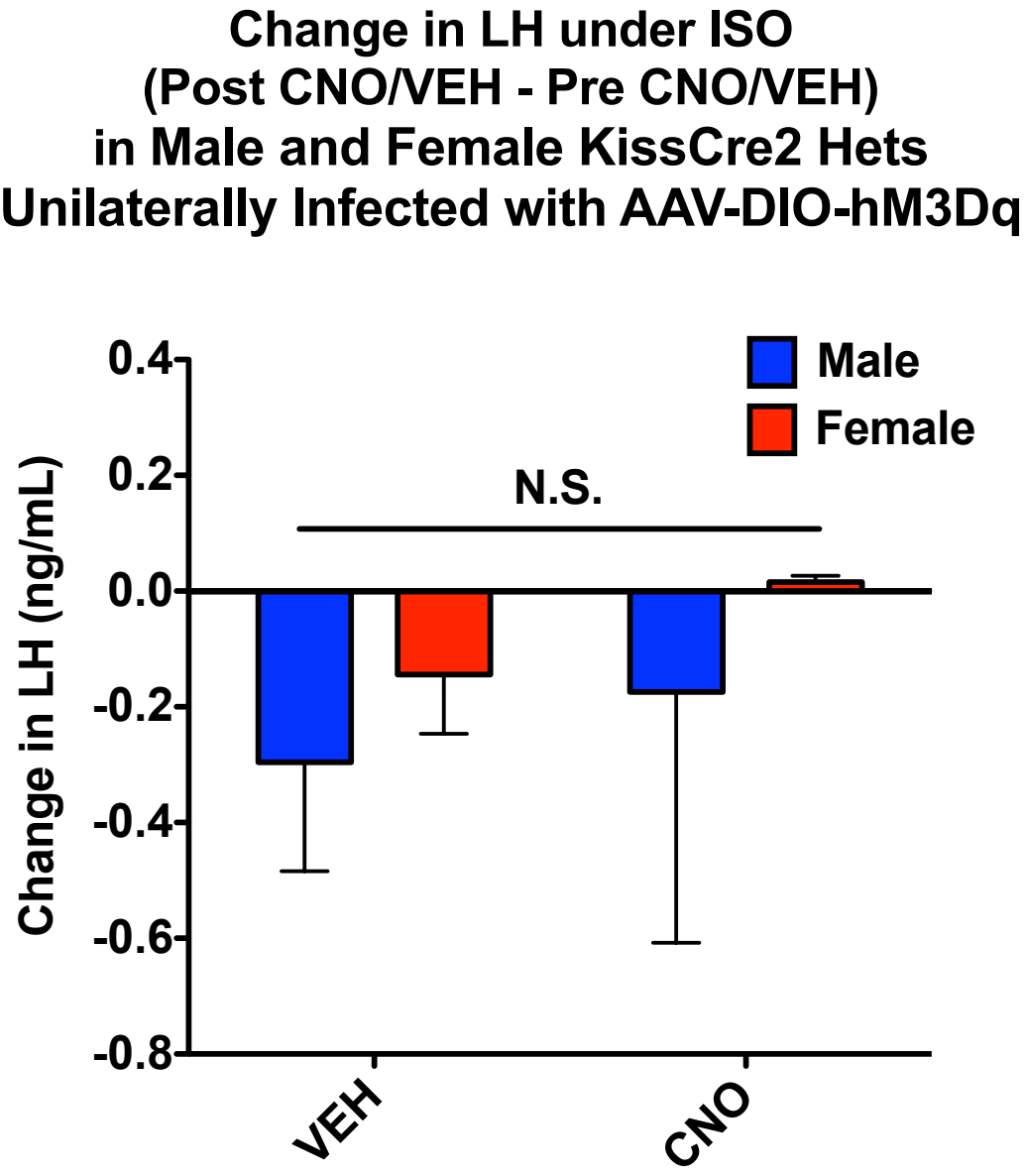

Figure 11. LH Response to CNO/VEH in Anesthetized Male and Female Mice Expressing AAV-DIO-hM3Dq. N.S. by 2way ANOVA.

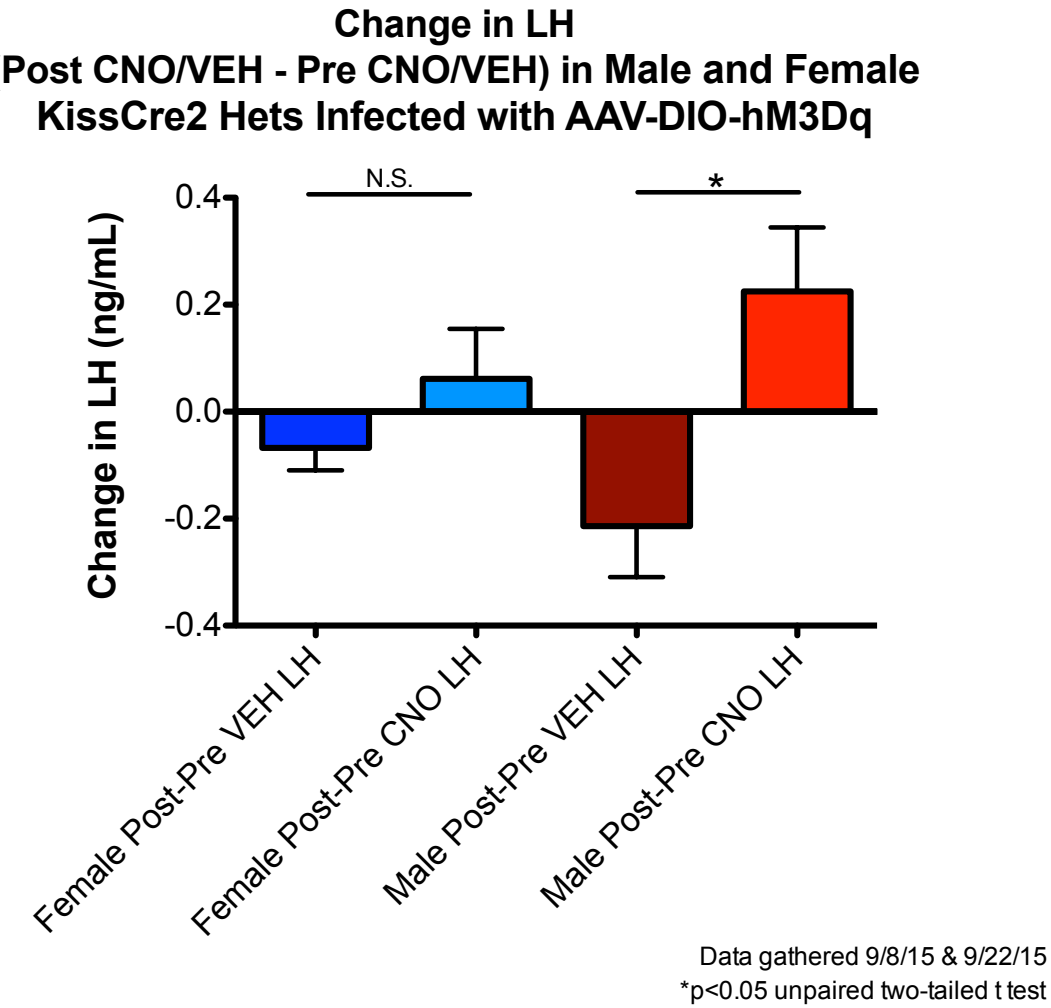

Figure 12. LH Response to CNO/VEH in Male and Female Mice Expressing AAV-DIO-hM3Dq.

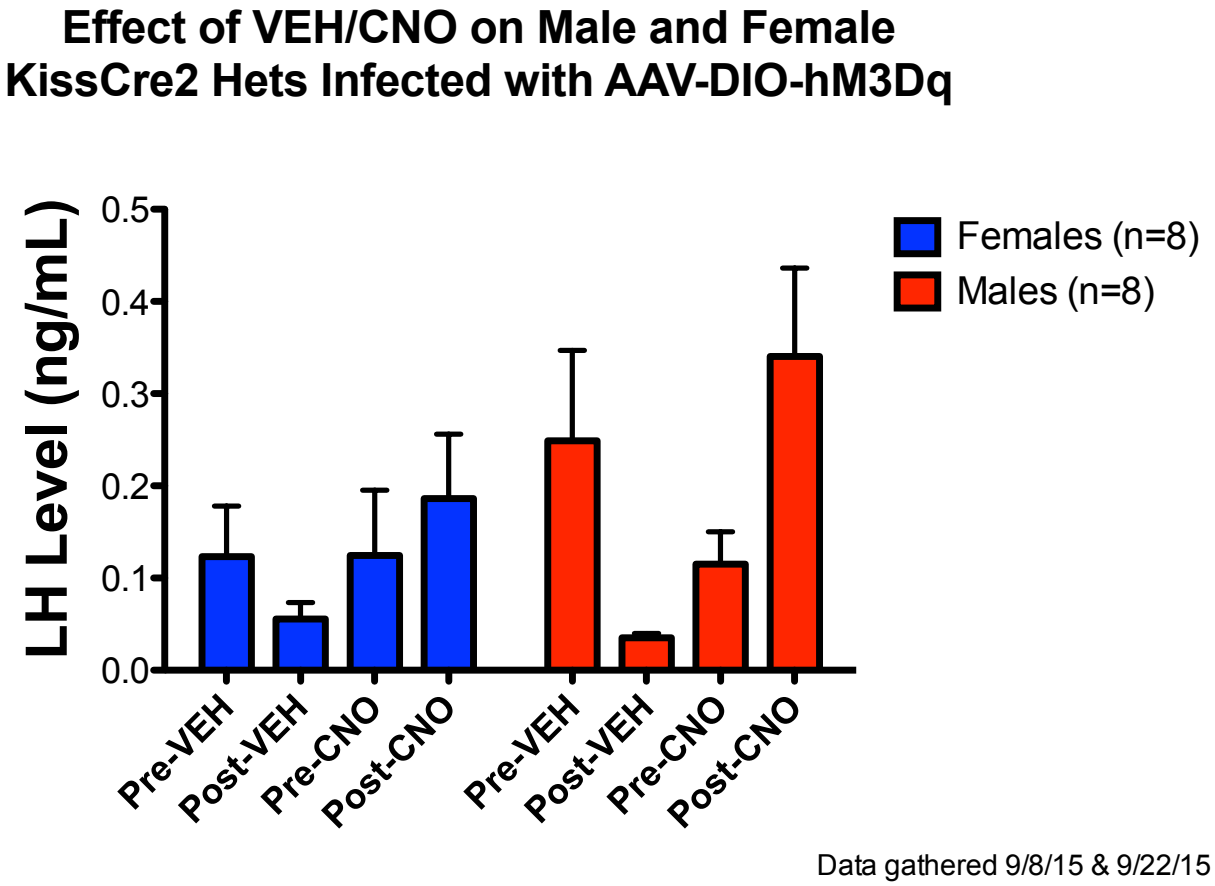

Figure 13. Mean LH Level in Response to CNO/VEH in Male and Female Mice Expressing AAV-DIO-hM3Dq. All group comparisons (Pre vs. Post VEH/CNO for males and females were N.S. by paired t-test.

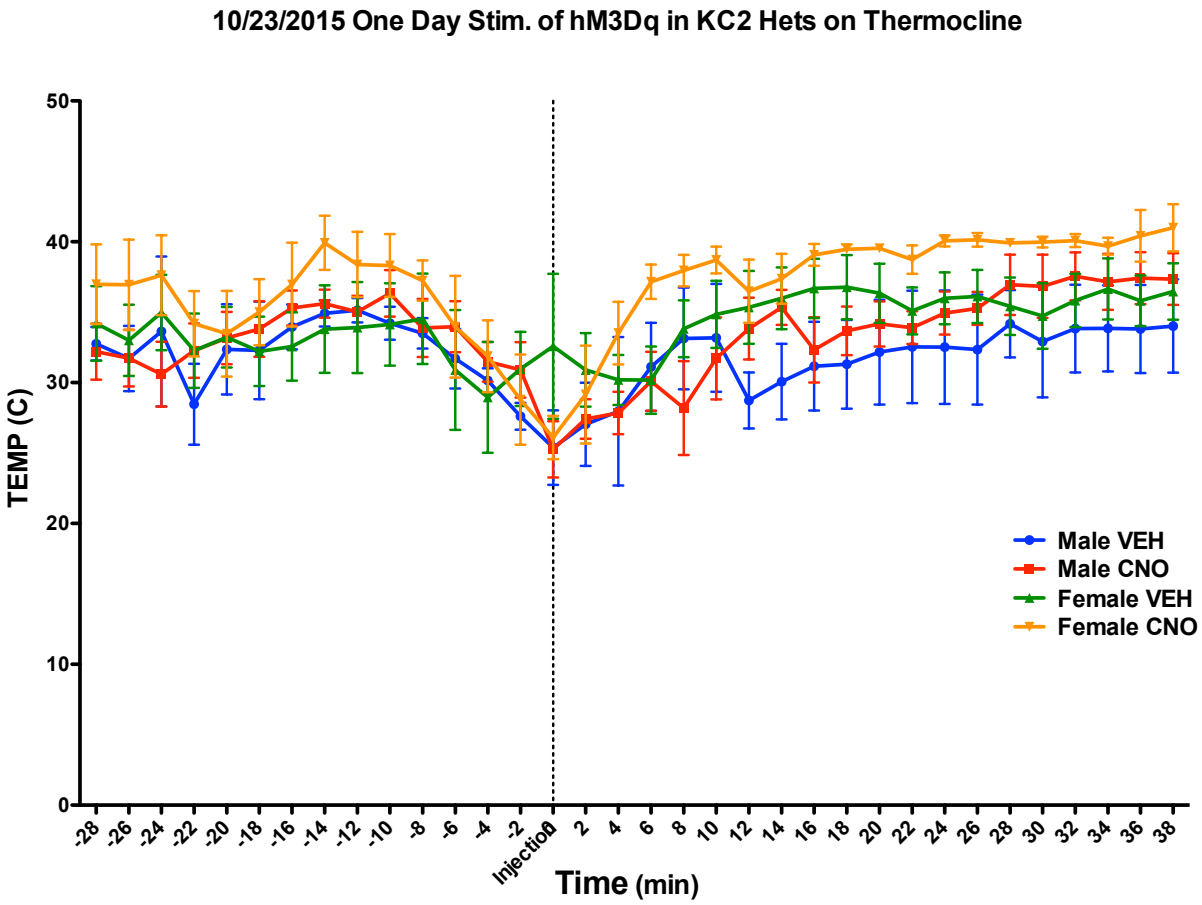

Figure 14. Preliminary Study of Thermocline Behavior of Male and Female Mice Expressing AAV-DIO-hM3Dq and Treated with CNO/VEH. Comparison of all groups by 2way ANOVA of AUC values showed that there were no significant effects of sex or treatment.

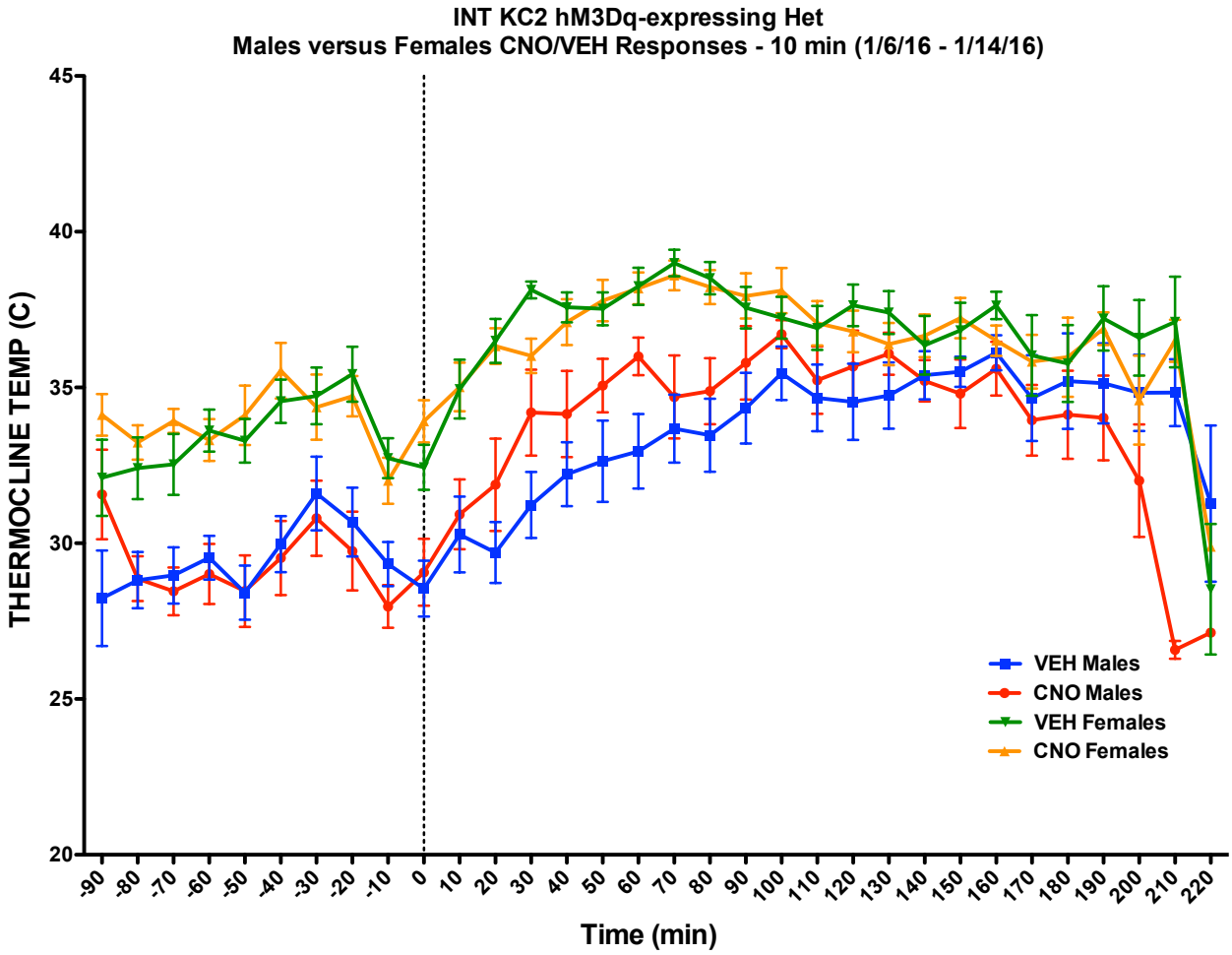

Figure 15. Thermocline Behavior of Intact Male and Female Mice Expressing AAV-DIO-hM3Dq and Treated with CNO/VEH. Analysis of the data by 2way ANOVA of AUC values yielded a significant effect of sex (***p=0.0002), but not of treatment, on the response of the mice.

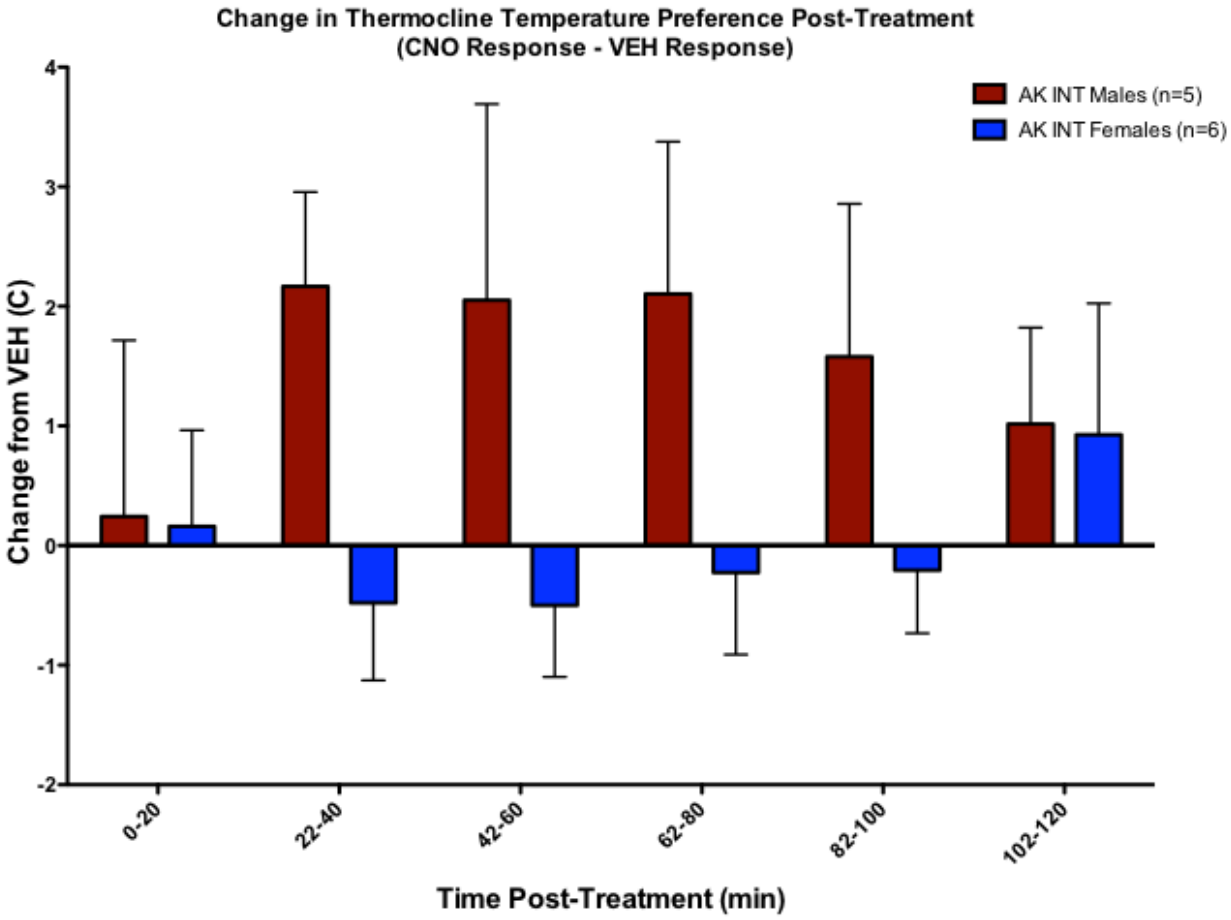

Figure 16. Change in Thermocline Temperature Preference in Response to CNO/VEH of Intact Male and Female Mice Expressing AAV-DIO-hM3Dq. When analyzed by 1way ANOVA, the change in temperature preference of males and females did not significantly differ over time.

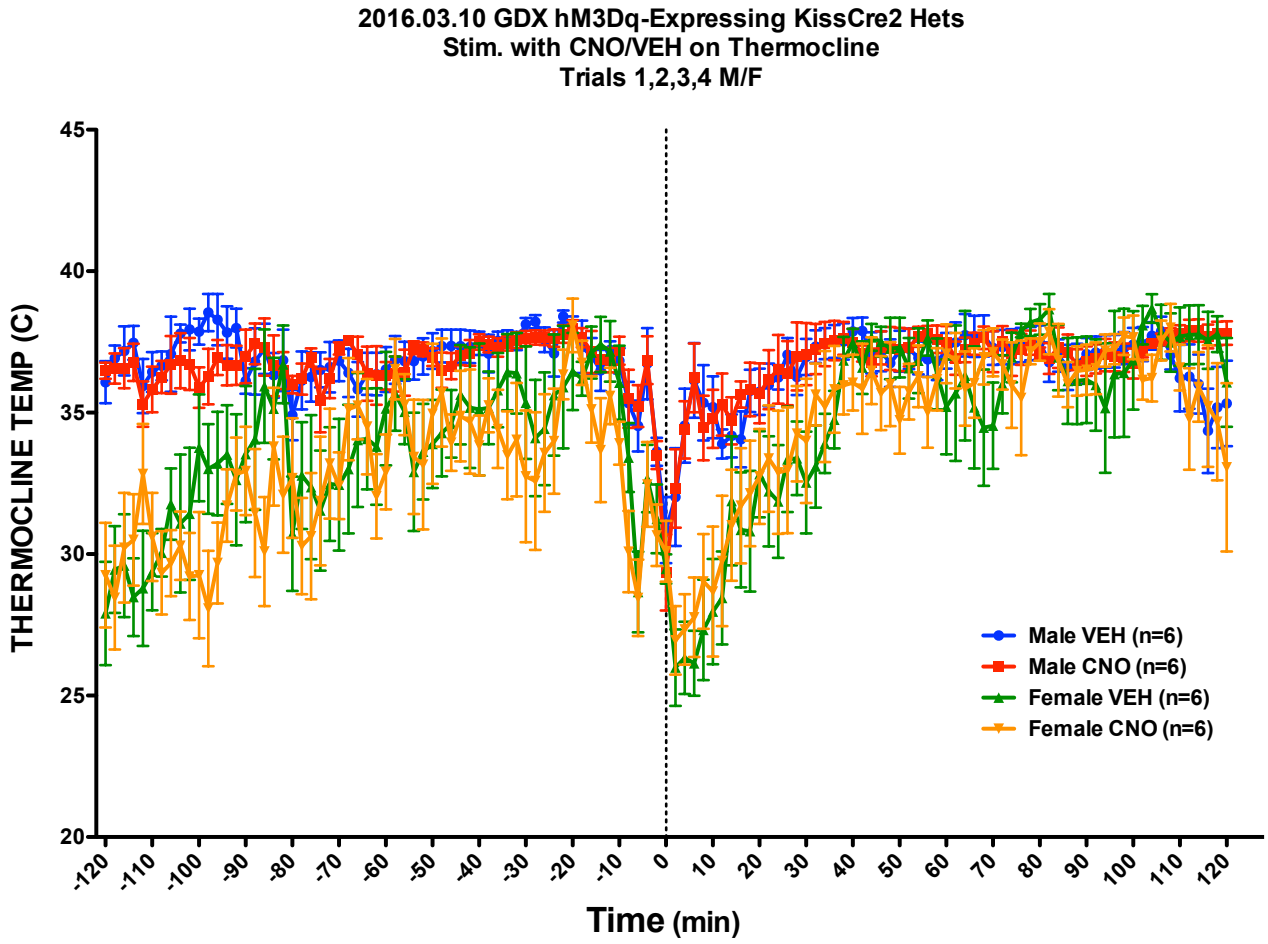

Figure 17. Thermocline Behavior of Gonadectomized Male and Female Mice Expressing AAV-DIO-hM3Dq and Treated with CNO/VEH. Analysis by 2way ANOVA of the AUC values revealed a significant effect of sex (***$p<0.0001$), but not of treatment, on the response of mice.

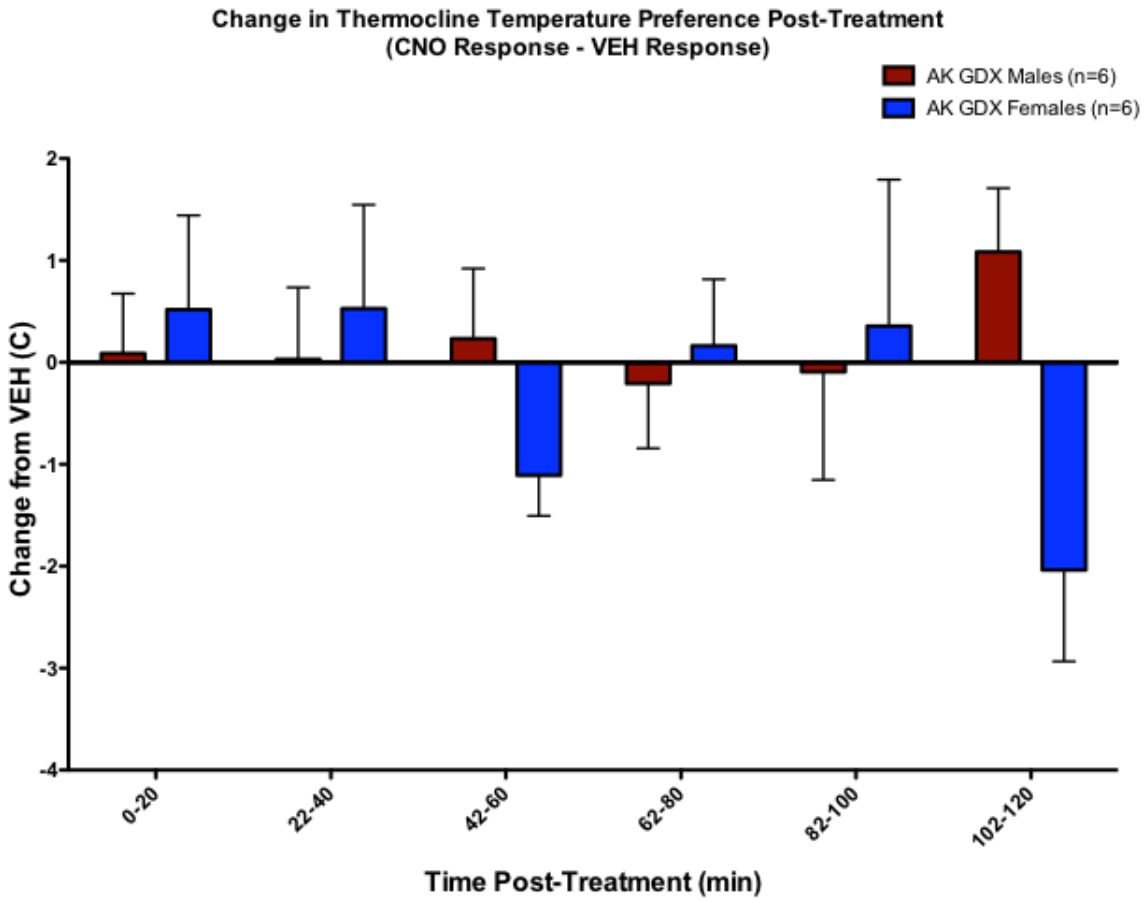

Figure 18. Change in Thermocline Temperature Preference in Response to CNO/VEH of Gonadectomized Male and Female Mice Expressing AAV-DIO-hM3Dq. Analysis of the male and female data, respectively, by 1way ANOVA revealed no significant change in thermocline temperature preference over time.

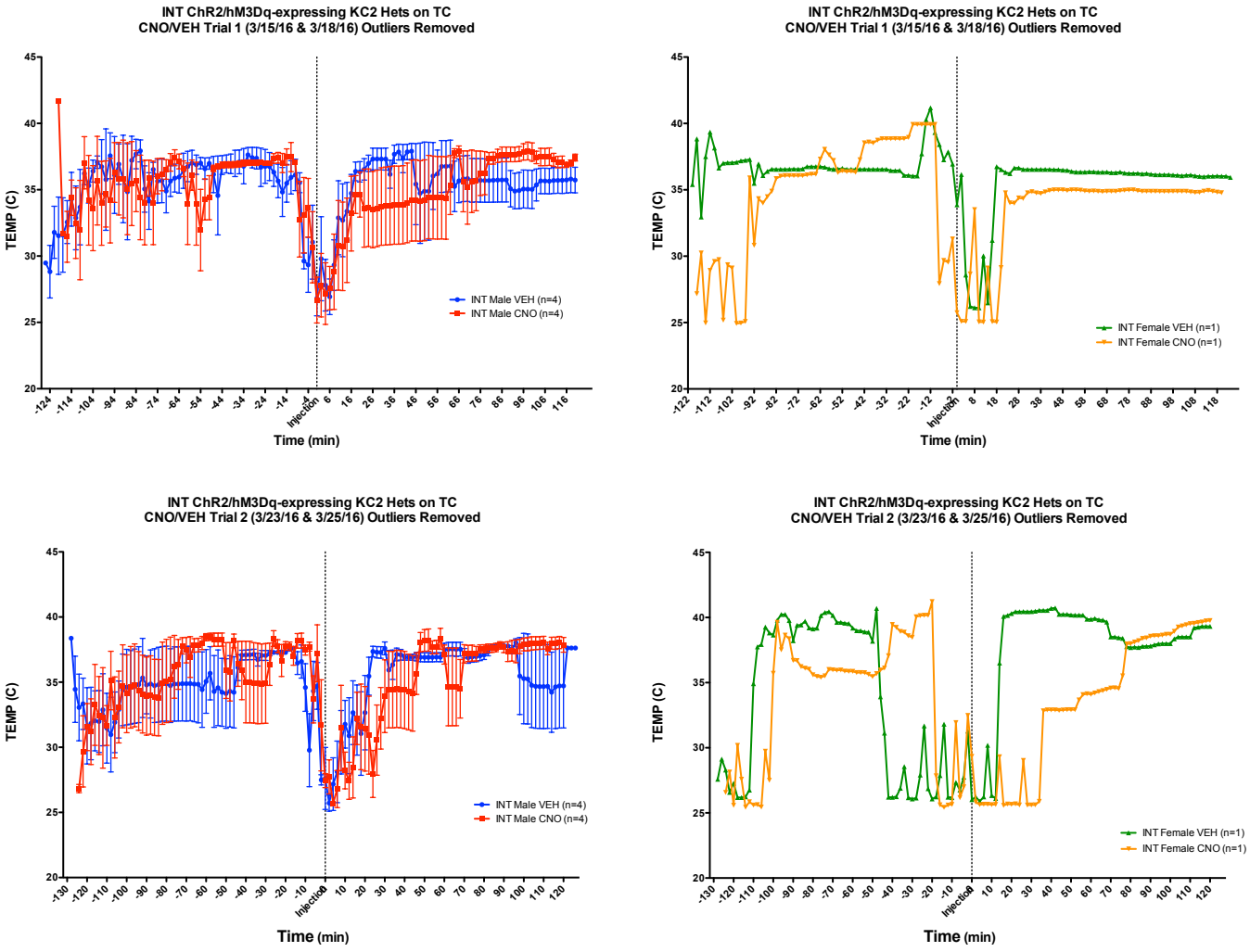

Figure 19. Thermocline Behavior of Intact Male and Female Mice Expressing hM3Dq and ChR2 and Treated with CNO/VEH. Male responses to VEH vs. CNO did not significantly differ by t-test of AUC. The single female's responses could not be analyzed in this way, but the qualitative and quantitative differences in her temperature preferences in trial 2 can be appreciated.

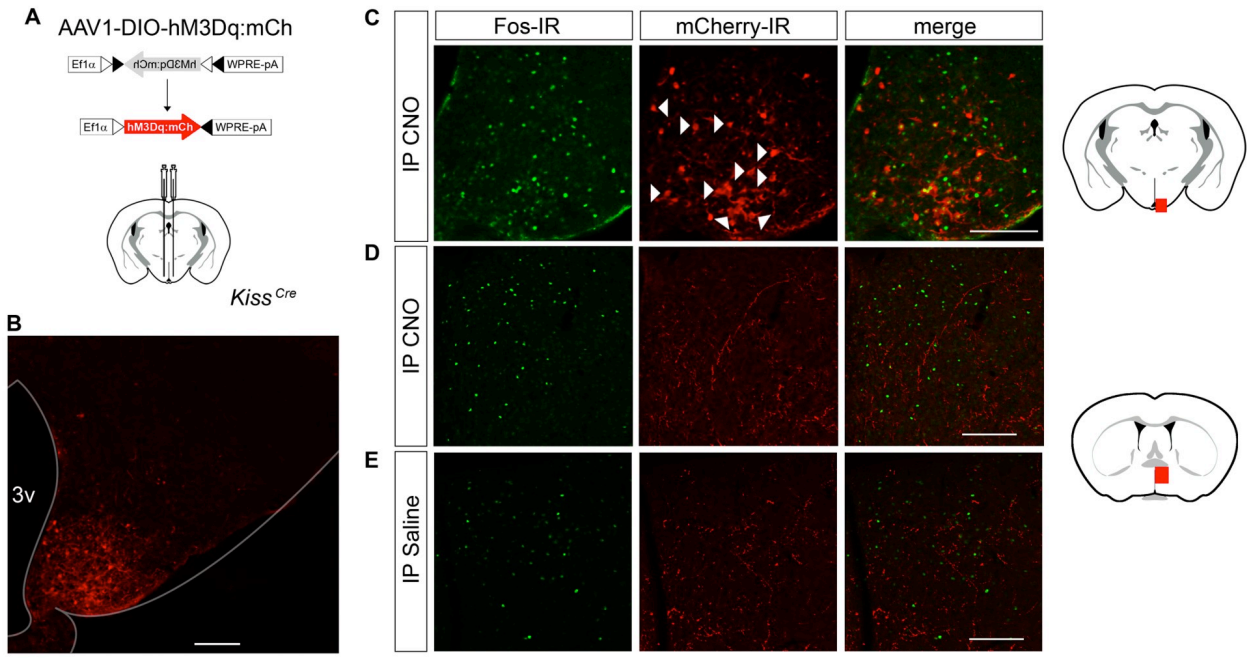

Figure 20. Fos expression in intact mice expressing hM3Dq and ChR2 and treated with CNO/VEH. (A) Schematic of viral injection strategy. (B) Representative image of viral marker expression in the arcuate nucleus. (C) Representative images demonstrating Fos expression in virus-expressing Kiss1 neurons (mCherry-labeled neurons) following CNO administration. Figure courtesy of Dr. Stephanie Padilla.

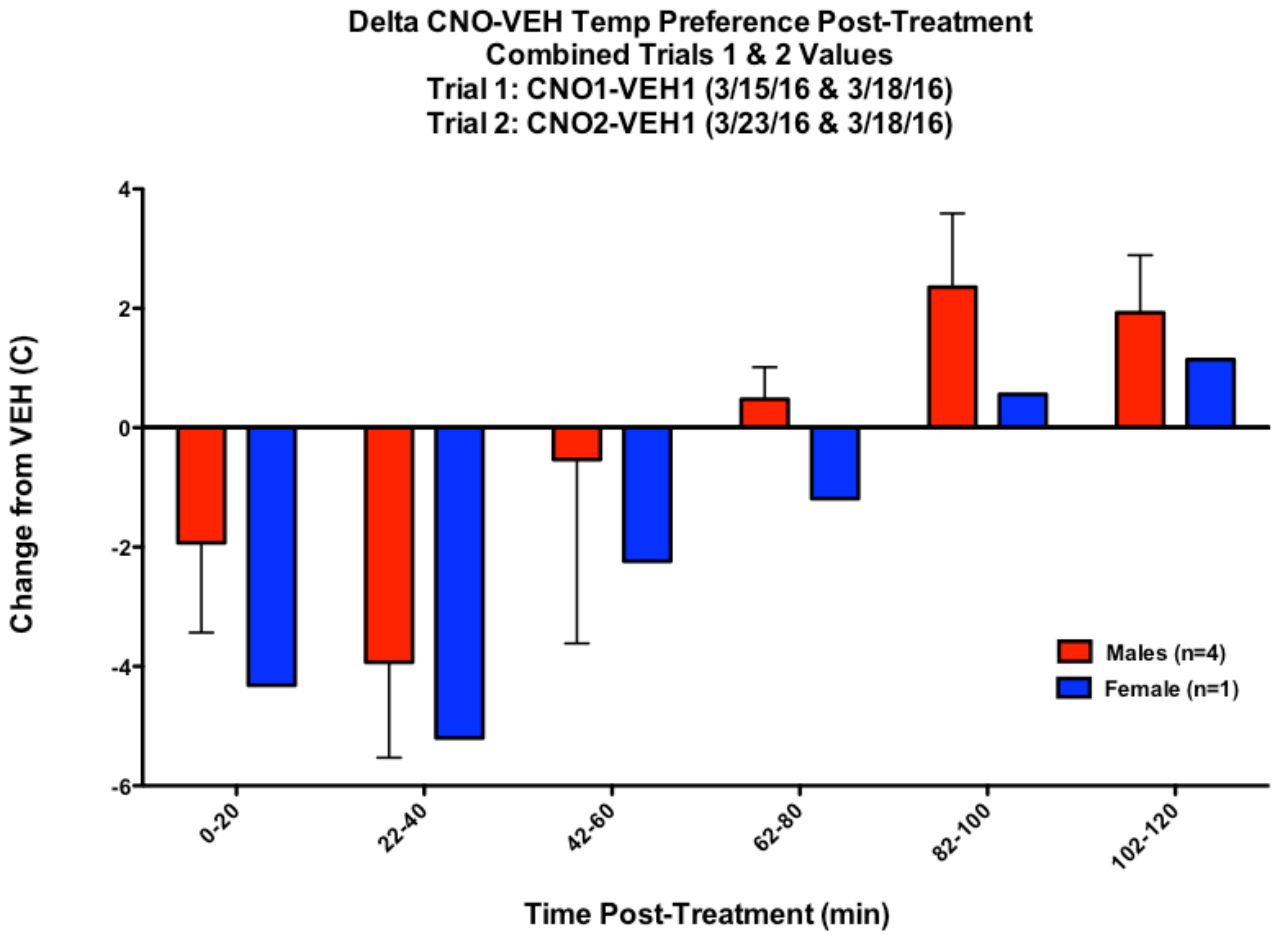

Figure 21. Change in Thermocline Temperature Preference in Response to CNO/VEH of Intact Male and Female Mice Expressing AAV-DIO-hM3Dq and AAV-DIO-ChR2. Analysis of the male data by 1way ANOVA revealed no significant change in thermocline temperature preference over time. The single female's responses could not be analyzed in this way, but the difference in her temperature preferences can be appreciated.

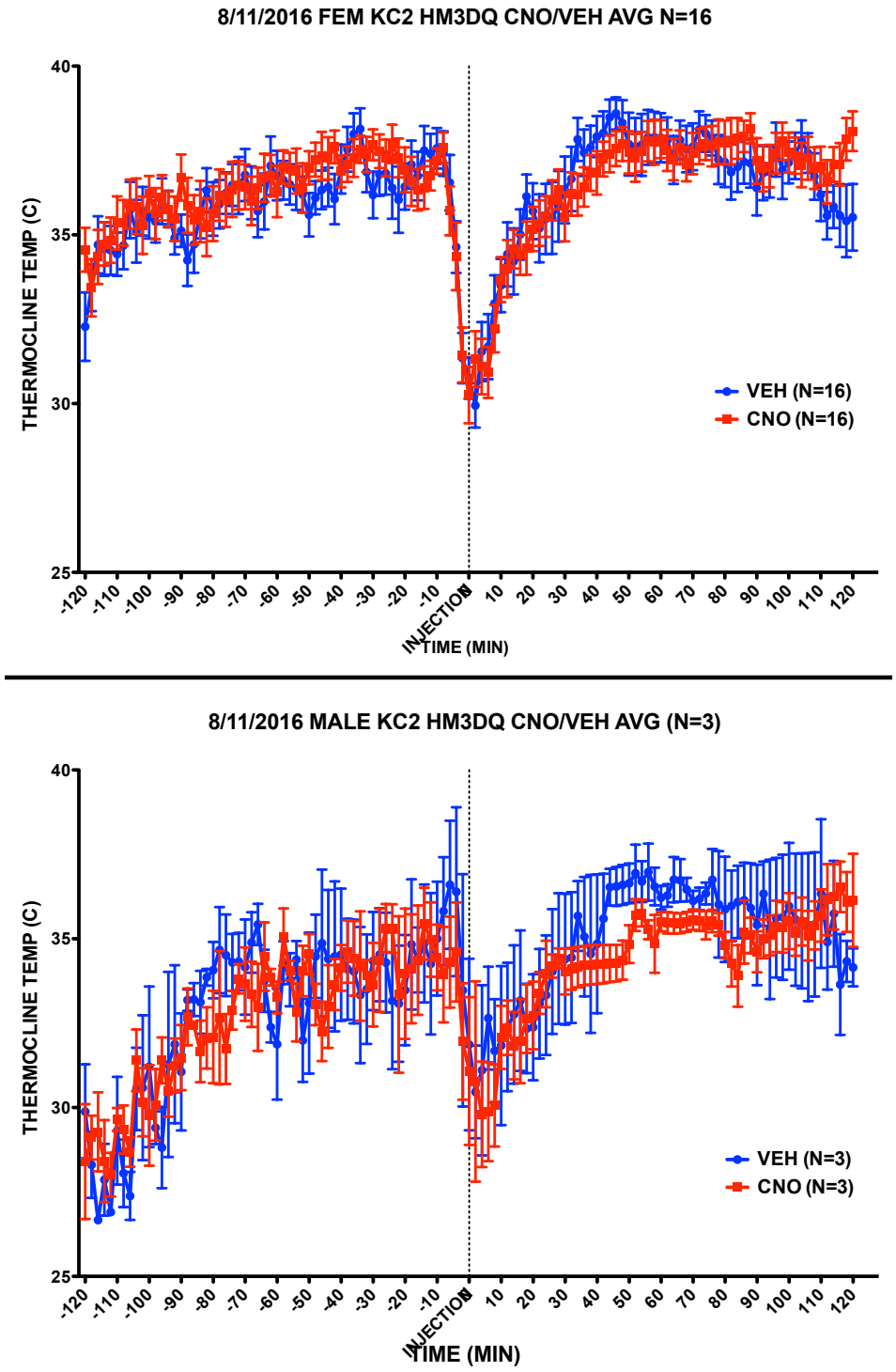

Figure 22. Thermocline Behavior of Intact Male and Diestrous Female Mice Expressing hM3Dq and Treated with CNO/VEH. Female and male responses, respectively, to VEH vs. CNO did not significantly differ by t-test of AUC.

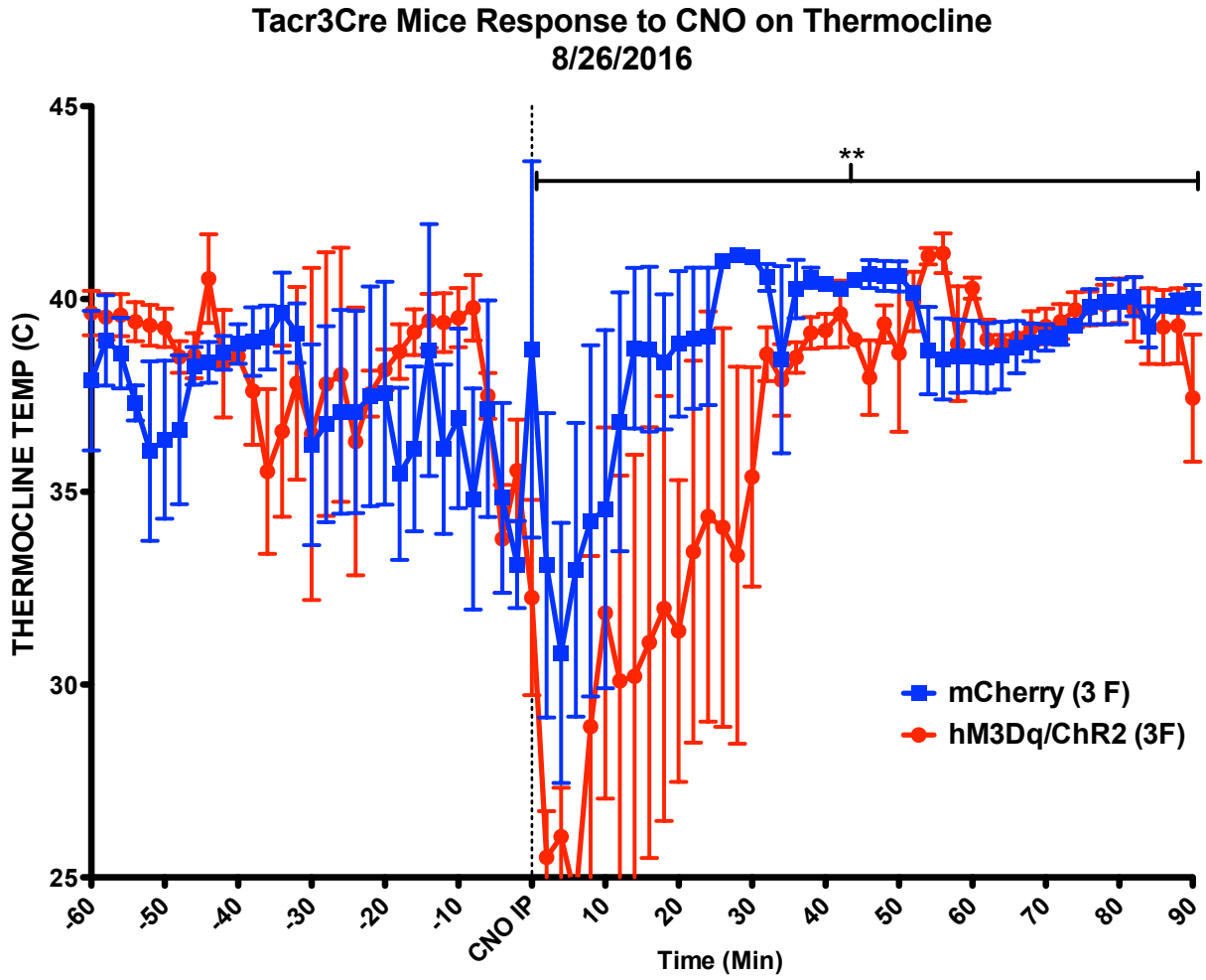

Figure 23. Thermocline behavior of female intact Tacr3Cre mice expressing AAV-DIO-hM3Dq/ChR2 or DIO-mCherry. Data presented as mean and SEM (**p<0.01).

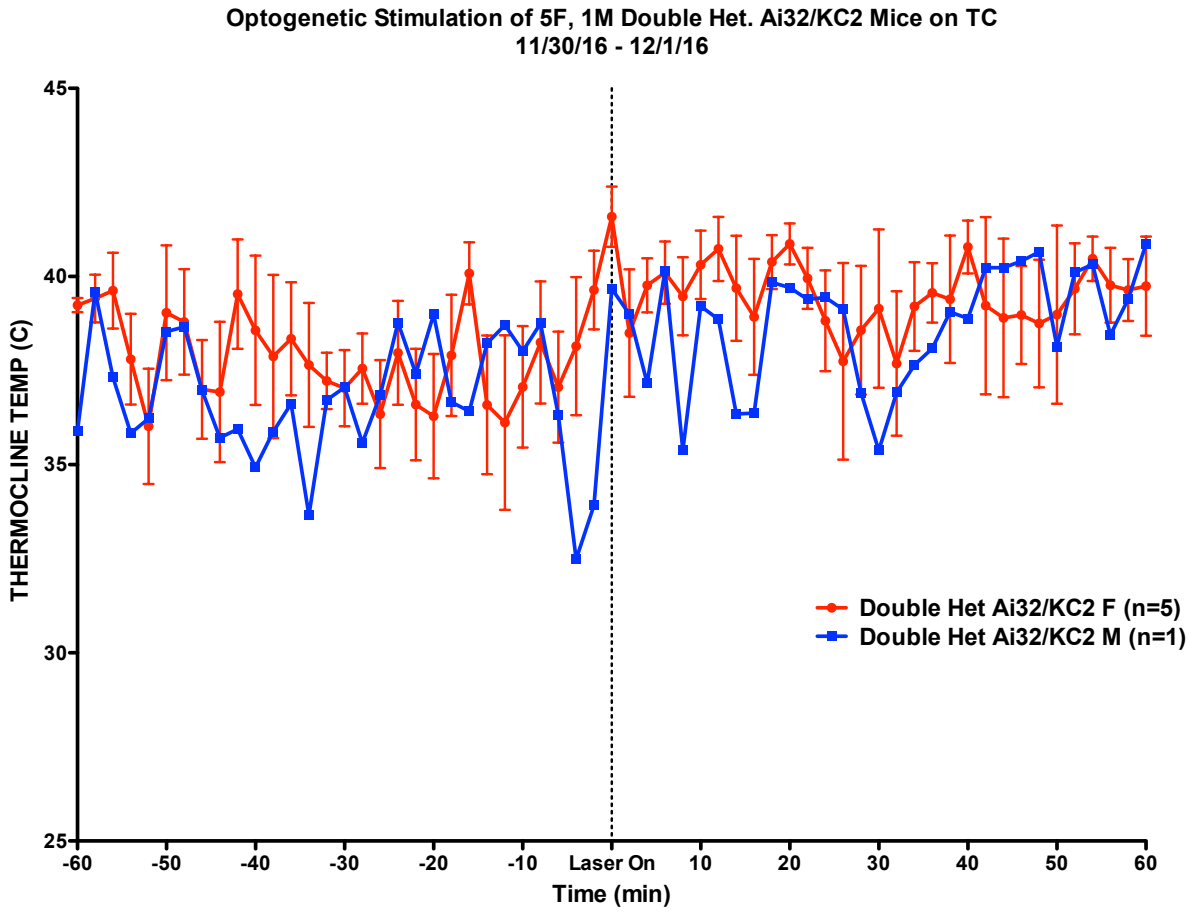

Figure 24. Thermocline Behavior following Optogenetic Stimulation of Intact KC2/Ai32 Male and Female Mice.

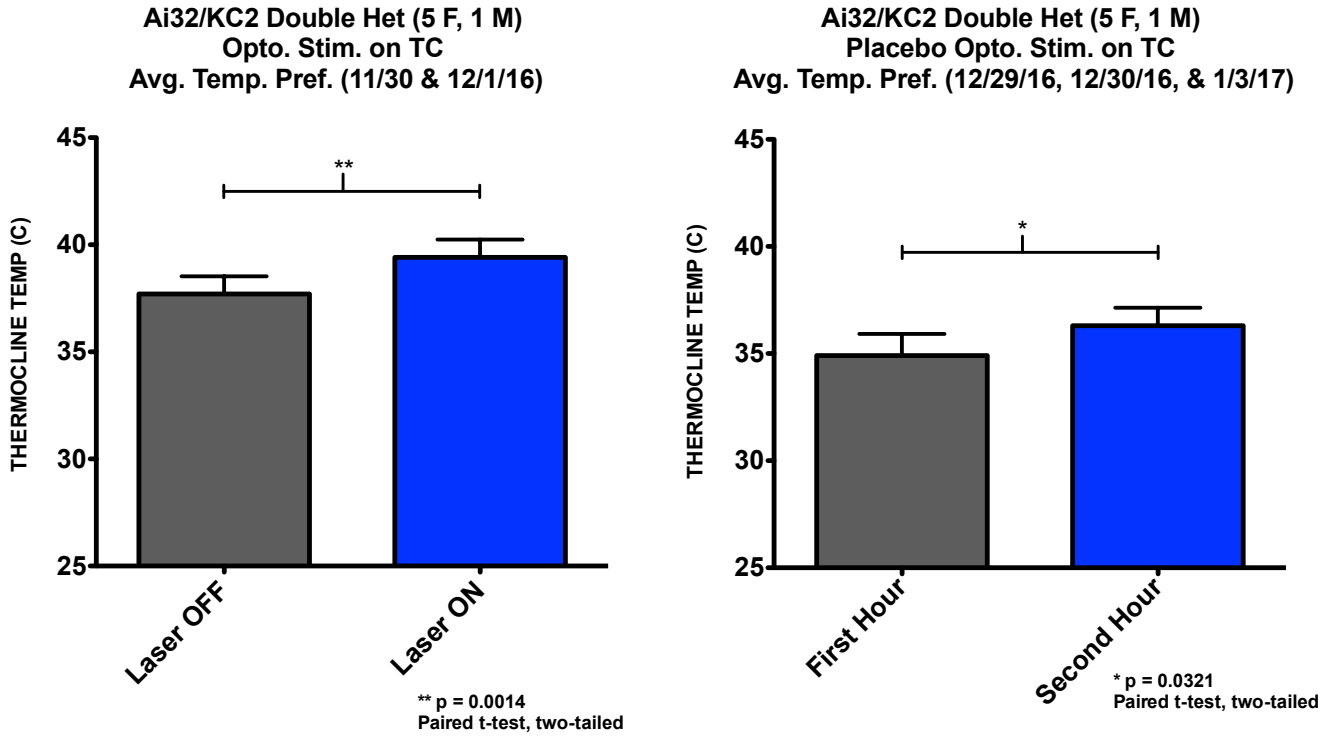

Figure 25. Average Temperature Preference With and Without Optogenetic Stimulation in Intact KC2/Ai32 Male and Female Mice.

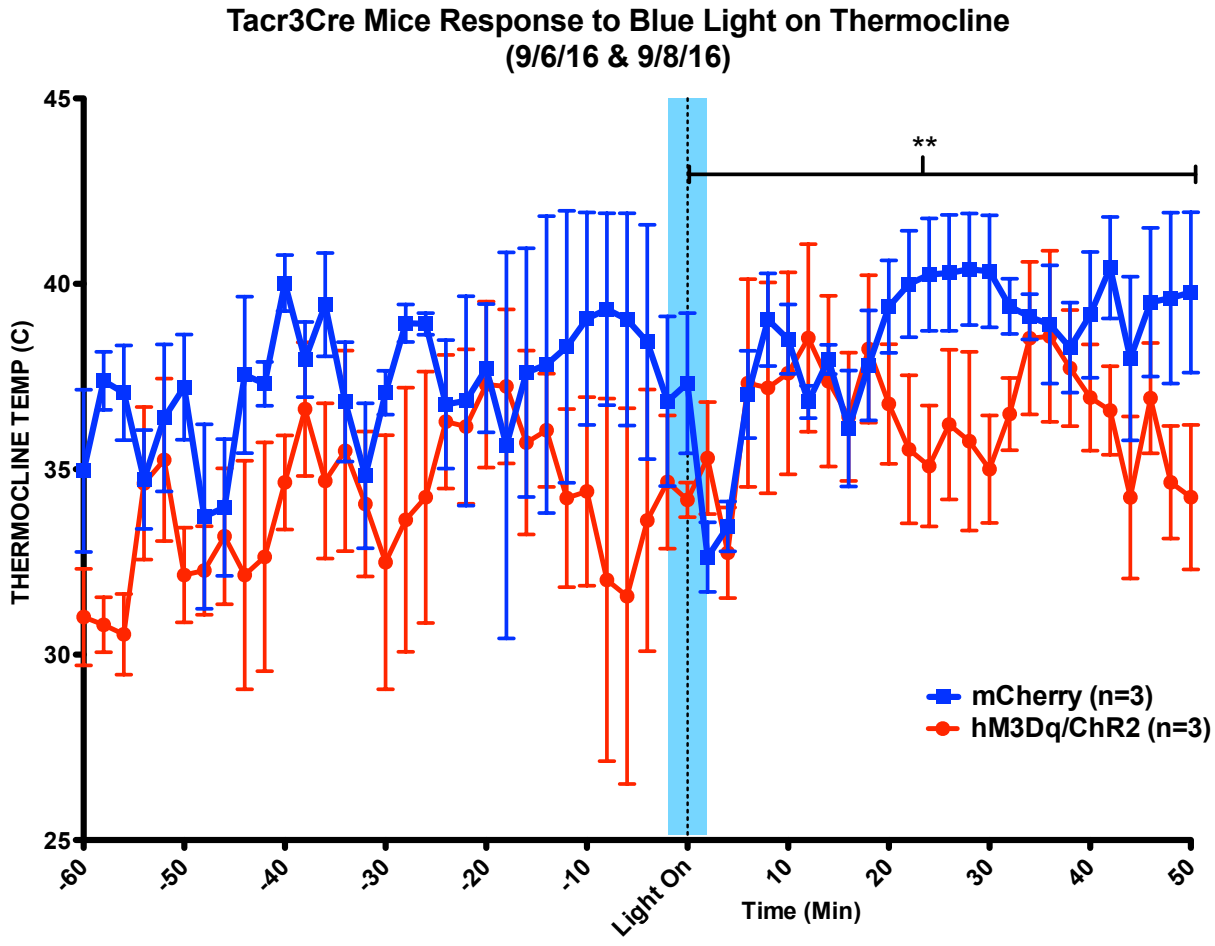

Figure 26. Thermocline behavior following optogenetic stimulation of intact Tacr3Cre mice expressing AAV-DIO-hM3Dq/ChR2 or DIO-mCherry. Data presented as mean and SEM (**$p<0.01$). There was no significant difference in temperature preference between the groups prior to the laser being turned on. The blue highlighted region indicates when the laser was turned on and the pulse train commenced. The laser was activated intermittently from time 0 until time +50 min.

Thesis Section	Experiment	Surgeon	Virus Injected (AAV-DIO)	Volume Injected (nL)	Receptor Marker Expression	Fos Induction	LH Response	Thermocline Response	Core Body Temp. Change	Tail Skin Temp. Change
2.10.4	Unilateral stim. under Isofluorane	AAK	hM3Dq-mCherry	500	ND	ND	Decrease	ND	ND	ND
2.10.5	Bilateral stim. of Awake Mice	AAK	hM3Dq-mCherry	500	ND	ND	Males only, subtle	None	ND	ND
2.10.6	Stim. of Intact Mice on Thermocline	AAK	hM3Dq-mCherry	500	2/11 mice	None	ND	None	ND	ND
2.10.7	Stim. of GDX Mice on Thermocline	AAK	hM3Dq-mCherry	500	6/6 Males 2/6 Females	None	ND	None	ND	ND
2.10.8	Stim. of Intact Mice on Thermocline	SLP	hM3Dq-mCherry; ChR2-YFP	500	9/9 mice	Present	ND	Varied	ND	ND
2.10.9	Stim. of Intact, Cycled Mice on Thermocline	SLP	hM3Dq-mCherry	500	0/3 Males 16/16 Females	None	ND	None	ND	ND
2.11.3	Stim. of Intact Ai32/KC2 Mice on Thermocline	CWJ	N/A	N/A	N/A	In Progress	ND	None	ND	ND

Table 1. Summary of Stimulation Experiments Involving Kiss1-Cre:GFP-v2 Mice. "Viral transduction" refers to the number of animals in which robust expression of the marker for receptor expression was observed in the arcuate nucleus, indicating that the virus had been taken up and expressed by neurons expressing Cre in the arcuate nucleus. "Fos induction" indicates whether Fos immunostaining was observed in neurons expressing the hM3Dq receptor. "LH Response" indicates if a change in LH concentration was observed following CNO administration. "Thermocline Response" indicates if mice in that experiments demonstrated behavioral alterations (e.g., change in temperature preference) following CNO administration.

VITA

Ashley was raised in Peoria, IL, where her parents and younger sister still live. She attended St. Philomena Grade School and earned her diploma at Peoria Notre Dame High School. Ashley completed her Bachelor of Science at the University of Iowa, majoring in Biochemistry and minoring in Biology and Spanish. In Iowa City, Ashley met and married Jordan Krull. Together they moved to Seattle, WA, where Ashley completed her Ph.D. in Neuroscience at the University of Washington.