Title
Examining Neurokinin Signaling in Etiology of Tamoxifen Induced Hot Flashes

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Examining Neurokinin Signaling in Etiology of Tamoxifen Induced Hot Flashes

A thesis submitted in partial satisfaction
of the requirements for the degree of Master of Science
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by

Weronika Budek

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ABSTRACT OF THE THESIS

Examining Neurokinin Signaling in
Etiology of Tamoxifen Induced Hot Flashes

by

Weronika Budek

Master of Science in Physiological Science
University of California, Los Angeles, 2023
Professor John Edward Van Veen, Co-Chair and Professor Stephanie Correa Van Veen, Co-Chair

Tamoxifen is a selective estrogen modulator used to treat breast cancer patients to reduce the reoccurrence of estrogen receptor-positive breast cancer. However, treatment with tamoxifen leads to serious side effects, including hot flashes. Hot flashes are a sudden feeling of warmth over the face, neck, and chest directly underneath the skin, but the mechanism of this thermal dysregulation is still unknown. Similar to what is observed in response to tamoxifen treatment, menopause is also associated with hot flashes. Menopausal hot flashes are thought to be caused by increased expression of neurokinin B in the infundibular (arcuate) nucleus of the hypothalamus. We hypothesized that a similar mechanism of increased neurokinin B expression might underlie tamoxifen-induced hot flashes. We compared neurokinin B expression in ovariectomized mice to control intact mice and intact mice treated with

tamoxifen. Surprisingly, tamoxifen treatment in intact mice over the course of 10 days induces thermal dysregulation but decreases the expression of neurokinin B in the arcuate nucleus when compared to ovariectomized mice. These results indicate that a neurokinin B-independent mechanism of tamoxifen-induced thermal dysregulation exists.

The thesis of Weronika Ewa Budek is approved.

Paul E Micevych

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Stephanie Correa Van Veen, Committee Co-Chair

John Edward Van Veen, Committee Co-Chair

University of California, Los Angeles

2023

Dedication:

I would like to dedicate this thesis to Dr. John Edward van Veen and my family. Your continuous support and encouragement has allowed me to grow and flourish as a scientist.

Acknowledgments:

I would like to thank my principal investigators Dr. John Edward van Veen and Dr. Stephanie Correa van Veen for all their guidance and support throughout my master's program. Their kindness and never-ending support helped me manage and successfully complete my project. Without them, I would not be the scientist I am today, nor would I have known how much I wanted to pursue academia in the future. I would also like to thank my committee members Dr. Paul Micevych and Dr. Pearl Quijada for all their help in my journey.

Additionally, thank you to all the members of the Van Veen and Correa lab and to my mentor Jae Park for all his support. Thank you for teaching me fundamental lab techniques necessary for my project.

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Chapter 1: Neurokinin signaling in etiology of tamoxifen-induced hot flashes changes with Tamoxifen and/or Ovariectomy.

I. Background

Tamoxifen is a selective estrogen modulator[1](SERM). Drugs that belong to the SERM family are known to act as estrogen receptor agonists or antagonists. Therefore, tamoxifen is a drug that acts like estrogen on some tissues but can also block the effect of estrogen on other tissues. Therefore, it is not like a pure estrogen receptor agonist or antagonist. A SERM's actions are different in various tissues that express estrogen-receptors, which provides the possibility to inhibit or stimulate estrogen action in tissues. SERMs can therefore be used to treat estrogen-related diseases[2]. Tamoxifen is used to treat and reduce the risk of breast cancer due to its anti-estrogen action in malignant breast epithelial tissue. Tamoxifen is a first-line hormonal treatment. It is used as a post-surgical adjuvant for hormone-receptor-positive breast cancer patients. Tamoxifen can decrease the incidence of breast cancer recurrence by up to 40%[1]. It is also used for patients diagnosed with advanced-stage or metastatic hormone-receptor-positive disease[2]. Tamoxifen can reduce breast cancer risk in women who have an above average risk for hormone-receptor-positive breast cancer but who haven't developed breast cancer. Therefore, tamoxifen can be taken as a preventative drug as well. Tamoxifen works by binding the estrogen receptors of different tissues, for example, breast tissue, to block estrogen from binding. Due to this, tamoxifen inhibits the ability of estrogen to stimulate the abnormal breast epithelial cells to divide and grow and therefore inhibits tumor growth and cancer progression[3].

Tamoxifen can cause serious side effects noted in patients. The more common side effects include induced hot flashes, changes in bone density, and fatigue[3]. Among female (commonly cited as women in the literature) patients using tamoxifen, the severity and frequency of these hot flashes can be overwhelming and can hinder women from functioning to the fullest capacity in their daily life. Hot flashes are experienced by 78% of women taking tamoxifen[4]. 25% of women fail to start, and complete treatment due to safety concerns[1], and 46% of women discontinue use due to the side effects[5]. But the exact cell types in the hypothalamus, a brain region known to regulate body temperature, that mediate these negative side effects remain unclear. Understanding these cells targeted by tamoxifen and the resulting cellular mechanisms is necessary for comprehending the effects of tamoxifen therapy on human physiology. Knowledge of this can lead to designing new drug therapies to improve the quality of life in these patients. Reduction of negative side effects will also potentially increase patient compliance.

It has recently been shown in mice that the hypothalamus is responsible for many of the physiological effects of tamoxifen administration[4]. Therefore, examining the effects of tamoxifen administration on the hypothalamus may shed new light on how the side effects of tamoxifen use manifest in human patients. In the brain, the hypothalamus and preoptic area express estrogen receptors, most notably estrogen receptor alpha. Estrogen receptor alpha (ERα) signaling regulates body temperature in different neuronal populations within the hypothalamus. A recent publication in the Correa and van Veen lab showed that tamoxifen induces gene expression changes in the hypothalamus and the preoptic area (hypothalamus-POA)[2]. In a 28-day experiment, tamoxifen was administered to mice based on human dosage

and monitored to see if mice experienced similar physiological effects to humans. The experiment found that tamoxifen treatment induced a decrease in body temperature and an increase in tail skin temperature in wild-type mice. This mirrored human hot flashes in a mouse model (REF PMID: 1150563). Hypothalamus-specific deletion of ERα inhibited the effects on thermoregulation caused by tamoxifen. This demonstrates that tamoxifen induces thermal dysregulation through its interaction with ERα positive nuclei in the hypothalamus, but the mechanism of how this occurs is still unknown.

The mechanism of how hot flashes manifest in the hypothalamus has been proposed[13], and a special subpopulation of KNDy neurons in the arcuate nucleus is believed to be the key[13]. KNDy neurons co-express Kisspeptin, neurokinin B, and dynorphin neurons in the hypothalamus of the brain. These neurons are central to the hormonal control of reproduction. These are involved in the negative feedback of GnRH (gonadotropin-releasing hormone) of the HPG (hypothalamus-pituitary-gonadal) axis[16, 20]. Sex steroids, like estrogens, released from the gonads (ovaries) act on KNDy neurons[7, 16, 20]. Estrogens will inhibit kisspeptin release, which provides negative feedback onto the HPG axis. NKB is the stimulating peptide that is shown to initiate the release of GnRH in a pulsatile fashion by activating NKB receptors (coded for by the *TACR3* gene) on connected KNDy neurons that will release kisspeptin[7]. Kisspeptin then activates GPR54 receptors on GnRH neurons, which induces the release of GnRH.

Estrogen withdrawal at menopause leads to hot flashes. In rats, estrogen-sensitive KNDy neurons project to preoptic areas of the brain. These pre-optic areas are thermoregulatory and express neurokinin 3 receptors (NK3R), the receptor for NKB. This led to the hypothesis that

these neurons could contribute to generating hot flashes[8]. Supporting this hypothesis, the ablation of KNDy neurons in the arcuate nucleus reduces tail skin temperature. Notably, this effect of KNDy ablation is insensitive to estrogen replacement, indicating that KNDy neurons are required for the effect of estrogens on tail skin temperature[8].

In post-mortem tissue from post-menopausal women, a subpopulation of neurons in the infundibular nucleus (equivalent to the arcuate in rodents) increased in size compared to pre-menopausal women[9]. These neurons were hypertrophied (larger) and had increased neurokinin B and kisspeptin gene expression[9]. This hypertrophy occurred secondary to estrogen withdrawal and was shown again in ovariectomized cynomolgus monkeys. These monkeys had hypertrophied neurons and increased Neurokinin B and Kisspeptin expression after ovariectomy. This was shown to be secondary to estrogen withdrawal as estrogen replacement in cynomolgus monkeys reversed this gene expression pattern[10]. Therefore, KNDy neurons are affected by estrogens in primates.

In current literature, it has been shown that KNDy neurons branch and project to GnRH terminals in the preoptic structures that regulate body temperatures. Secretion of GnRH into capillaries stimulates LH secretion, which in turn stimulates the secretion of estrogen E2 from the ovaries. E2 provides negative feedback and reduces LH secretion, thus decreasing NKB and kisspeptin mRNA in KNDy neurons. ERα is the isoform required for estrogen-mediated negative feedback and is expressed in the arcuate KNDy neurons. Median preoptic nucleus (MnPO) neurons express Nkr3 and receive information from ER-sensitive thermoreceptors that project

to the CNS to modulate heat-dissipation effectors. The activation of Nkr3 in the MnPO reduces body temperature.

Kiss1 neurons were activated using a combination of genetic and viral technologies[11]. They found that NKB-expressing neurons (that are negatively regulated by sex hormones[11]), when artificially activated, cause a heat-dissipation effect. This heat dissipation results in vasodilation of the vessels to the skin with decreased core body temperature in both males and females. Therefore, it resulted in responses that are similar to a hot flash. The paper also found that this response was sensitized by ovariectomy[11]. When KNDy neurons were blocked with a cocktail of neurokinin receptor antagonists the paper saw that hot flashes were abolished[13]. This paper concluded that activation of KNDy neurons after estrogen withdrawal from ovariectomy still causes hot flashes because of NKB release in the preoptic area[13]. KNDy neurons play a role in this mechanism which needs to be better understood in relation to tamoxifen. A recent FDA-approved drug, fezolinetant, is the first neurokinin 3 receptor antagonist primarily treating vasomotor symptoms in menopausal women[14]. It is a neurokinin 3 receptor antagonist that blocks NKB signaling from occurring[22]. Therefore, the activity of KNDy neurons in the thermoregulatory part of the arcuate does not function and reduce vasomotor symptoms (hot flashes). KNDy neurons play a great role in the induction of hot flashes and are a potential non-hormonal therapeutic target.

II. Research Question

Estrogen signaling via ERα in the hypothalamus mediates tamoxifen-induced temperature dysregulation, but the molecular mechanism is unknown. We aim to identify the

hypothalamic signaling molecules required for tamoxifen-induced hot flashes. Drugs inhibiting these molecules could treat hot flashes; therefore, the neurokinin-expressing circuit could maintain temperature homeostasis. The question I aim to answer is: how does tamoxifen administration affect the expression of NKB in the arcuate nucleus? This advances the current understanding of tamoxifen action in the hypothalamus. I aim to analyze the role of neurokinin signaling in tamoxifen-induced hot flashes and the hypothalamic circuit regulating temperature homeostasis.

IV. Hypothesis

Tamoxifen administration affects hypothalamic neurokinin expression in the arcuate nucleus (ARC).

Specific Aim 1: *To determine the effects ovariectomy has on Tac2 and Esr1.*

To address this specific aim, ovaries were removed from wild-type female mice. The mice were then injected with the vehicle for tamoxifen. Multi-color fluorescent in-situ hybridization (RNAscope™) was used to see the effects of tamoxifen administration on *Esr1* and *Tac2*. Microscopy analysis was used to image slides. The brains that were compared are ovariectomized mice to control wild-type female mice that undergo sham ovariectomy surgery. The effects of temperature were recorded for both groups.

Specific Aim 2: *To determine the effects tamoxifen administration has on Tac2* and *Esr1*.

To address this specific aim, tamoxifen was injected into wild-type female mice. Multi-color fluorescent in-situ hybridization (RNAscope™) was used to see how tamoxifen administration

affects the expression of *Esr1* and *Tac2*. Microscopy analysis was used to image slides. The brains were compared to ovariectomized mice and control wild-type female mice undergoing sham or ovariectomy surgery. The effects of temperature and significant hot flash symptoms were also recorded.

V. Methods

Mice

Wildtype agouti Esr1 fl-fl were bred in our colony at the University of California, Los Angeles. All mice were housed under a 12:12 hour light/dark schedule at room temperature (22-23 degrees C). They were provided food and water. A total of nineteen female mice were used for the study. Mice were 10-16 weeks old at the onset of any injections or surgeries. All studies were carried out in accordance with recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. UCLA is AALAS accredited. The UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. During ovariectomy surgery, mice were anesthetized with isoflurane and were treated with analgesics pre-and post-surgery.

Mouse results are the combination of two experiments: a first trial examining the effect of ovariectomy versus sham surgery (Mice N1-N8) and a second trial examining the effect of tamoxifen treatment versus vehicle treatment (Mice N9-N19). All ovariectomized animals were also given vehicle injections on the same schedule for comparability. All ovariectomized mice were left to heal for 15 days before vehicle injections began. All three experimental groups were included in the second experiment.

Tamoxifen administration

Tamoxifen was dissolved in ethanol and then diluted in corn oil at a final concentration of 100ug/mL and 0.5% ethanol. The vehicle oil was prepared in corn oil (5% ethanol). Daily subcutaneous injections of tamoxifen were given at a dose of 0.1mg/kg (or equal volume) for 10 days between 2 hours after lights on and 3 hours after lights on. The dosage models of human tamoxifen exposure are based on studies performed by the Van Veen lab.

Brain Processing

Deeply anesthetized mice were perfused with ice-cold PBS (pH=7.4) followed by 4% paraformaldehyde (PFA) in PBS. The brains were fixed in 4% PFA overnight and dehydrated in 30% sucrose for 24 hours. The brains were embedded post-sucrose treatment and in optimal cutting temperature (OCT) compound. Brains were stored at -80 degrees Fahrenheit before sectioning. Coronal sections were cut using a cryostat into 16 equal series at 18um.

Imaging

Images were taken by Nikon Eclipse Ti2 in 20X magnification. Exposure was set at the same level for each brain after the brightest slide was found. UV was set at 150ms, GFP was at 60ms, and CY5 was at 23ms. *Esr1* was used as a landmark for the Bregma location independent of NKB. Sections were then selected blindly.

In-situ hybridization

Single RNA in-situ hybridization was performed using 2.5 HD Reagent Kit-RED (REF No. 322381) according to the manufacturer's protocol. The sections were washed in PBS and dried in a 60 °C

incubation chamber for 30 minutes. The slides were incubated in a boiling (100 °C) solution of 1X Target Retrieval for 5 minutes. The slides were washed in PBS and were dehydrated in graded EtOH (50%, 75%, and 100%) for 3 minutes each. A hydrophobic barrier was drawn around the tissues using an ImmEdge Pen. The tissues were incubated in proteinase solution at 40 °C for 30 min. Then the tissues were washed in PBS, and amplification steps Amp 1-3 in the kit were performed. ISH signals for channels 1-2 were performed. *Esr1* catalog probe was channel 1 with red fluorescence, and the *Tac2* probe was channel 2 with green fluorescence. Both were incubated at 40 °C for 2 h. All remaining washing steps were performed using 1× wash buffer in the kit. Slides were cover-slipped with Gold Mounting Media.

Cell Profiler

Tac2 and *Esr1* were determined using CellProfiler (Mac IOS version 4.2.1) with a custom pipeline. First, images were cropped to include only the arcuate nucleus. DAPI-stained nuclei were detected, and intensity was used to measure *Tac2* mRNA (NKB) cells.

Statistics

Data were analyzed for statistical significance using a one-way ANOVA followed by a post-hoc Tukey test to compare groups. Significance was defined at a level of $\alpha < 0.05$. Statistics were performed using RStudio.

VI. Data

To examine *Tac2* mRNA (NKB) expression in ovariectomized mice, four mice were ovariectomized, and four underwent sham surgery. All were injected with oil for 10 days. After

perfusion, cryosection, and RNAscope™ were performed, image quantification was performed. It appeared that the highest expression of NKB in neurons was found in ovariectomized mice, as shown in Figure 1. This result is shown throughout all eight mice when comparing Figure 1.2 to Figure 1.3. However, when comparing the mean NKB fluorescence per animal, no significant difference was observed between the ovariectomy and sham groups.

After this experiment, tamoxifen administration was included in a second experiment. 2 mice were ovariectomized and given a vehicle for tamoxifen for 10 days. 4 wild-type mice underwent sham surgery and were given a vehicle for 10 days. 5 mice underwent sham surgery and were injected subcutaneously with tamoxifen for 10 days. One-way ANOVA demonstrates that the treatment group significantly affects the mean NKB expression in the arcuate per mouse ($p = 0.024$). The post-hoc comparison shows that mice given tamoxifen mice had significantly **decreased** *Tac2* mRNA (NKB) expression in the arcuate compared to ovariectomized (OVX) mice. In summary, this was shown with representative images in Figure 1.1. Figures 1.2-1.5 shows the images for all individual mice that underwent this experiment. Figure 1.6 shows *Esr1* mRNA expression, and no significant differences were observed between treatment groups by one-way ANOVA statistical analysis.

Therefore, the main findings are that *Tac2* mRNA (NKB) expression was **significantly decreased** in the arcuate nucleus in tamoxifen-injected mice compared to ovariectomized mice, shown in Figure 1.7. As expected and seen in figure 1.8, ovariectomy notably increases body weight, and a 10-day course of tamoxifen injection daily similarly increases body weight

compared to control mice. Figure 1.8 shows uterine weight in tamoxifen-injected and control mice is significantly larger than in ovariectomized mice.

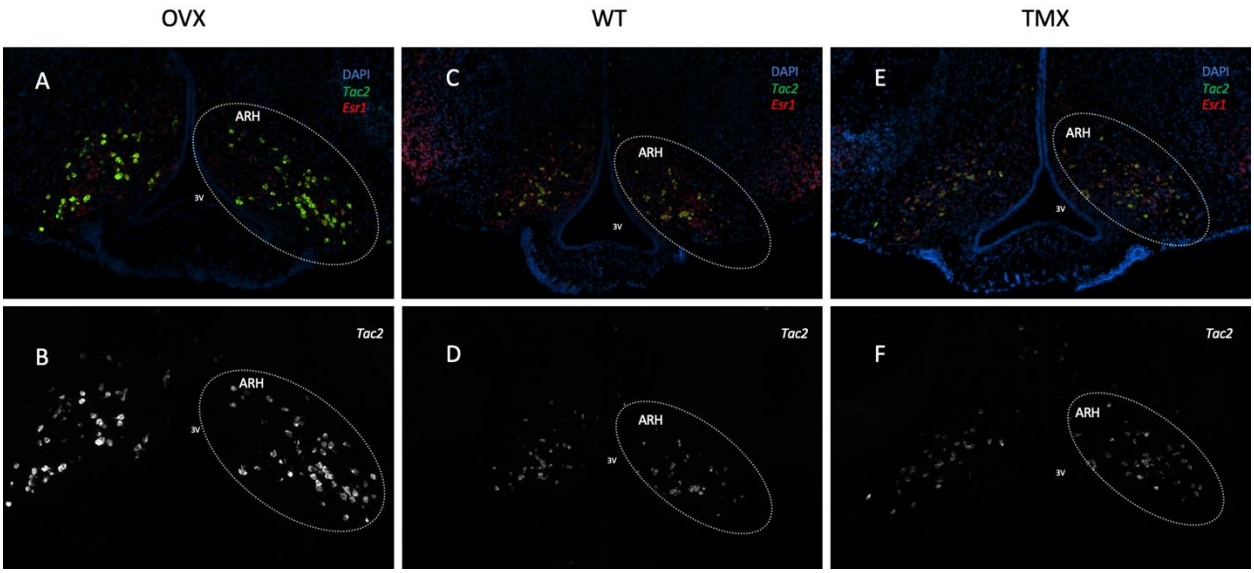

Figure 1.1- Representative Images of *Tac2* mRNA (NKB) expression. Top Row (A, C, & E) shows in-situ hybridization, RNAscope, in the arcuate (ARH) nucleus of the hypothalamus of mice from three different groups. Blue shows DAPI stained nuclei, green shows *Tac2* mRNA (NKB), and red shows *Esr1* mRNA for estrogen receptor alpha. **A:** Image representation of in-situ hybridization for mice that underwent ovariectomy and after 15 days were injected with vehicle for 10 days. **C:** Image representation of in-situ hybridization for mice that underwent sham surgery and then were injected with vehicle for 10 days. **E:** Image representation of in-situ hybridization for mice that underwent sham surgery and then were injected with tamoxifen for 10 days. **Bottom Row (B, D, & F)** is in-situ hybridization in the arcuate (ARH) of mice from three different groups. *Tac2* mRNA (NKB) expression is shown in black and white for clarity. **B:** In-situ hybridization for *Tac2* mRNA (NKB) expression for mice that underwent ovariectomy with vehicle injection for 10 days. **D:** In-situ hybridization for *Tac2* mRNA (NKB) expression for mice that were injected with vehicle for 10 days. **F:** In-situ hybridization for *Tac2* mRNA (NKB) expression for mice that were injected with tamoxifen for 10 days. Arcuate nucleus, ARH. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX).

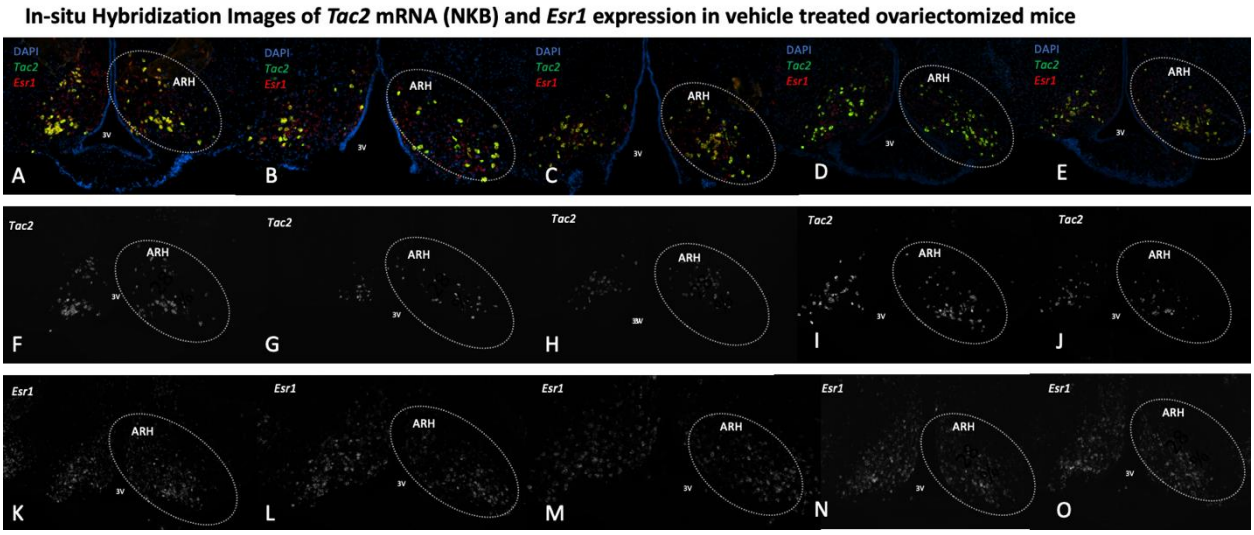

Figure 1.2- In-Situ Hybridization Images of *Tac2* mRNA (NKB) and *Esr1* expression in vehicle treated ovariectomized mice. Figure shows in-situ hybridization of images taken at 20X of brain sections from the arcuate (ARH) nucleus of the hypothalamus of five different mice that were all ovariectomized and after 15 days were injected with vehicle for 10 days. **Row (A, B, C, D, E)** shows merged RGB staining from in-situ hybridization (RNAscope). Blue shows DAPI stained nuclei, green shows *Tac2* mRNA (NKB), and red shows *Esr1* mRNA for estrogen receptor alpha**. Middle row (F, G, H, I, J)** shows *Tac2* mRNA (NKB) expression in black and white for all OVX mice. **Bottom row (K, L, M, N, O)** shows *Esr1* mRNA (NKB) expression in black and white for all OVX mice in this experiment. Arcuate nucleus, ARH. Ovariectomized mice, OVX. Third Ventricle, 3V.

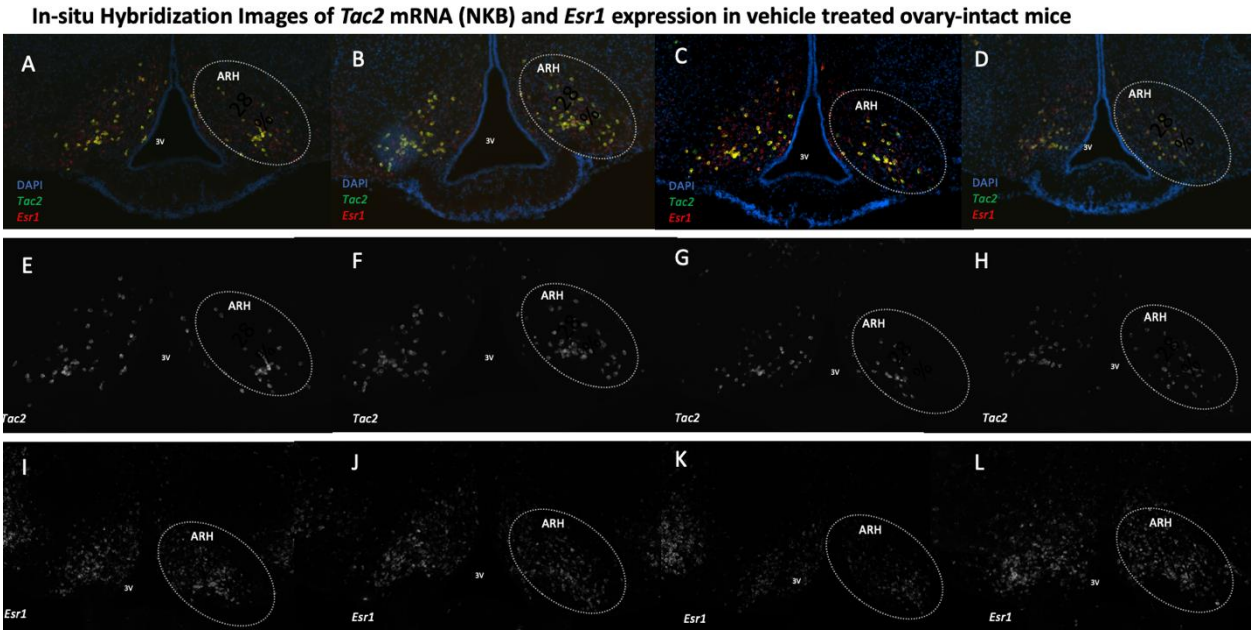

Figure 1.3- In-situ Hybridization Images of *Tac2* mRNA (NKB) and *Esr1* expression in vehicle treated ovary-intact mice. 20X images taken from the arcuate (ARH) of the hypothalamus of four different mice in the same treatment condition from the first experiment. All mice were injected with vehicle for 10 days in the first experiment. Top Row (A, B, C, D) shows merged RGB staining from RNAscope in-situ hybridization for nuclei (blue), *Tac2* mRNA (NKB) expression (green), and estrogen receptor (*Esr1*) mRNA expression (red). A, E, and I are mouse N3. B, F, and J are mouse N4. C, G, and K are mouse N7. D, H, and L are mouse N8. **Middle row (E, F, G, H)** are only showing the channel for *Tac2* mRNA (NKB) expression in black and white for the respective wild type mice. **Bottom row (I, J, K, L)** is showing *Esr1* mRNA expression in black and white for respective wild type mice of the first experiment. Arcuate nucleus, ARH. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX). Third Ventricle, 3V.

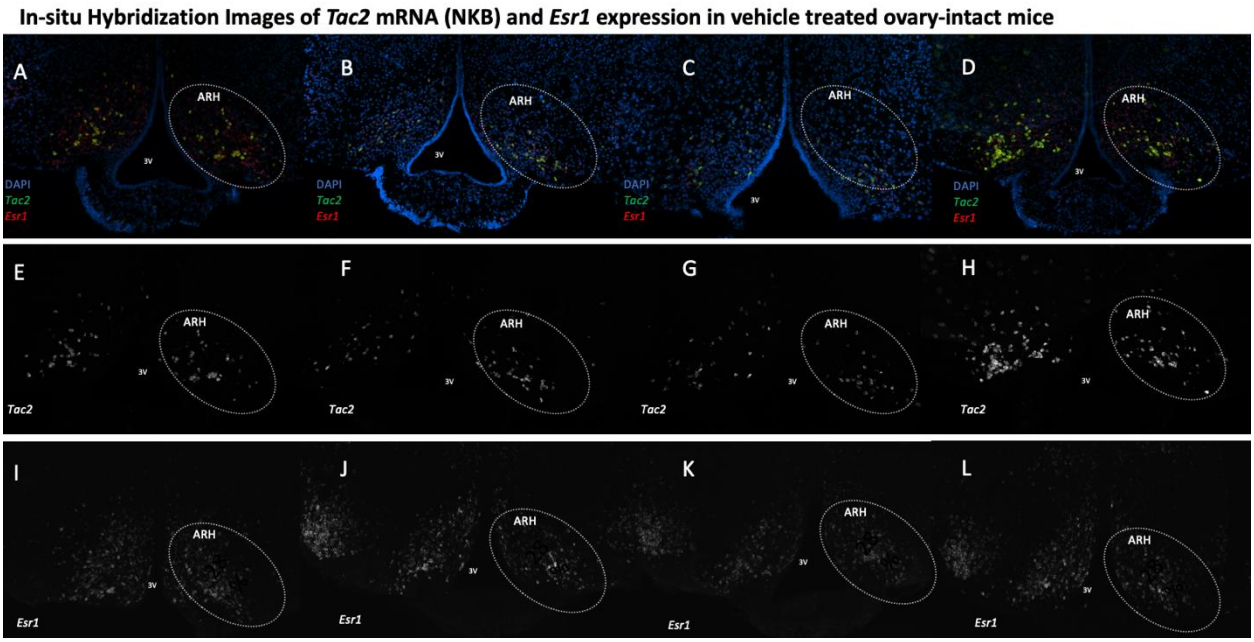

Figure 1.4- In-situ Hybridization Images of *Tac2* mRNA (NKB) and *Esr1* expression in vehicle treated ovary-intact mice. In-situ hybridization (rnascope) shows images that were taken of the arcuate (ARH) of the hypothalamus of four wild type (control) mice in the second experiment at 20X. All mice underwent sham surgery and were injected with vehicle for 10 days. Top Row (A, B, C, D) shows merged RGB staining from RNAscope in-situ hybridization for nuclei (blue), T*ac2* mRNA (NKB) expression (green), and estrogen receptor (*Esr1*) mRNA expression (red). **Middle row (E, F, G, H)** shows *Tac2* mRNA (NKB) expression in black and whtie. **Bottom row (I, J, K, L)** shows *Esr1* mRNA expression in black and white for the four wild-type mice in the second experiment. Arcuate nucleus, ARH. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX). Third Ventricle, 3V.

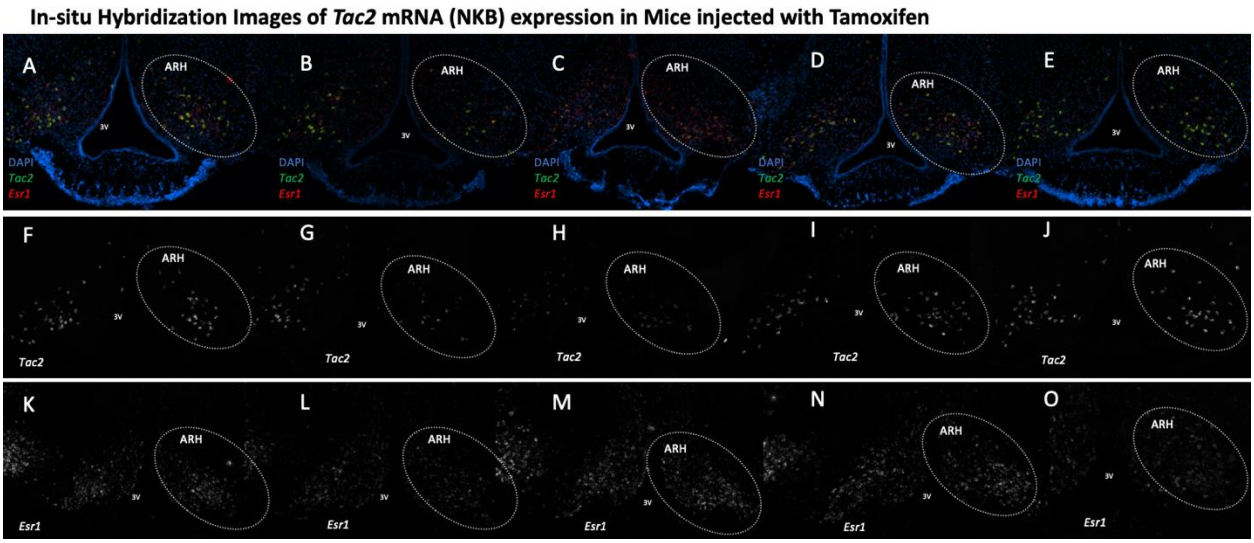

Figure 1.5- In-situ Hybridization Images of *Tac2* mRNA (NKB) expression in Mice injected with Tamoxifen. In-Situ hybridization of five mice brains that were imaged for the arcuate nucleus of hypothalamus (ARH) at 20X. All mice were injected for 10 days with tamoxifen. Top tow (A-E) shows merged RGB staining from RNAscope in-situ hybridization for nuclei (blue), *Tac2* mRNA (NKB) expression (green), and estrogen receptor (*Esr1*) mRNA expression (red). Middle row (F-J) shows *Tac2* mRNA (NKB) expression in these tamoxifen injected mouse brains. Bottom row (K-O) shows *Esr1* mRNA expression in black and white for clarity. Arcuate nucleus, ARH. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX). Third Ventricle, 3V.

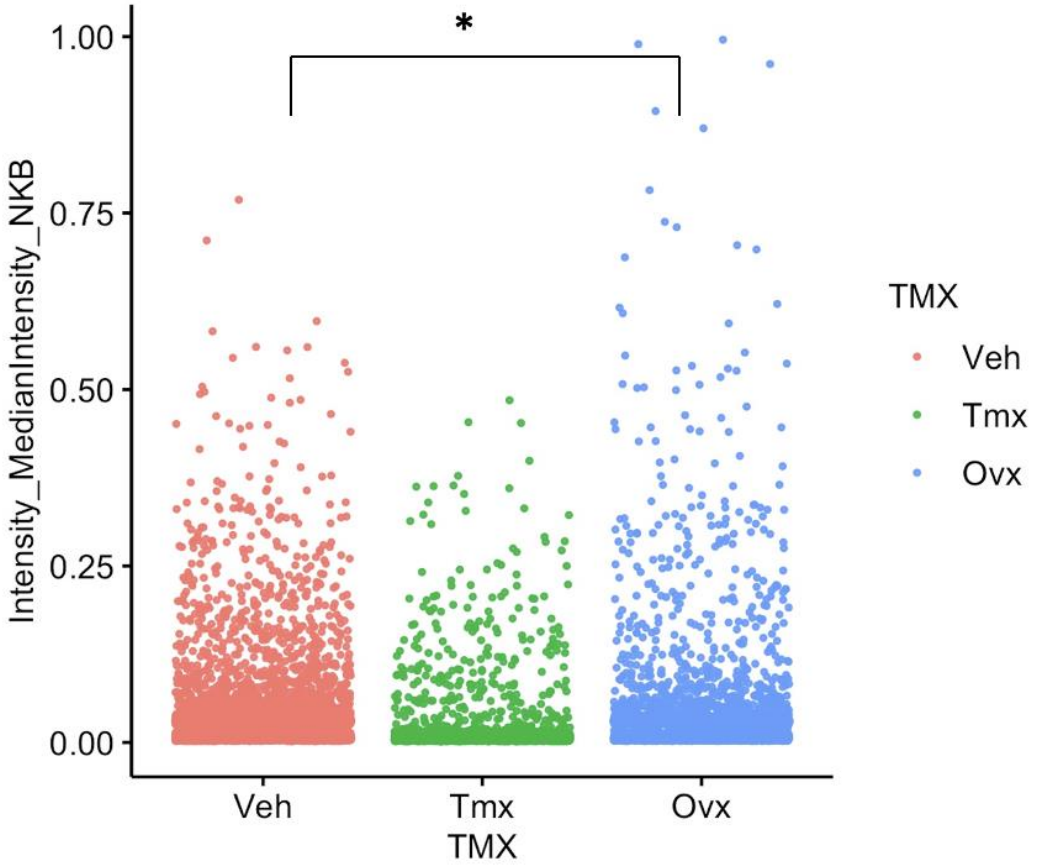

Figure 1.6 *Tac2* mRNA median intensity in control compared to Tamoxifen or OVX group. Cell profiler was used to analyze images of the arcuate of all 19 mouse brains. Each dot represents a single neuron. Normalized median intensity values of *Tac2* transcript for each neuron are colored by treatment group. One-way ANOVA demonstrates that there is a significant effect of treatment group on NKB expression ($p < .024$), after averaging fluorescence per mouse and treating those as biological replicates. Post-hoc Tukey test indicates a significant difference between Tmx and Ovx groups. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX).

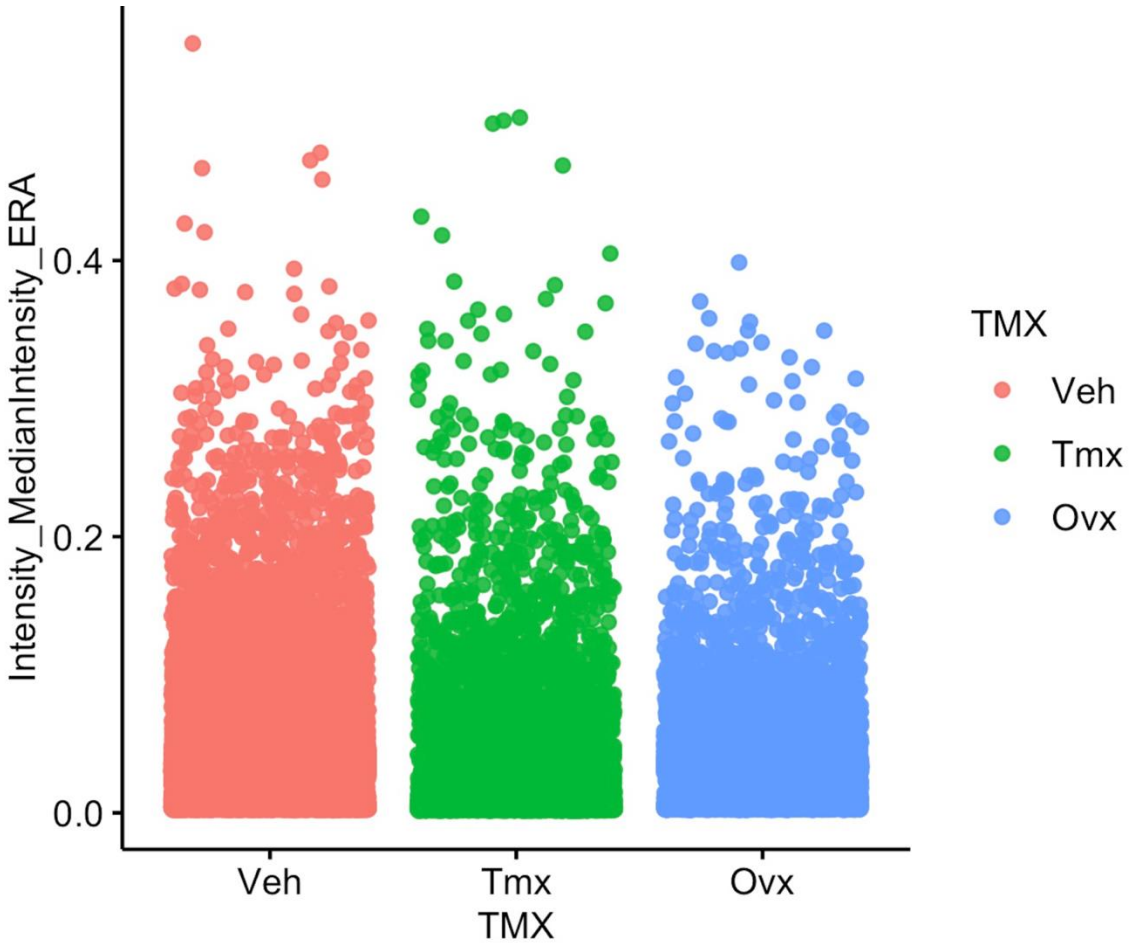

Figure 1.7 *Esr1* mRNA median intensity across three trial groups
Cell profiler was used to analyze images of the arcuate of all 19 mouse brains. Each dot represents a single neuron. Normalized median intensity values of *Esr1* transcript for each neuron are colored by treatment group. One-way ANOVA demonstrates that there is no significant effect of treatment group on *Esr1* expression ($p < .18$). ERα is the protein, Esr1 is the gene. Ovariectomized mice, OVX. Wilt-type (WT). Tamoxifen-treated mice (TMX).

Mouse Number	Treatment group	Body Weight (g)	Uterus Weight (mg)
N1	**OVX + VEH**	**23.1**	**21.7**
N2	**OVX + VEH**	**23.0**	**25.4**
N3	SHAM + VEH	20.7	74.1
N4	SHAM + VEH	21.9	77.6
N5	**OVX + VEH**	**23.0**	**30.7**
N6	**OVX + VEH**	**22.8**	**25.7**
N7	SHAM + VEH	22.1	148.1
N8	SHAM + VEH	21.6	170.2
N9	**OVX + VEH**	**23.6**	**36.1**
N10	**OVX + VEH**	**24.2**	**33.4**
N11	SHAM + VEH	20.1	66.2
N12	SHAM + VEH	22.0	172.3
N13	SHAM + VEH	22.1	94.0
N14	SHAM + VEH	21.9	76.9
N15	**SHAM + TMX**	**24.5**	**134.1**
N16	**SHAM + TMX**	**25.5**	**141.9**
N17	**SHAM + TMX**	**23.2**	**129.2**
N18	**SHAM + TMX**	**23.3**	**137.0**
N19	**SHAM + TMX**	**22.8**	**111.2**

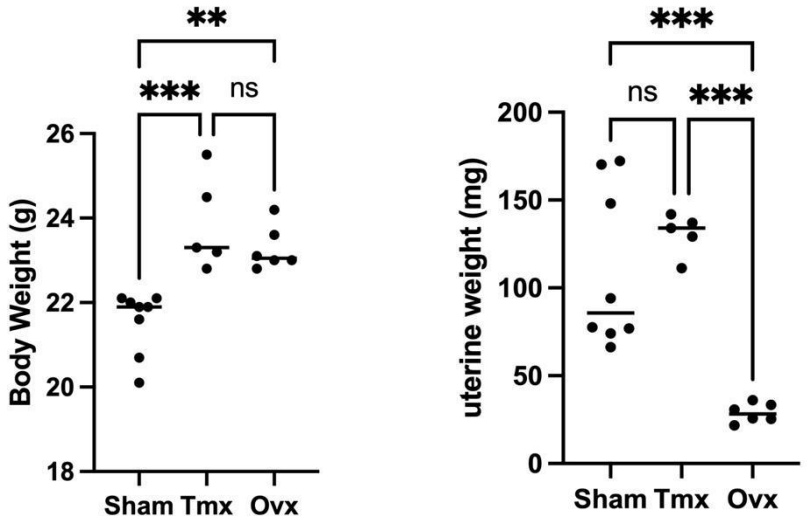

Figure 1.8 Uterus and Bodyweight of Mice Table 1.1 shows raw body and uterine weight data for all groups (n=19): Tamoxifen injection for 10 days, control injection with the vehicle for 10 days, and ovariectomized mice post 15 days and injected with the vehicle for 10 days. Bodyweight was taken before perfusion. Uterus weight was taken after perfusion to aid in

estrus cycle staging. Figure 1.8.1 shows one-way ANOVA p = .0002, post hoc Tukey ** p = .0027, *** p = .0003 of body weight for all groups. Figure 1.8.2 shows one-way ANOVA p < .0001, post hoc turkey *** p < .0005.

VII. Discussion

The circuit for hot flashes has been well studied. Prolonged decreases in estrogen cause decreased expression of NKB in KNDy neurons, which express Estrogen receptor alpha. These neurons signal to the MnPO, which has NKB receptors located on it, which causes efferent neural pathways to fire to control heat-defense effectors[5], otherwise known as a hot flash. I hypothesized that ovariectomy of mice, which mimics post-menopausal conditions in women due to the decrease in estrogen, would have increased *Tac2* mRNA (NKB) expression in the arcuate nucleus. I also hypothesized that tamoxifen treatment of mice would increase *Tac2* mRNA (NKB) in the arcuate nucleus, contributing to thermal dysregulation. Women who take tamoxifen experience hot flashes similar to post-menopausal women, even if they are still cycling[12].

We found tamoxifen *decreased* rather than increased *Tac2* mRNA (NKB) expression. This was interesting because the results contradict the prevailing hypothesis that increased Tac2 expression is the principal mechanism for the induction of thermal dysregulation. The mice still show thermal dysregulation, but *Tac2* mRNA (NKB) expression does not increase. If confirmed, these results indicate there is more to thermal dysregulation than NKB expression regarding the mechanism of hot flashes. Our research separated thermo-dysregulation from changes in *Tac2* mRNA (NKB) expression. Thermo-dysregulation may occur before changes in NKB are seen. This does not mean that NKB is not involved; rather, the results show that something else may also be happening. The order of occurrence may be different than first anticipated. Thermo-dysregulation may occur after 10 days of tamoxifen injection, but since women can take

tamoxifen for up to 10 years, longer injections might show changes in NKB expression as a secondary effect. The receptors may show changes as well in the MnPO. Other peptides may be cooperating with NKB in the creation of hot flashes. For example, the Pituitary adenylate cyclase-activating polypeptide (PACAP) is expressed in the arcuate nucleus and is another gene associated with thermal regulation[21]. Future studies will examine the timing and regulation of arcuate nucleus transcripts in response to tamoxifen administration.

This is consistent with previous findings that estrogen acts and its effects on tachykinins (NKB) are more complex than an "on-off" response'[13]. In an experiment where all mice were ovariectomized (no circulating ovarian estrogens), one group was left as a control, a second ground was reintroduced to estrogen by short-term injection, and a third group was reintroduced to estrogen as a capsule with more continuous exposure. The paper found that when estradiol benzoate was reintroduced in capsule format (continuous estradiol exposure) the number of tachykinin-positive cells (through immunoreactive cells) showed a 'substantial decrease'[13]. On the other hand, short-term estrogen exposure in ovariectomized rats did not show a significant change in the population of these tachykinin-positive cells. Therefore, this paper showed that the timing and dose of exogenous estrogen is important. The specific period of exposure is also important. The paper showed that prolonged estrogen exposure downregulates NKB.

My data here indicate that there may be a *Tac2*-independent mechanism of tamoxifen induced thermal dysregulation. This unknown mechanism happens either independently of or preceding changes in the better characterized *Tac2* induction in response to lack of estrogen. It

is also formally possible that tamoxifen administration causes thermal dysregulation using an entirely different mechanism than menopause. In the mechanism of hot flashes, many effectors may represent clinically actionable targets to provide hot flash symptom relief.

VIII. Future Directions

The data I gathered in this project have further advanced our understanding of tamoxifen-induced effects on hypothalamic neurokinin signaling. A future direction for tamoxifen research and neurokinin signaling is to determine whether prolonged tamoxifen exposure causes increases in NKB expression and whether this is causative in tamoxifen-induced thermal dysregulation. To address this, neurokinin signaling agonists/antagonists can be used to study the function of hypothalamic neurokinin signaling in regulating temperature homeostasis in the context of tamoxifen treatment. Further, my data imply that signaling molecules and pathways other than Tac2 are involved in generating thermal dysregulation; indicating that there may be many unexplored targets for exploration.

IX. References

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