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Tamoxifen-mediated changes in estrogen-dependent gene expression in hypothalamic neurons responsible for hot flashes

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by

Amanda Marie Misquez

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ABSTRACT OF THE THESIS

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by

Amanda Marie Misquez

Master of Science in Physiological Science

University of California, Los Angeles, 2023

Professor Stephanie Correa Van Veen, Co-Chair

Professor John Edward Van Veen, Co-Chair

Tamoxifen (Tmx) is a selective estrogen receptor modulator widely used as a chemotherapeutic drug in the treatment regimen for estrogen-sensitive breast cancer. Tmx treatment leads to many side effects; the most prominently reported side effect is hot flashes. Hot flashes are episodes of thermodysregulation and have been shown to be caused by estrogen-receptor α (ERα) signaling in the hypothalamus. In this thesis, we delivered a custom, bi-functional fluorescent reporter to three estrogen-sensitive nuclei in the hypothalamus that regulate body temperature: the medial preoptic area (MPA), the arcuate nucleus (ARC), and the ventrolateral region of the ventromedial hypothalamus (VMHvl). The reporter labels estrogen-

sensitive neurons that express ERα and reports whether neurons alter estrogen-dependent gene transcription and expression in response to Tmx treatment. Upon delivering the fluorescent reporter to the three thermoregulatory and estrogen-sensitive regions of interest (the MPA, ARC, and VMHvl), we observed that Tmx treatment in *Esr1 Cre* and *Kiss1 Cre* mice showed no significant differences in ERE-dependent gene expression in the MPA, ARC, and VMHvl compared to control, Oil-treated mice. Therefore, we are unable to detect Tmx-induced changes in gene expression in estrogen-sensitive neurons of the MPA, ARC, or VMHvl. We conclude that Tmx does not alter estrogen-dependent gene expression in the hypothalamus under the conditions we used. As our Tamoxifen treatment paradigm is sufficient to induce thermodysregulation, this implies the intriguing possibility that Tamoxifen induces hot flashes without widespread changes in hypothalamic gene expression.

The thesis of Amanda Marie Misquez is approved.

Paul 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 my mother, Guadalupe Robles. Your strength is deeply aspirational, and you have shown me what it means to find and follow your passion boldly. I am the woman I am today because of your sacrifices and unconditional support.

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Lastly, I would like to express gratitude to Rocket, Claire, and Luna for their invaluable emotional support.

BACKGROUND

Tamoxifen (Tmx) is an accessible and common chemotherapeutic and chemopreventive for cases of estrogen-receptor-positive breast cancer [1]. Tmx's effects across systemic tissues that express estrogen receptors vary due to its multifaceted nature [2]. As a selective estrogen receptor modulator (SERM), Tmx can act as an antagonist [3] or agonist [4, 3] to the cellular signaling mediated by estradiol upon binding its receptor in specific tissues [3]. The literature cites that self-reported women undergoing Tmx treatment for breast cancer are prone to various side effects, including hot flashes, changes in bone density, lethargy, and deep vein thrombosis [5]. However, the most prominent and disruptive side effect among self-reported women [6] and men [2] taking Tmx therapy for breast cancer treatment is hot flashes, a form of thermodysregulation.

Hot flashes are a sudden, transient increase in skin temperature leading to flushing of the skin and a decrease in core body temperature [5, 7, 8]. Hot flashes are periodic and are reported as an overwhelming sensation of heat, sweating, and flushing of the skin [7,8]. Hot flashes are observed in perimenopausal and menopausal women when the follicular cells of the ovaries begin to reduce the amount of 17β-estradiol (E2) produced every month [9, 10]. The occurrence of hot flashes in breast cancer patients appears slightly higher in women than in men [2], such that 40% of post-menopausal women reported experiencing hot flashes upon starting Tmx treatment [11, 12, 13, 14].

Breast cancer manifests with the uncontrollable and abnormal growth of mammary cells within the breast tissue. These rapidly dividing abnormal cells may express steroid hormone receptors on their surface, including estrogen receptors (ERs) or progesterone receptors (PRs) [15]. When abnormal cells express one or both types of receptors, the specific incidence of breast

cancer can be classified as hormone-sensitive [15]. If breast cancer cells exclusively express ERs, the binding of circulating E2 to ERs on breast cancer cells can amplify their growth and proliferation. This cancer can be further classified as estrogen-sensitive or estrogen-receptor-positive. Approximately 67–80% of breast cancer cases in women are ER-positive [15, 16], and approximately 90% of breast cancer cases in men are also ER-positive [17]. ER-positive breast cancer is most common in middle-aged and older women [16].

The literature cites that women with early ER-positive breast cancer are commonly prescribed Tmx [18] as the standard of care to prevent breast cancer cell proliferation. Tmx inhibits ERα transcriptional activity in mammary cells and effectively reduces the risk of recurrence of invasive or in situ ER-positive breast cancer, independent of age [19]. Since it acts as an ER antagonist on breast cancer cells to prevent tumor growth [3], it effectively reduces the likelihood of breast cancer cell proliferation [19]. Tmx can be taken every day for up to 10 years as it is the predominant adjuvant therapy that clinicians use after primary treatment (such as surgical interventions including lumpectomy, mastectomy, or ovariectomy) [20]. Despite its efficacy in reducing the risk of breast cancer cell progression, Tmx has the potential to act as an ER-agonist or antagonist in other tissues in the body that express ERs [2]. ERs are ubiquitously expressed in various tissues as there are two classes of ERs: nuclear and membrane estrogen receptors [21].

Estrogen-receptor α (ERα) and estrogen-receptor β (ERβ) are part of the nuclear estrogen receptor family [10]. G-protein coupled estrogen receptor-1 (GPER1) is a membrane estrogen receptor, and it is responsible for mediating the rapid effects of estradiol signaling through G-protein coupled and protein kinase cascades upon estradiol binding. This thesis will focus on the transcriptional effects of Tmx on ERα-expressing neurons exclusively. To better understand the

potential effects of Tmx on gene expression in ERα-expressing cells, it is first important to indicate the mechanism in which estradiol, and then subsequently Tmx, interacts with ERα.

ERα is 595 amino acids in length and weighs 67kDa. As a member of the nuclear hormone receptor superfamily of transcriptional regulators, ERα has the following functional domains: A/B, C, D, and E/F [10]. The A/B region, known as the amino-terminal domain (NTD), is responsible for gene transcription transactivation. This region contains a zinc finger that assists in the binding of ERα to the target gene sequence on DNA. The C region, known as the DNA-binding domain (DBD), contributes to ERα dimerization and binding to specific sequences on the chromatin. These canonical sequences on the chromatin are collectively known as estrogen response elements (EREs) [10, 22, 23]. The D domain, the hinge domain, connects the C and E domains and can bind to chaperone proteins. This region also contains the nuclear localization signal. This is revealed when 17-β estradiol (E2) binds to ERα, allowing the receptor-ligand complex to either translocate to the nucleus or closer to the chromatin region of interest if ERα is present in the nucleus already. The E/F region, known as the ligand binding domain (LBD), contains the estrogen-binding area and binding sites for coactivators or corepressors. There are two additional regulators of the estrogen receptor transcriptional activity known as activation function (AF) domains: AF1 and AF2 [10]. AF1 is located within the NTD in the A/B region, and AF2 is positioned within the DBD in the C region [24]. Unlike AF2, AF1 does not require binding to hormones or steroids to be activated [25].

E2 can readily cross the plasma membrane because it is a steroid hormone; it can interact with intracellular and nuclear ERα to exert its direct effects on gene transcription once the ERα-E2 complex binds to DNA sequences of interest, known as estrogen response elements (EREs) [10]. E2 can also initiate intracellular signaling cascades through interactions with GPER1 and

ERα or ERβ. Estrogen-mediated signaling events can then be categorized into genomic or nongenomic effects [10].

Genomic effects of E2 include the dimerization of E2-ERα complexes and the direct interaction with the chromatin at specific DNA sequences known as EREs [21]. Despite EREs being identified in several gene promoters and regulatory regions, it has been reported that more than ⅓ of human genes regulated by estrogen receptors do not contain ERE sequence elements [26]. Non-genomic signaling effects can also involve the indirect regulation of gene expression via various intracellular signaling events [10].

Direct genomic signaling is commonly referred to as the classical mechanism of estrogen signaling; in this process, ERα can act as a ligand-activated transcription factor [27, 28]. Upon binding of E2 to ERα, the estradiol-receptor complex will dimerize [29]. This subsequent dimerized complex can bind to the chromatin at ERE sequences in DNA, or enhancer regions within or close to promoters, and/or 3'-untranslated regions of target genes [30]. However, the ERα binding affinity for the canonical ERE sequence may differ depending on the gene of interest, and there are estrogen-regulated genes reported in the literature that do not require EREs to have an effect on gene transcription [10, 25]. The transcription and subsequent expression of several genes that do not contain EREs in their promoter regions can also be regulated by estradiol without directly binding estrogen receptors to DNA. An estimated 35% of estrogen-targeted genes lack ERE-like sequences [27, 10]. In these cases, estrogen-receptor complexes act on gene expression through activation initiated by protein-protein interactions with other transcription factors and response elements [31, 32]. This is known as indirect genomic signaling. Under this mechanism, estrogen-receptor complexes signal indirectly to influence the activation or suppression of target genes and their expression. Nuclear estrogen receptors like

ERα also induce the expression of genes containing the activator protein-1 (AP-1) sites through protein-protein interactions. AP-1 is a transcription factor that regulates key cellular processes such as cell differentiation, proliferation, and apoptosis [10].

Although estrogen signaling is complex, here I will focus solely on direct genomic signaling of E2 when circulating E2 (or an E2 agonist/antagonist) can bind to ERα to exert changes in gene transcription at the ERE of estrogen-regulated genes. The binding of E2 to ERα triggers the dissociation of heat shock proteins typically associated with unbound ERα [10]. This dissociation then allows for the dimerization of two estradiol-estrogen receptor complexes. The dimerized complex can then bind to EREs found in the promoter regions of DNA that regulate the expression of ER-target genes and activate transcription [10]. As mentioned previously, two transcriptional activation functions in the estrogen receptor contribute to this process: AF-1 in the receptor amino-terminal domain is hormone-independent, whereas AF-2 in the C-terminal ligand binding domain functions only when estrogen or another agonist is bound [10].

Tmx is a ligand for nuclear and membrane estrogen receptors [33]. Individuals undergoing Tmx treatment for breast cancer are prone to an array of side effects due to its ability to act as an agonist or antagonist to the typical mechanism of E2 signaling in systemic tissues expressing estrogen receptors [33, 25]. There are cell-specific outcomes of Tmx binding to ERα, including a variation in Tmx's effect on the subsequent activation or repression of estrogen-dependent gene transcription [33]. Gene transcription and, subsequently, gene expression can be regulated by classical EREs or promoters regulated by AP-1 sites when Tmx binds to ERα. When acting like an E2 antagonist, Tmx allows ERα binding to the ERE, but it inhibits gene expression because it does not allow AF-2 to carry out its typical function [33]. When Tmx acts like an agonist to E2 signaling, AF-1 can become active since the Tmx-ERα dimer can also bind

to the ERE and upregulate gene transcription [33]. In trying to determine which effect Tmx is exerting on ERE-dependent gene expression in the three estrogen-sensitive and thermoregulatory hypothalamic nuclei of interest, a novel bi-functional fluorescent reporter can serve to report the duality of the phenomenon of interest: it must report the presence of ERα and qualify the extent of ERE-dependent gene expression in estrogen-sensitive neurons.

The custom reporter engineered exclusively in our lab (Figure 1), once delivered to a brain region of interest via stereotaxic surgery, can function as a dual estrogen reporter. It can successfully detect ERα expression and estrogen-response element (ERE) dependent gene expression in neurons after injection in *Esr1 Cre* female mice.

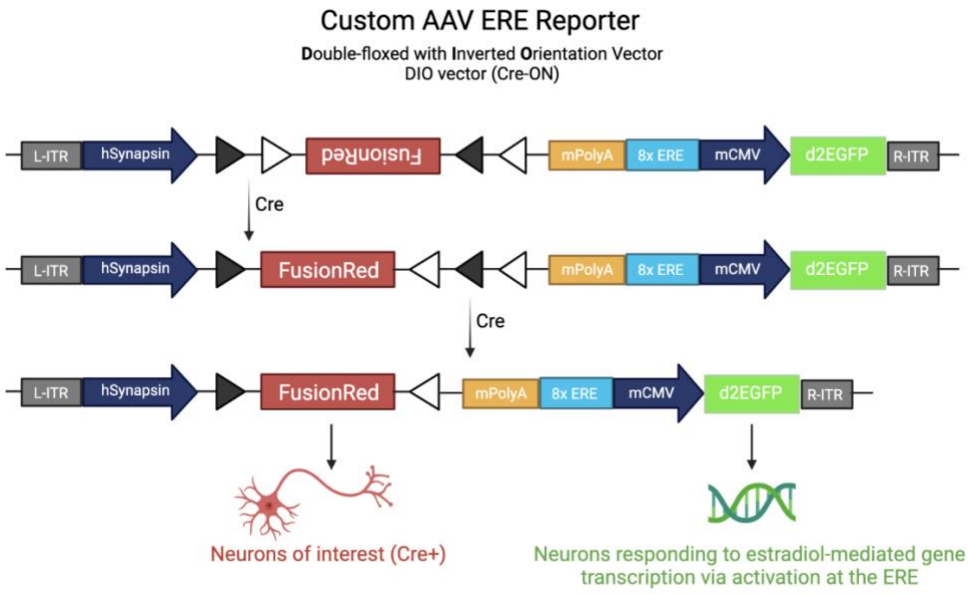

Figure 1: Viral construct of the custom, bi-functional fluorescent reporter. This schematic shows the virus encoding Cre-dependent expression of FusionRed in ERα+ neurons while synchronously reporting ERE-dependent gene expression in neurons via destabilized GFP (d2EGFP).

This fluorescent reporter utilizes a FLip Excision switch, which relies on Cre-Lox recombination. The viral construct initially has the red fluorophore FusionRed in a double-floxed

with an inverted orientation position, such that region-specific Cre-mediated recombination will allow for the selective expression of FusionRed. FusionRed is downstream of a human synapsin 1 gene promoter, which restricts transgene expression from the adenoviral vector exclusively to neurons [34]. In the presence of Cre, recombination will occur at the loxP sites surrounding FusionRed such that FusionRed will be properly flipped and subsequently expressed in cells. This project utilizes both *Esr1 Cre* and *Kiss1 Cre* female mice as the mouse models of interest, the purpose being that Cre will be selectively expressed in neurons that either express the *Esr1* gene (*Esr1 Cre* mice) or in neurons that selectively express the *Kiss1* gene (*Kiss1 Cre* mice). For the majority of this project, *Esr1 Cre* mice will be the primary mouse model of interest as we want to observe estrogen-sensitive neurons in the hypothalamus that express ERα with FusionRed fluorescence. This reporter thus allows us to permanently label estrogen-sensitive neurons of interest in the hypothalamus with the red fluorophore FusionRed, which is a low-toxicity derivative of RFP. For simplicity, we will refer to these cells as RFP+ throughout this thesis. Synchronously, destabilized green fluorescent protein (GFP) is placed under the control of 8 repeats of the conserved ERE upstream of a minimal CMV promoter. The ERE is found in the promoter regions of DNA that are responsible for estrogen-regulated genes [25]. If the ERE-dependent gene expression increases in ERα-expressing neurons, those neurons will fluoresce green to show the extent of ERE-dependent gene expression [10]. The destabilized GFP has a half-life of two hours, so it can provide a realistic temporal representation of increases or decreases in gene expression when mice are sacrificed 6-hours after the tenth and final Vehicle (Oil) or Tmx injection. In the microscopy analysis of DAPI-stained coronal mouse brain sections, the GFP signal intensity can vary to delineate the extent of ERE-dependent gene expression (and is considered GFP+).

The primary research question posed in this thesis is: how does Tmx alter gene expression in thermoregulatory ERα+ neurons in the hypothalamus to cause thermodysregulation in mice? This thesis uses rigorous techniques, including stereotaxic surgery, where region-specific stereotaxic microinjections will deliver a dual-fluorescent reporter to report ERα-sensitive neurons and ERE-dependent gene expression in the hypothalamic nuclei of interest. This thesis aims to reveal the hypothalamic nuclei in which Tmx-treatment initiates differences in ERE-dependent gene expression in thermoregulatory and estrogen-sensitive neurons compared to Oil-treated mice: namely by assessing changes in gene expression in the medial preoptic area (MPA), arcuate nucleus (ARC), and the ventrolateral aspect of the ventromedial hypothalamus (VMHvl).

CHAPTER 1: Tmx does not induce statistically significant differences in ERE-dependent gene expression in thermoregulatory neurons in the ventrolateral region of the ventromedial hypothalamus (VMHvl) compared to controls.

Introduction

Reported side effects of Tmx treatment for estrogen-sensitive breast cancer include hot flashes, changes in bone density in postmenopausal women, lethargy, and deep vein thrombosis [5]. The most prominently reported and disruptive side effect, especially in women, is hot flashes [2].

In the literature, hot flashes are hypothesized to be an inappropriate recruitment of thermoregulatory heat-effector responses to a perceived warmth [35]. The recruitment of thermoregulatory responses within the brain, specifically within the hypothalamus, leads to an acute episode of vasodilation of the vessels underneath the skin (which causes a rush of heat and flushing of the skin) and a decrease in core body temperature as heat is lost to the environment. Researchers are beginning to identify the neuronal subtypes and resulting neuronal circuits of how hot flashes occur when caused by decreased levels of circulating E2 [35], similar to what occurs during menopause in humans [9, 35] or ovariectomy in mice [5]. One potential mechanism pioneered by Naomi Rance describes that upon a gradual decrease in circulating E2, a population of *Kiss1* neurons in the arcuate nucleus (ARC) of the hypothalamus that coexpresses kisspeptin, neurokinin B (NKB), dynorphin, and ERα hypertrophy over time [9]. Upon E2 depletion from the circulation, these neurons release significantly more neurokinin B onto thermoregulatory neurons in the medial preoptic area (MPA) to induce a hot flash [9]. As it

is widely known that Tmx-treatment induces hot flashes in breast cancer patients taking the drug, it is important to study the mechanism by which Tmx induces hot flashes, which has not been delineated thus far. The mechanism in which Tmx exacerbates hot flashes through potentially acting on hypothalamic estrogen-sensitive neurons is an important scientific advancement to pursue as many women and men prescribed Tmx for estrogen-sensitive breast cancer report disturbances in daily function and quality of life due to an increase in the occurrence of hot flashes [2]. To learn how Tmx may induce hot flashes in humans, we must use a mouse model of equivalent thermodysregulation in response to Tmx treatment.

Work from our lab has shown successful induction of thermodysregulation equivalent to a hot flash in humans through a decrease in core body temperature and an increase in tail skin temperature in mice upon a 28-day Tmx treatment. Specifically, upon giving control *Esr1* Cre female mice a 28-day Tmx treatment, a statistically significant decrease in core body temperature and an increase in tail skin temperature was observed during the light phase. The combined decrease in core body temperature and increase in tail skin temperature in mice are equivalent phenomena of temperature dysregulation observed in a human hot flash. To confirm the receptor through which Tmx was exerting its effects, ERα was conditionally knocked out in the hypothalamus of *Esr1*f/f, *Nkx2-1*-Cre mice [5]; these mice exhibit a selective loss of ERα immunoreactivity in the hypothalamus and pre-optic area in the mouse brain. Upon giving this cohort of ERα knockout mice either a daily Vehicle (Oil) or Tmx injection, the thermodysregulation previously observed in the ERα-intact Tmx-treated mice was now ablated in the ERα knockout mice. Specifically, the decrease in core body temperature and increase in tail skin temperature previously observed in the ERα-expressing Tmx group was not detected and Tmx-treated ERα-/- mice showed no temperature dysregulation. These findings suggest that

Tmx likely induces thermodysregulation in mice through ERα signaling, specifically in the hypothalamus. This Tmx-ERα interaction likely occurs within estrogen-sensitive nuclei in the hypothalamus that have a role in regulating body temperature.

Based on this preliminary data from our lab, the question this thesis aims to answer is, "How does Tmx alter ERE-dependent gene expression in thermoregulatory regions of the hypothalamus?". The cellular signaling mechanisms of direct genomic signaling involving E2 and ERα are better understood. This thesis focuses on the notion that gene transcription and expression are typically upregulated upon E2 binding to ERα and causes activation or suppression of estrogen-response-element (ERE)-dependent gene expression. However, the way in which Tmx affects ERE-dependent gene expression to cause the observed thermodysregulation in our mouse model is not well understood.

The first estrogen-sensitive hypothalamic region of interest to be investigated is the ventromedial hypothalamus (VMH), specifically the ventrolateral region of this nucleus which is known as the VMHvl. The VMHvl is rich in ERα expression, and it is a brain region that plays an important role in fear, aggression [36], and thermogenesis [37]. Previous work in our lab has established that ERα is largely expressed in VMH neuronal populations with a sex-biased expression of the reprimo gene (*Rprm*). *Rprm* is a TP53- and ERα-regulated gene, and its expression can regulate the core temperature of mice in a sex-specific manner such that female mice, but not male mice, that received an *Rprm*-siRNA injection into the VMH showed an increased core body temperature [37]. Knocking down *Rprm* expression did not reduce physical activity in female mice, indicating that the *Rprm* gene in neurons in the VMHvl selectively regulate temperature. Therefore, we want to determine if Tmx is exerting agonistic or antagonistic effects on ERα neurons in the VMHvl to induce changes in temperature, namely

through a decrease in core body temperature and an increase in skin temperature. To determine this effect, we will observe any differences in the population of GFP+ cells in the VMHvl (exhibiting ERE-dependent gene expression) that are estrogen-sensitive and fluoresce with RFP (RFP+ cells) between Vehicle (Oil)-treated mice.

Materials and Methods

Animals

To address the research question, we did two rounds of analysis to optimize where the fluorescent reporter was delivered in the mouse brain via stereotaxic surgery injections. Both sets of *Esr1 Cre* female mice were maintained on a C57BL/6 genetic background and bred in our lab's colony at the University of California, Los Angeles (UCLA). All mice were maintained under a 12:12 light/dark schedule and given food and water ad libitum. Mice were 10-16 weeks old at the start of all experiments.

Mouse Procedures

All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. UCLA is AALAS accredited, and the UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

Stereotaxic Surgery

The fluorescent reporter was delivered to the brain regions of interest via stereotaxic surgery. Here, we used two approaches to target reporter delivery to the VMHvl.

First, we tested to determine if delivering a large amount of the fluorescent reporter to the medial basal hypothalamus was sufficient to permeate the MPA, ARC, and VMH. Based on the results from the first cohort of mice (denoted as Cohort 1), we used a more direct delivery of the fluorescent reporter to the caudal arcuate nucleus, which is surrounded by the VMH and VMHvl.

For all stereotaxic procedures, mice were anesthetized with isoflurane. The first batch of *Esr1 Cre* female mice (n=3-4), known as Cohort 1, received bilateral injections of the fluorescent reporter to the medial basal hypothalamus. 1500 nL of the reporter were delivered at the following injection coordinate: X = +/- .45, Y -1.3, Z -5.7 from Bregma. To confirm specificity in where the reporter was being delivered, we did a second round of surgeries and injections to another batch of *Esr1 Cre* female mice (n=3-4), known as Cohort 2. These *Esr1 Cre* female mice received bilateral injections of the fluorescent reporter to the caudal arcuate nucleus, which diffused to the neighboring VMH and VMHvl. 500 nL of the reporter were delivered at the following coordinates: AP -1.56, ML +/- .2, DV -5.9 (Caudal ARC).

After surgery, mice recovered for 2-3 weeks before a 10-day treatment plan was implemented in which mice either received daily Tmx or Vehicle (corn oil and ethanol) injection. Mice treated with vehicle injections will be referred to as "Oil-treated" mice through all 3 chapters of this thesis. Mice were sacrificed 6 hours after the last injection of Tmx or Oil at the conclusion of the 10-day injection schedule.

Perfusion, Embedding, Cryosectioning

After the 10-day treatment with Oil or Tmx, mouse brains were perfused, collected, embedded in OCT, and frozen at -80°C after one overnight post-fixation in 4% PFA and another overnight incubation in 30% sucrose to cryopreserve the tissue. Coronal mouse brain sections were obtained with a Vibratome cryostat at 18 µm for Cohort 1 *Esr1 Cre* VMHvl mice. Coronal

mouse brain sections were obtained on a Leica CM1680 cryostat at 20 µm for Cohort 1*Esr1 Cre* VMHvl mice. Brain sections across the VMH and VMHvl were collected on slides for all mice from both groups and cohorts (n=3-4). Slides were stained with a 1:1000 dilution of stock DAPI Hoechst (DAPI 1,000x) and PBS for 15 minutes, then washed with PBS and coverslipped with 180 µL of DAPI-free Fluoromount-G. Slides were housed in a 4°C fridge before microscopy analysis.

Microscopy

Images were taken with a Nikon Eclipse Ti2 Series Inverted Epifluorescence microscope. All images were taken utilizing the Large Image feature on the accompanying NIS-Elements AR 5.30.04 64-bit software. This software takes images at 20x magnification and stitches together all images across a designated area displayed at 4x magnification. All images were taken using consistent sets of exposures across all channels (UV for DAPI, GFP, and RFP). Images were exported appropriately for quantification. Quantification was performed via a custom CellProfiler pipeline using CellProfiler software for macOS 4.2.1.

Statistics

Statistics utilized to analyze cells in the VMHvl of both cohorts of *Esr1 Cre* female mice were completed such that we did not pseudo-replicate the data; we did not consider each individual virally infected neuron to be an independent biological replicate, but rather a technical replicate. Quantification of fluorescence in neurons was completed using single-cell resolution from a custom CellProfiler pipeline. We measured the median intensity of GFP and RFP in cells across all animals (n=3-4) and subsequently removed the cells that did not express the reporter by removing cells below a set lower limit of normalized median RFP fluorescence determined by examining the raw images. We then calculated the per-animal mean of all median fluorescence

values in the VMHvl; each value counted as 1 individual biological replicate. To test for significant differences in GFP fluorescence between groups, we used the non-parametric Wilcoxon rank-sum test.

Statistics for the second cohort of *Esr1 Cre* female mice were completed such that we quantified cells over the whole region of the VMHvl for all mice instead of a single-cell approach. From the quantified regions, we then calculated the per-animal mean of all median fluorescence values in the VMHvl, such that values from each animal counted as 1 individual biological replicate. To test for significant differences in GFP fluorescence between groups, we used the non-parametric Wilcoxon rank-sum test.

Results

Analysis of the microscopy images across the hypothalamus for the first set of *Esr1 Cre* female mice (Cohort 1) showed that the fluorescent reporter most drastically accumulated in the VMHvl. Both Oil (n=3) and Tmx (n=4) groups had an appropriate expression of ERα-positive neurons in the VMHvl, confirming that the *Esr1 Cre* mice were appropriately causing recombination in *Esr1*-expressing cells in the VMHvl (Figure 2.2 B, E). This means that these neurons may be responding to circulating E2 and/or Tmx in the intact female mice either receiving a Tmx or Oil injection for 10 days. Figure 2.1 shows microscopy images across the VMHvl of Oil and Tmx-treated *Esr1 Cre* female mice with merged GFP/RFP channels. We can then see GFP only, RFP only, and GFP/RFP fluorescence across the VMHvl of both experimental groups.

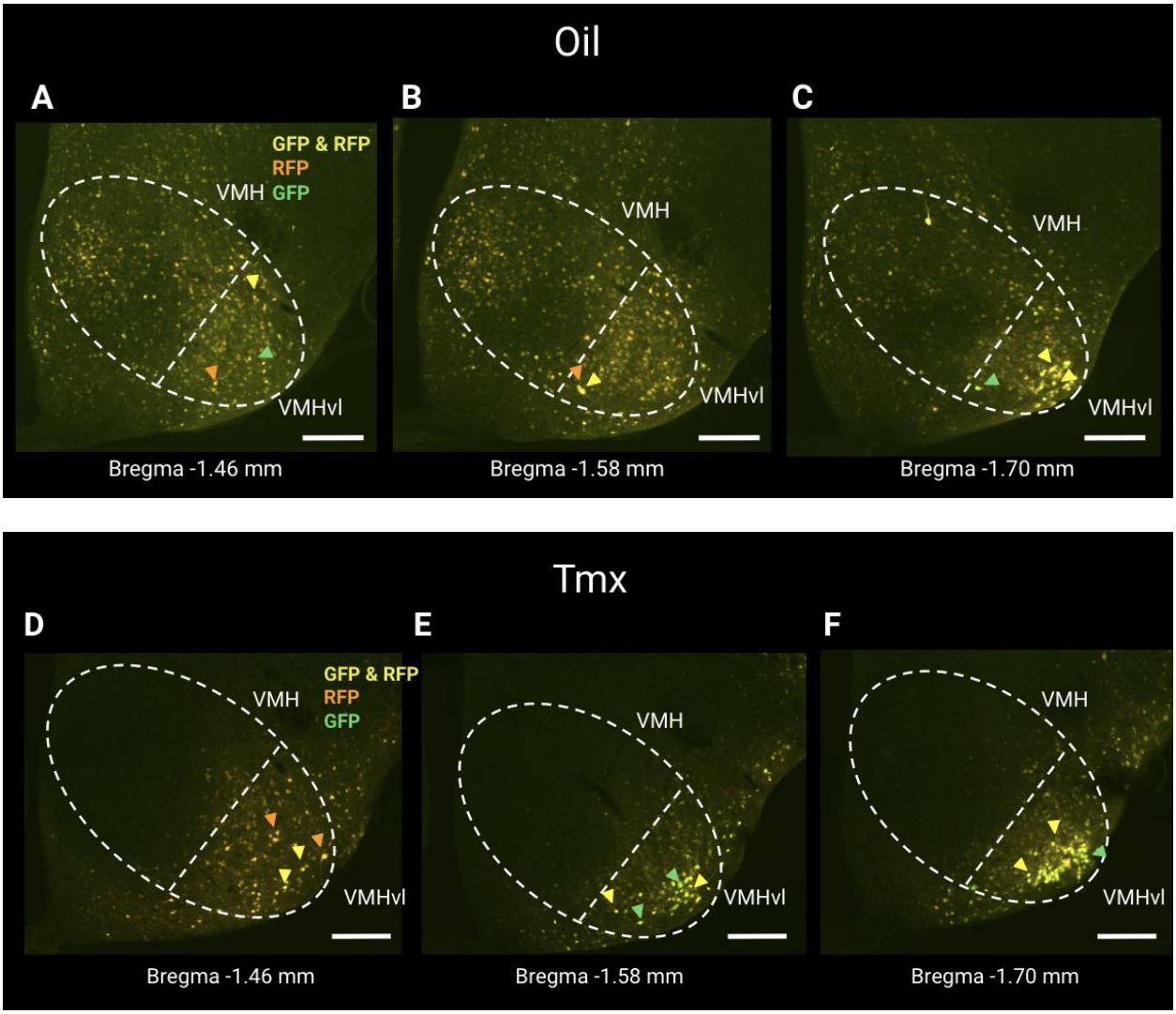

Figure 2.1: GFP+/RFP+ cells in the VMHvl of Oil and Tmx-treated *Esr1 Cre* female mice (Cohort 1). This figure shows merged images of the RFP and GFP channel from a DAPI stain at 20x magnification in both Oil and Tmx-treated mice across the VMHvl. Cells that fluoresce orange are RFP+, indicating that the cell expresses Cre in *Esr1*-expressing neurons. The *Esr1* gene codes for ERα. Cells that fluoresce green are GFP+, indicating ERE-dependent gene expression occurring within that neuron. VMHvl neurons that co-express GFP+ and RFP+ cells fluoresce yellow. VMH, ventromedial hypothalamus. VMHvl, ventrolateral region of the ventromedial hypothalamus. **A-C.** Merged GFP/RFP channel across the VMHvl of Oil-treated Esr1 Cre female mice shows GFP/RFP fluorescence, indicating that there are estrogen-sensitive (RFP+) neurons in that VMHvl that displayed ERE-dependent gene expression (GFP+) at the time of sacrifice. **D-F.** Merged GFP/RFP channel across the VMHvl of Tmx-treated *Esr1 Cre* female mice shows GFP/RFP fluorescence, indicating that there are estrogen-sensitive (RFP+) neurons in that VMHvl that displayed ERE-dependent gene expression (GFP+) at the time of sacrifice. Scale bar: 400 µm.

GFP fluorescence indicates ERE-dependent gene expression in neurons, and RFP fluorescence indicates the presence of the Cre in neurons that express the *Esr1* gene, which codes for ERα. Therefore, RFP-positive (RFP+) cells in the VMH fluoresce orange if ERα is expressed in that neuron. Due to the nature of the fluorescent reporter, it is likely that some cells may fluoresce green and are, therefore, GFP-positive (GFP+) without expressing ERα. This could be due to signaling and ERE-dependent gene transcription and expression via estrogen-receptor β (ERβ). To ensure that we were quantifying the differences in the population of GFP+ neurons that were also RFP+ and just RFP+, we merged the GFP and RFP channels to identify the cells in the VMHvl that fluoresce yellow, which indicated that the cell was both RFP+ and GFP+ (Figure 2.1). In Figure 2.1A-C, we observed neuronal populations in the VMHvl of Oil-treated mice that were either only RFP+ or GFP+, and we observed a fair amount of GFP+/RFP+ cells that were likely responding to circulating E2 from the ovary of the intact *Esr1* Cre female mice that received the vehicle treatment. Figure 2.1D-F demonstrates the same fluorescence metrics as Figure 2.1A-C; however, this figure shows GFP and RFP fluorescence in the VMHvl of a Tmx-treated mouse. In the Tmx-treated mouse, we observed neuronal populations in the VMHvl that were RFP+ and GFP+, as we observed a fair amount of GFP+/RFP+ cells that were likely responding to circulating Tmx potentially acting as an E2 agonist by enhancing ERE-dependent gene expression at the onset of binding to ERα. Figure 2.2 compares GFP & RFP, GFP only, and RFP only fluorescence in the Oil and Tmx-treated *Esr1 Cre* female mice to better visualize the cells that are specifically responding to either circulating E2 (Oil) or Tamoxifen (Tmx) through changes in ERE-dependent gene expression. Figure 2.2 C, F shows that there appears to be little to no difference in the extent of ERE-dependent gene expression (GFP+ cells) in the VMHvl

between Tmx-treated and control (Oil) mice, as both groups contain ERα+ neurons that are displaying ERE-dependent gene expression.

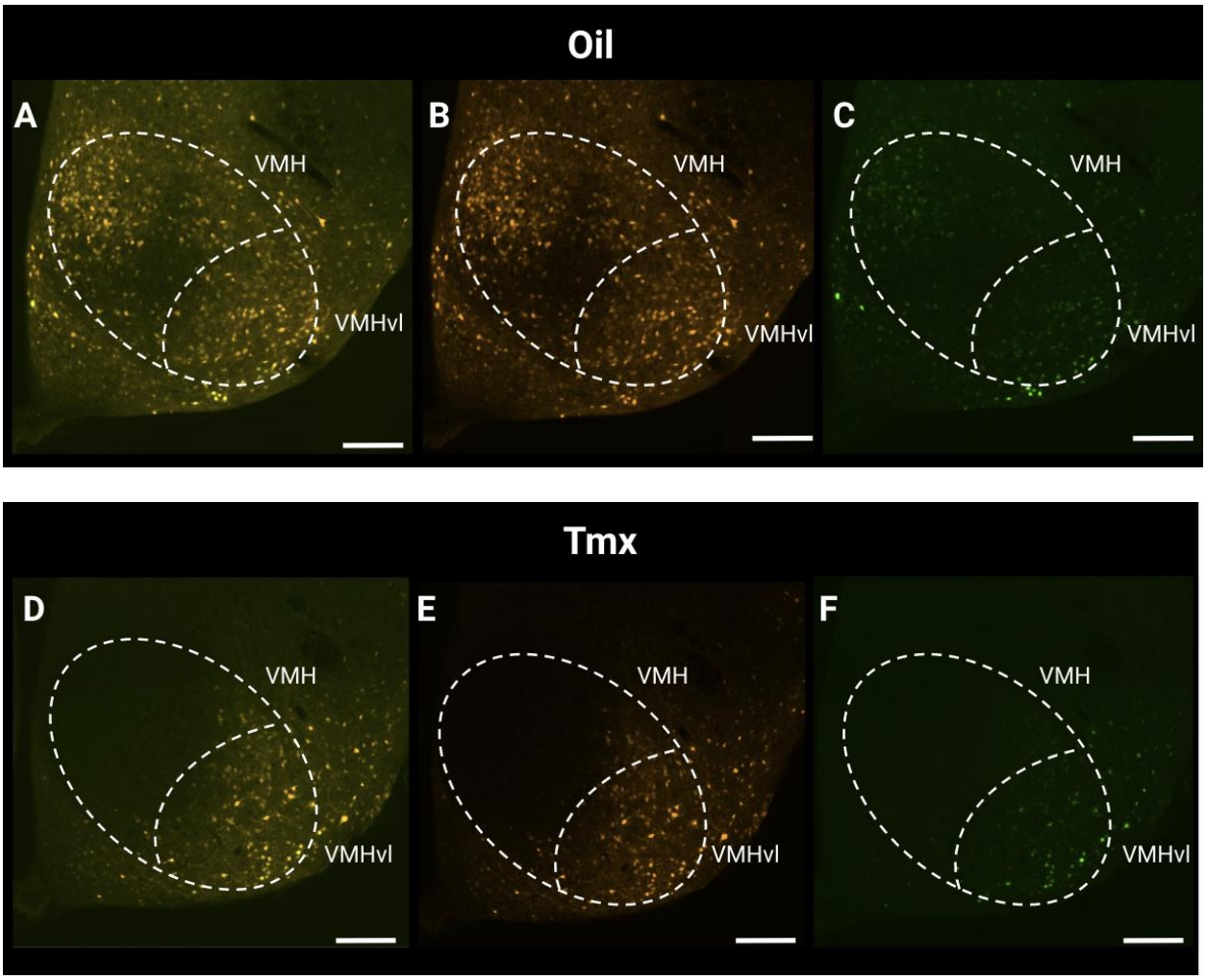

Figure 2.2: Microscopy analysis of ERα presence and ERE-dependent gene expression in the VMHvl of Cohort 1 *Esr1 Cre* female mice given Oil and Tmx-treatment. A. Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Oil-treated mouse at Bregma -1.58 mm. Cells in the VMHvl that fluoresce yellow both express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). VMH, ventromedial hypothalamus. VMHvl, ventrolateral region of the ventromedial hypothalamus. **B.** RFP-positive (RFP+) cells in the VMHvl express ERα. **C.** GFP-positive (GFP+) cells in the VMHvl displayed ERE-dependent gene expression at the time of sacrifice. **D.** Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Tmx-treated mouse at Bregma -1.58 mm. Cells in the VMHvl that fluoresce yellow both express ERα (RFP-positive) and exhibit ERE-dependent gene expression (GFP-positive). **E.** RFP-positive (RFP+) cells in the VMHvl express

ERα. **F.** GFP-positive (GFP+) cells in the VMHvl displayed ERE-dependent gene expression at the time of sacrifice. Scale bar: 400 μm.

We then compiled all of the images of the VMHvl from all Tmx-treated and Oil-treated mice from the Cohort 1 *Esr1 Cre* female mice into a custom CellProfiler pipeline (n=3-4). We used a non-parametric Wilcoxon rank-sum test to quantify the mean fluorescence of GFP in cells that were also RFP+ across both groups of mice in the VMHvl. We carefully discarded the cells that were GFP+ but not RFP+, as those neurons did not express ERα. We kept the quantification data of the cells that were RFP+ but not GFP+, as those cells were not displaying ERE-dependent gene expression. This distinction in the analysis of the quantification data is important such that we wanted to capture and observe differences in ERE-dependent gene expression in neurons that were RFP+, and therefore estrogen-sensitive, as we wanted to observe any potential changes or differences caused by Tmx-ERα signaling, specifically. Figure 2.3 illustrates that upon comparing the mean fluorescence ratio of GFP/RFP cells per mouse, there was no statistically significant difference in the number of GFP+ cells that were also RFP+ in the VMHvl of both groups of mice; therefore, we were unable to conclude whether ERE-dependent gene expression was more or less prevalent in VMHvl neurons in Tmx-treated mice based on the fluorescent reporter delivery injection coordinates and experimental parameters in Cohort 1.

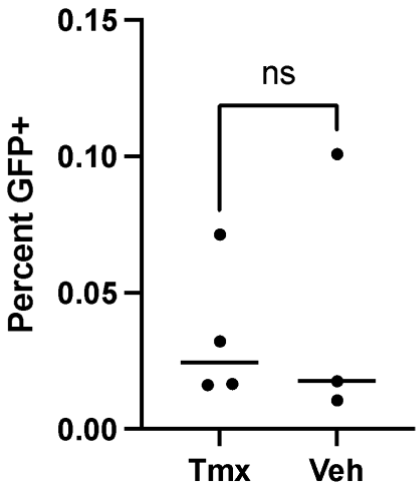

Figure 2.3: Quantifying GFP+ cells in the VMHvl that concurrently expressed RFP between Oil and Tmx-treated *Esr1 Cre* female mice (Cohort 1). This quantification compares the fraction of GFP/RFP fluorescence ratio per mouse between the Tmx and Vehicle (Oil) treated *Esr1 Cre* female mice. There is no statistical significance in GFP+ cells in the VMHvl between Tmx and Oil-treated mice. Tmx, Tamoxifen. Veh, Vehicle. ns = not significant, $P > 0.05$.

Microscopy analysis from the second cohort of *Esr1 Cre* female mice (Cohort 2) that had the fluorescent reporter delivered to the caudal arcuate nucleus showed that, likewise, there seemed to be no significant differences in ERE-dependent gene expression (GFP+ cells) of estrogen-sensitive (RFP+ cells) in the VMHvl between Tmx (n=3) and Oil-treated (n=4) mice. Figure 2.4 shows the merged GFP/RFP, GFP only, and RFP only channels across the VMHvl of both groups. Figure 2.4 A-C shows that the VMHvl of Oil-treated mice shows an appropriate expression of ERα+ neurons (RFP+), as well as activation of ERE-dependent gene expression of those estrogen-sensitive neurons (GFP+). Figure 2.4 D-F shows similar findings, such that the VMHvl of Tmx-treated mice shows an appropriate expression of ERα+ neurons (RFP+), as well as activation of ERE-dependent gene expression in the estrogen-sensitive neurons (GFP+).

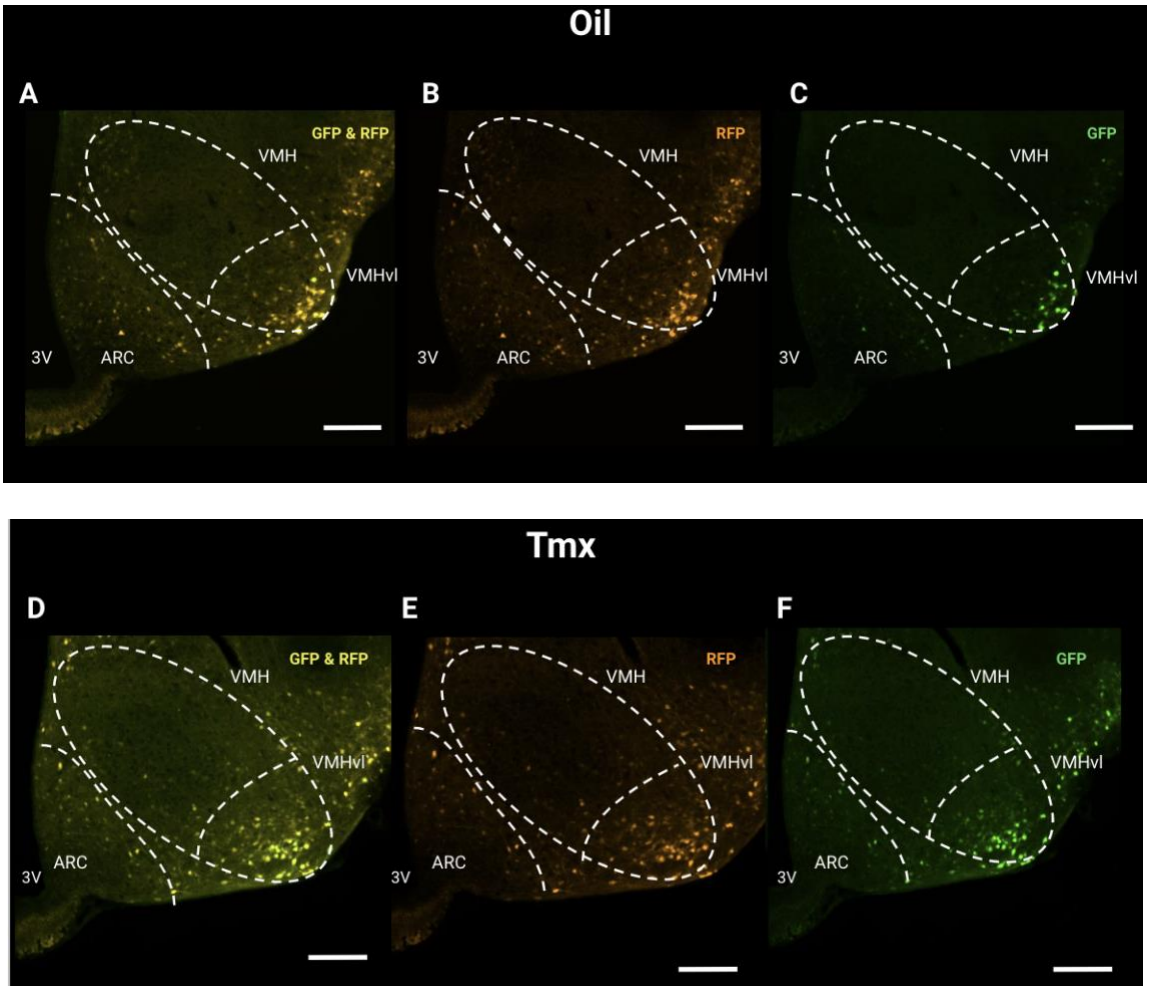

Figure 2.4: Microscopy analysis of ERα presence and ERE-dependent gene expression in the VMHvl of Cohort 2 *Esr1 Cre* female mice given Oil and Tmx-treatment. A. Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Oil-treated mouse at Bregma -1.58 mm. Cells in the VMHvl that fluoresce yellow both express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). 3V, third ventricle. ARC, arcuate nucleus. VMH, ventromedial hypothalamus. VMHvl, ventrolateral region of the ventromedial hypothalamus. **B.** RFP-positive (RFP+) cells in the VMHvl express ERα. **C.** GFP-positive (GFP+) cells in the VMHvl displayed ERE-dependent gene expression at the time of sacrifice. **D.** Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Tmx-treated mouse at Bregma -1.58 mm. Cells in the VMHvl that fluoresce yellow both express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). **E.** RFP-positive (RFP+) cells in the VMHvl express ERα. **F.** GFP-positive (GFP+) cells in the VMHvl displayed ERE-dependent gene expression at the time of sacrifice. Scale bar: 400 µm.

To test for significant differences in GFP fluorescence in the VMHvl between the two groups in Cohort 2, we quantified the number of GFP/RFP positive cells and RFP+ only cells

across the VMHvl of both the Oil and Tmx-treated mice and compared the GFP/RFP fluorescence ratio per mouse. The results of our quantification and the non-parametric Wilcoxon rank-sum test determined that Tmx treatment in our experimental design does not induce statistically significant differences in ERE-dependent gene expression in ERα+ neurons in the VMHvl of *Esr1 Cre* female mice compared to controls (P = 0.8571, Figure 2.5).

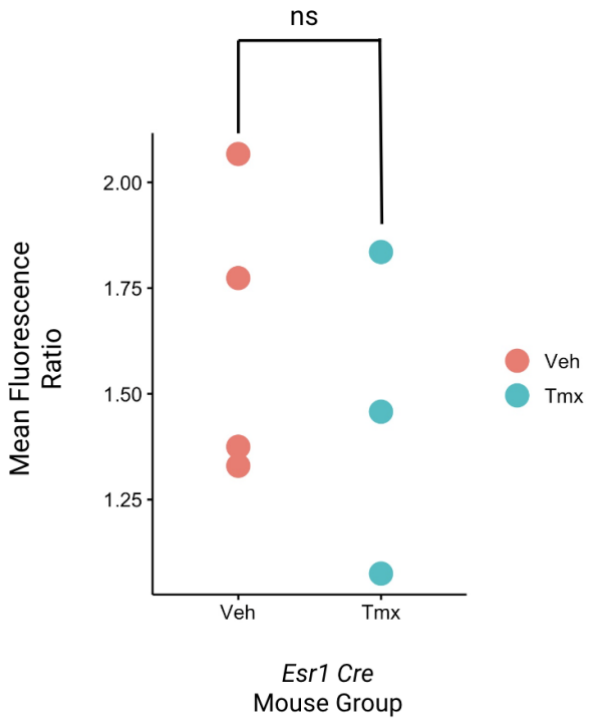

Figure 2.5: Quantifying GFP+ cells in the VMHvl of Cohort 2 *Esr1 Cre* female mice that concurrently expressed RFP between Oil and Tmx-treated mice. This quantification compares the mean GFP/RFP fluorescence ratio per brain region of interest within each mouse, between Tmx and Vehicle (Oil)-treated *Esr1 Cre* female mice. Here, the fluorescent reporter was delivered to the caudal arcuate nucleus, which is medial to the VMHvl. This ratio determines the significance of the fraction of GFP+ cells per mouse that were also RFP+ between the second cohort of Tmx and Vehicle (Oil) treated mice when the fluorescent reporter was stereotaxically injected in the caudal arcuate nucleus. There is no statistically significant difference in GFP+ cells in the VMHvl between Tmx and Oil-treated mice. Tmx, Tamoxifen. Veh, Vehicle. ns = not significant, P = 0.8571 (Non-parametric test).

CHAPTER 2: Tmx does not induce statistically significant differences in ERE-dependent gene expression in thermoregulatory neurons in the hypothalamic arcuate nucleus (ARC) compared to controls.

Introduction

Kisspeptin-expressing neurons in the hypothalamic arcuate nucleus (ARC) have been shown to regulate core body temperature in mice. The toxin-mediated silencing of *Kiss1* neurons led to attenuated locomotor activity and temperature rhythms [35].

A specific population of *Kiss1* neurons that co-express kisspeptin, NKB, and ERα (known as KNDy neurons) have been implicated in temperature dysregulation (specifically hot flashes) in postmenopausal women when there is a decrease in circulating levels of E2. Advancements by Naomi Rance [9] utilized a rat model to show that estrogen-sensitive KNDy neurons in the hypothalamus play a significant role in body temperature regulation and may activate thermoregulatory vasodilation (which triggers the sensation of flushing or throwing off heat of the skin) through projections to the preoptic regions of the hypothalamus that express a receptor for Neurokinin-3 (NK3). This data suggests that a prolonged decrease in circulating levels of estradiol exacerbates a neuronal projection pathway that leads to the over-expression and production of neurokinin B from KNDy neurons in the arcuate nucleus, which leads to downstream thermodysregulation in the rat.

However, little is known about how Tmx as a chemotherapeutic drug given to women or men with breast cancer amplifies or dampens this cellular process studied and reported by Naomi Rance in the arcuate nucleus (ARC). As of now, it is unclear whether the process of Tmx-induced hot flashes is the same or at least similar to what occurs when post-menopausal women

experience hot flashes, which are caused by the depletion of circulating estradiol. Specifically, I want to determine if Tmx could potentially up-regulate ERE-dependent gene expression in these estrogen-sensitive neurons in the arcuate nucleus to cause hot flashes in humans and thermodysregulation in mice.

The goal of this chapter was to observe and quantify the extent of ERE-dependent gene expression in estrogen-sensitive neurons in *Kiss1 Cre* female mice in response to Tmx or Vehicle treatment (n=2). To confirm the validity of our observations in the *Kiss1 Cre* mice, we used data from the Cohort 2 *Esr1 Cre* female mice from Chapter 1 to observe and quantify the number of ERα+ neurons undergoing ERE-dependent gene expression in the arcuate nucleus. Specifically, we analyzed the images of the arcuate nucleus of *Esr1 Cre* female mice (n=3-4) and quantified the mean fluorescence ratio of GFP/RFP-positive cells per mouse across Vehicle (Oil) and Tmx-treated mice. I hypothesize that Tmx may induce hot flashes in breast cancer patients by acting like an estrogen antagonist to ERE-dependent gene expression in the arcuate nucleus such that Tmx mimics conditions that match an estradiol-depleted state. Tmx treatment may bind to ERα in the KNDy neuronal population and eventually increase gene expression of Neurokinin B, which would presumably be released in higher quantities and subsequently bind on preoptic neurons in the hypothalamus.

Materials and Methods

Animals

Kiss1 Cre (n=2) and *Esr1 Cre* (n=3-4) female mice were maintained on a C57BL/6 genetic background and bred in our lab's colony at the University of California, Los Angeles

(UCLA). All mice were maintained under a 12:12 light/dark schedule and given food and water ad libitum. Mice were 2-3 weeks old at the start of all experiments.

Mouse Procedures

All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. UCLA is AALAS accredited, and the UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

Stereotaxic Surgery

The fluorescent reporter was delivered to the brain regions of interest via stereotaxic surgery. For all stereotaxic procedures in the *Kiss1 Cre* cohort (n=2), mice were anesthetized with isoflurane and received bilateral injections of the fluorescent reporter to the arcuate nucleus. 500 nL of the reporter were bilaterally delivered at the following injection coordinate: X = +/- .2, Y -1.56, Z -5.9 from Bregma.

For all stereotaxic procedures in Cohort 2 *Esr1 Cre* (n=3-4) female mice, mice were anesthetized with isoflurane and received bilateral injections of the fluorescent reporter to the caudal ARC. 500 nL of the reporter were delivered at the following coordinates: AP -1.56, ML +/- .2, DV -5.9 (Caudal ARC).

After surgery, mice recovered for 2-3 weeks before a 10-day treatment plan was implemented in which mice either received daily Tmx or vehicle (corn oil and ethanol) injection. Mice were then sacrificed at the conclusion of the 10-day Oil or Tmx injection schedule.

Perfusion, Embedding, Cryosectioning

After the 10-day treatment, mouse brains were perfused, collected, embedded in OCT, and frozen at -80℃ after one overnight post-fixation in 4% PFA and another overnight

incubation in 30% sucrose to cryopreserve the tissue. Coronal mouse brain sections were obtained with a Leica CM1680 cryostat at 20 µm. The ARC was collected on slides for all mice (*Kiss1 Cre* n=2, Cohort 2 *Esr1 Cre* n=3-4). The slides were stained for 15 minutes with a 1:1000 dilution of DAPI Hoechst stock (DAPI 1,000x) in PBS, then washed with PBS and coverslipped with 180 µL of DAPI-free Fluoromount-G. Slides were housed in a 4℃ fridge.

Microscopy

Images were taken with a Nikon Eclipse Ti2 Series Inverted Epifluorescence microscope. All images were taken utilizing the Large Image feature on the accompanying NIS-Elements AR 5.30.04 64-bit software. This software takes images at 20x magnification and stitches together all images across a designated area displayed at 4x magnification. All images were taken using a consistent set of exposures across all channels (UV for DAPI, GFP, and RFP). Images were exported appropriately for quantification. Quantification was performed via a custom CellProfiler pipeline using CellProfiler software for macOS 4.2.1.

Statistics

We were not able to reliably quantify the mean fluorescence intensity of cells in the arcuate nucleus (ARC) of the *Kiss1 Cre* cohort because the experiment was underpowered, the observed fluorescence was dim, and visible fluorescence appeared only in scattered cells.

Statistics for the arcuate nucleus of the Cohort 2 *Esr1 Cre* female mice were completed such that we measured the mean fluorescence across each channel (DAPI, GFP, RFP) over the entire arcuate nucleus on both sides of the mouse brain. We used the same statistical analyses used to analyze the VMHvl of the Cohort 2 *Esr1 Cre* female mice in Chapter here in our analysis of the ARC. To test for significant differences in GFP fluorescence between groups, we used the non-parametric Wilcoxon rank-sum test (n=3-4).

Results

To determine whether Tmx induced a significant increase or decrease in ERE-dependent gene expression in ERα+ neurons of the arcuate nucleus (ARC), we selectively delivered the fluorescent reporter to the arcuate nucleus of *Kiss1 Cre* female mice (n=2). Mice were randomly assigned to receive a 10-day Tamoxifen (Tmx) or Vehicle (Oil) injection regimen.

The arcuate nucleus was imaged for all mice, and the images with the merged GFP and RFP channels were compiled (Figure 3.1) to display the presence of ERα+ neurons (RFP+ signal) and any changes in ERE-dependent gene expression (GFP+ signal) occurring within neurons of the arcuate nucleus. All mice exhibited a mostly unilateral delivery of the reporter, indicating that the bilateral delivery did not permeate both sides of the mouse brain.

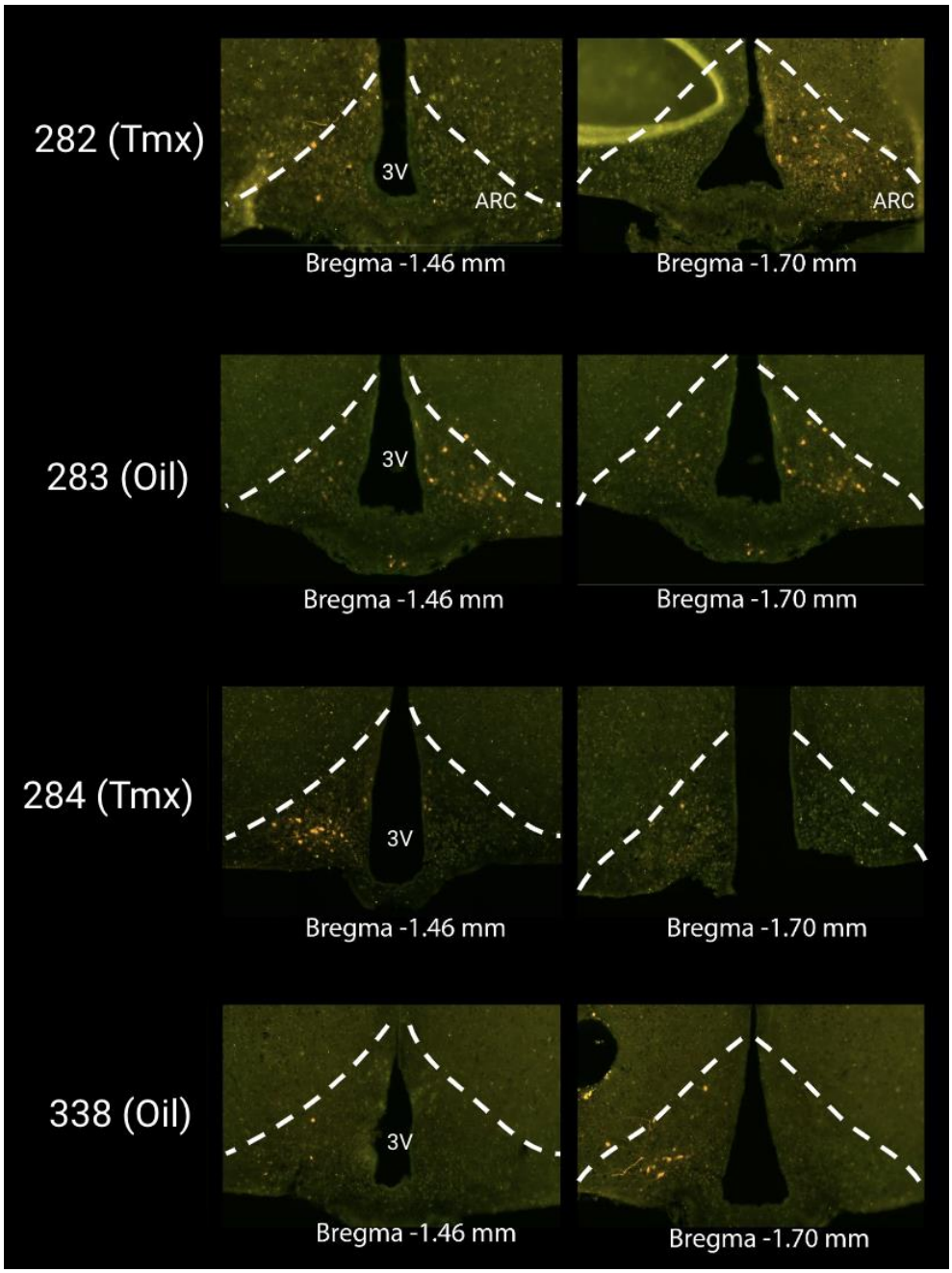

Figure 3.1: Representative montage of merged GFP/RFP channels across the ARC of *Kiss1 Cre* female mice given daily Oil or Tmx-treatment. 20x microscopy fluorescence from a DAPI stain of the virally-treated *Kiss1 Cre* mice shows no significant differences in ERE-dependent gene expression between Tamoxifen (Tmx) and Vehicle (Oil)-treated groups. 3V, third ventricle. ARC, arcuate nucleus. Numbers 282, 283, 284, and 338 indicate mouse number.

Microscopy analysis across the ARC for each Tmx and Oil-treated mouse shows similarities between both experimental groups. Figure 3.2A shows a merged GFP/RFP image of

the ARC of both Oil and Tmx-treated mice. It appears as though there are few GFP/RFP positive cells in the ARC, and there are cells that appear to only be RFP+. Both Tmx and Oil-treated groups expressed similar amounts of RFP fluorescent cells in the arcuate nucleus (Figure 3.2B), which is consistent with what we would expect to see as neurons that express the *Kiss1* gene express ERα [9]. Since we are targeting a specific subpopulation of neurons in the arcuate nucleus (that express Kiss1 and ERα), we do not expect to see widespread RFP fluorescence throughout the arcuate nucleus; and this is observed in Figure 3.2. Interestingly, there was not much visible ERE-dependent gene expression observed in the ERα-expressing neurons in the ARC of both groups, as there were few detectable GFP+ cells in the arcuate nucleus across both Oil and Tmx-treated mice (Figure 3.2C).

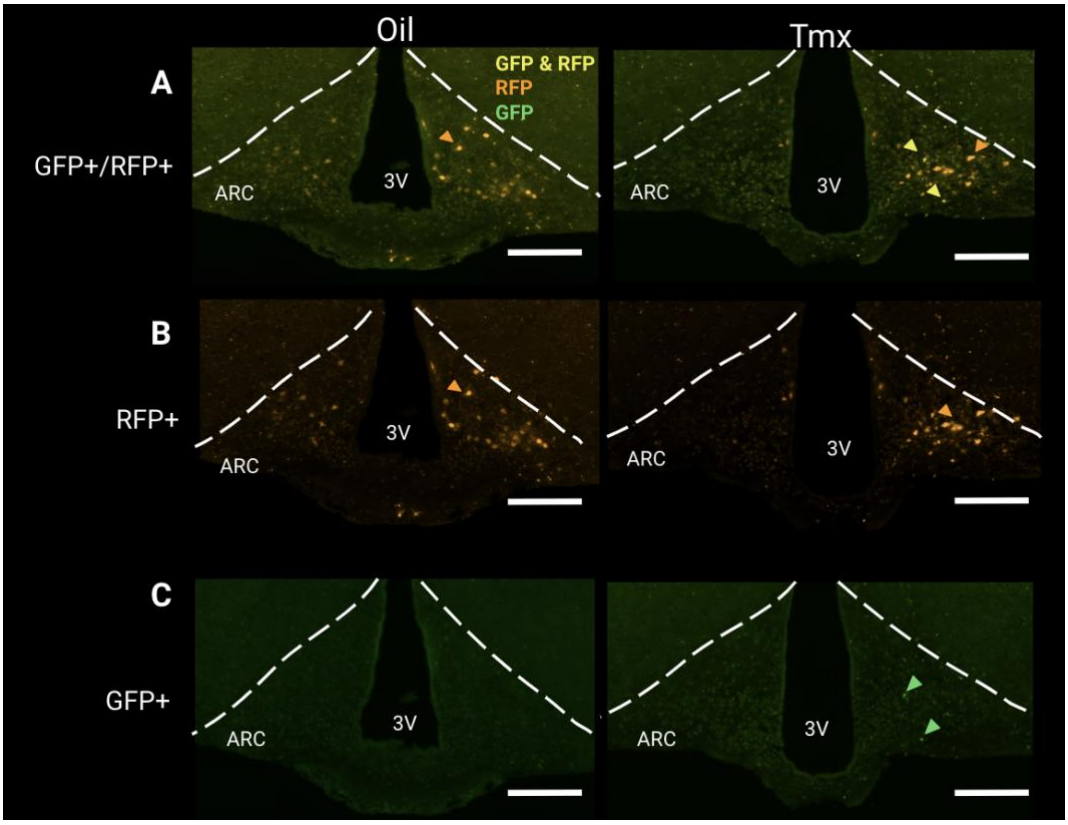

Figure 3.2: Microscopy analysis of ERα presence and ERE-dependent gene expression in the ARC of *Kiss1 Cre* female mice given Oil or Tmx-treatment. A. Merged images of the GFP and RFP channel from a DAPI stain at 20x magnification in the Oil-treated (left) and Tmx-treated (right) mouse at Bregma -1.46 mm. Cells in the ARC that fluoresce yellow express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). ARC, arcuate nucleus. Oil-treated and Tmx-treated mice exhibit GFP/RFP fluorescent cells in the ARC. **B.** RFP-positive (RFP+) cells in the ARC express ERα in both Oil-treated (left) and Tmx-treated (right) mice. **C.** GFP-positive (GFP+) cells in the ARC of Tmx-treated mice (right) displayed ERE-dependent gene expression at the time of sacrifice, whereas the Oil-treated mice (left) do not have GFP+ cells in the ARC. Scale bar: 400 µm.

We could not reliably quantify the median intensity fluorescence of GFP and RFP in the arcuate nucleus of the *Kiss1 Cre* cohort due to a small sample size (n=2) and due to the unreliability of reporter function in the *Kiss1 Cre* mouse model. Therefore, through the microscopy analysis, we can determine that the *Kiss1 Cre* model is challenging for analyzing ERE-dependent gene expression fluorescence in the arcuate nucleus.

We then selectively delivered the fluorescent reporter to the caudal ARC of *Esr1 Cre* female mice (n=3-4). The same analysis was utilized such that the ARC was imaged, montaged (Figure 3.3), and images were quantified using a custom CellProfiler pipeline. Microscope images across the ARC of both groups of mice showed that the reporter mostly appeared unilaterally on the left side of the mouse brain (Figure 3.3).

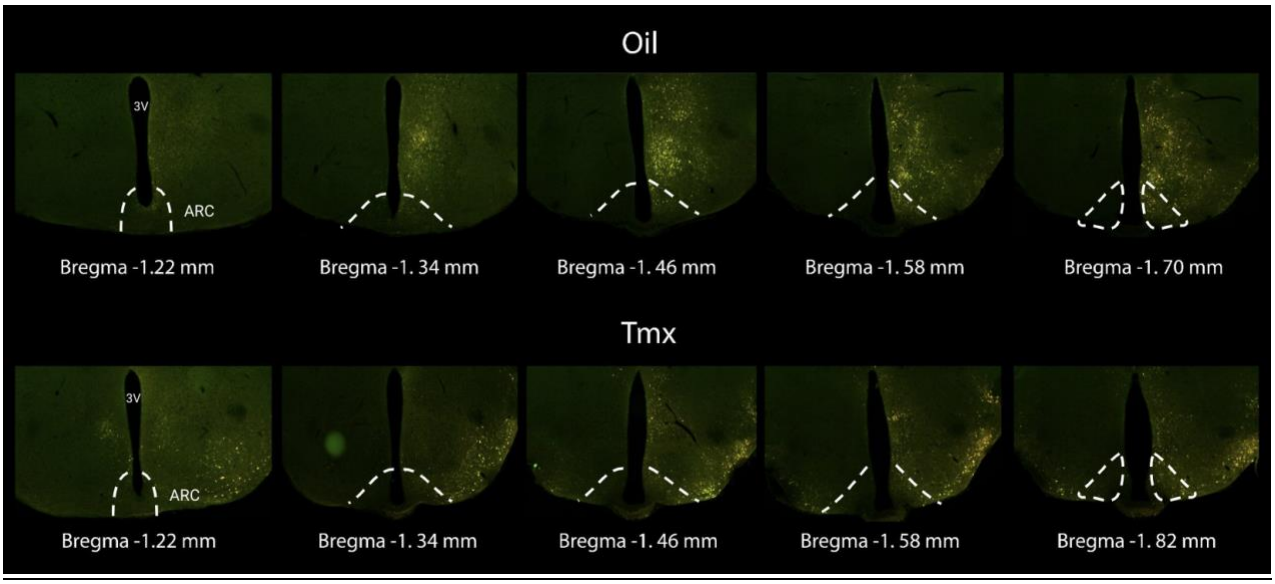

Figure 3.3: Representative montage of merged GFP/RFP channels across the ARC of *Esr1 Cre* female mice given daily Oil or Tmx-treatment. Microscopy images taken at 4x magnification of DAPI-stained slides shows the ARC nucleus across Tmx and Oil-treated mice. The results of stereotaxic surgery indicate a unilateral delivery of the fluorescent reporter to the caudal arcuate nucleus. 3V, third ventricle. ARC, arcuate nucleus.

Upon comparing the fluorescence of cells with the merged GFP and RFP channels (Figure 3.4 A, D), with RFP only (Figure 3.4 B, E), and with GFP only (Figure 3.4 C, F) between the Oil-treated (n=4) and Tmx-treated (n=3) mice, it appeared that the Oil-treated mice underwent more ERE-dependent gene expression in ERα+ neurons compared to the Tmx-treated group. Unilateral sections are shown in Figure 3.4 as the reporter delivery appeared to most efficiently permeate the left side of the mouse brain, which is also evident in Figure 3.3. Mice

from both groups had similar numbers of RFP+ neurons, indicating relatively equal expression of ERα.

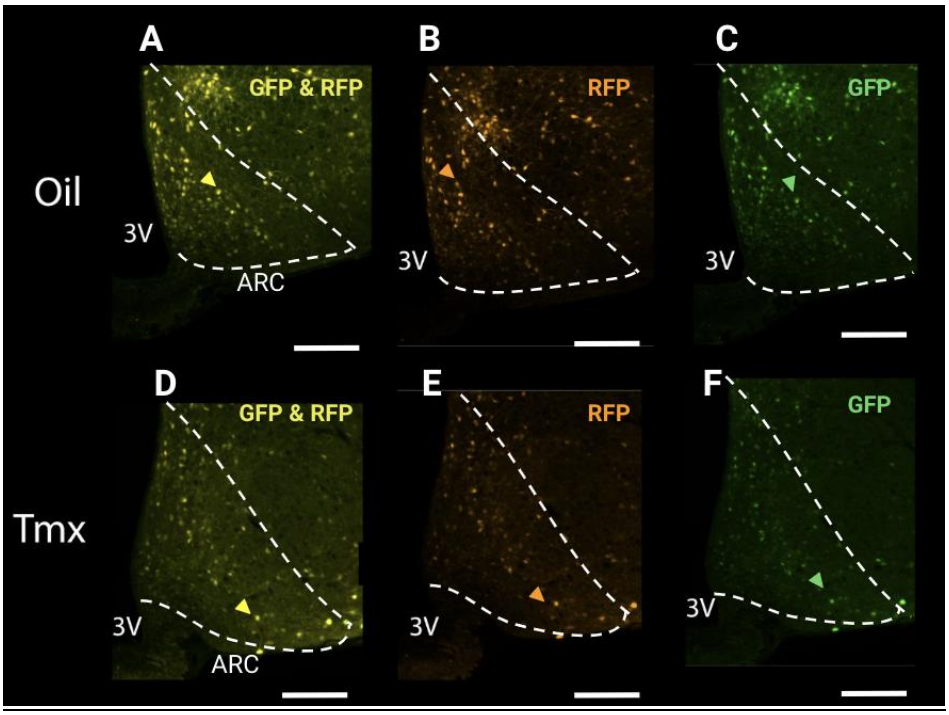

Figure 3.4: Microscopy analysis of ERα presence and ERE-dependent gene expression in the ARC of *Esr1 Cre* female mice given Oil and Tmx-treatment. A. Merged image of the GFP and RFP channel from a DAPI stain at 20x magnification in the Oil-treated mouse at Bregma -1.58 mm. Cells in the ARC that fluoresce yellow express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). ARC, arcuate nucleus. **B.** RFP-positive (RFP+) cells in the ARC express ERα. **C.** GFP-positive (GFP+) cells in the ARC displayed ERE-dependent gene expression at the time of sacrifice. **D.** Merged image of the RFP and GFP channel from a DAPI stain at 20X magnification in the Tmx-treated mouse at Bregma -1.58 mm. Cells in the ARC that fluoresce yellow express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). **E.** RFP-positive (RFP+) cells in the ARC express ERα. There was visible RFP fluorescence in the ARC **F.** Cells in the ARC displayed ERE-dependent gene expression at the time of sacrifice. Scale bar: 400 µm.

Interestingly, these observations contradict what was previously observed in the *Kiss1 Cre* cohort of mice. The results of the *Kiss1 Cre* cohort are underpowered (due to a limit in the number of *Kiss1 Cre* mice used as subjects) since we deemed that the fluorescent reporter does

not optimally perform Cre-Lox recombination in female *Kiss1 Cre* mice. We concluded that it is very likely that utilizing the fluorescent reporter in *Kiss1 Cre* mice is not optimal and cannot represent the extent of ERE-dependent gene expression occurring in estrogen-sensitive neurons of the arcuate nucleus.

The quantification results from images across the ARC of the female *Esr1 Cre* cohort determined that there was no statistically significant difference in ERE-dependent gene expression in the estrogen-sensitive neurons of the arcuate nucleus of Oil and Tmx-treated mice (Figure 3.5). This indicates that Tamoxifen treatment in our experimental design does not induce statistically significant differences in ERE-dependent gene expression in ERα+ cells in the ARC of *Esr1 Cre* female mice compared to controls (P = 1, Figure 3.5).

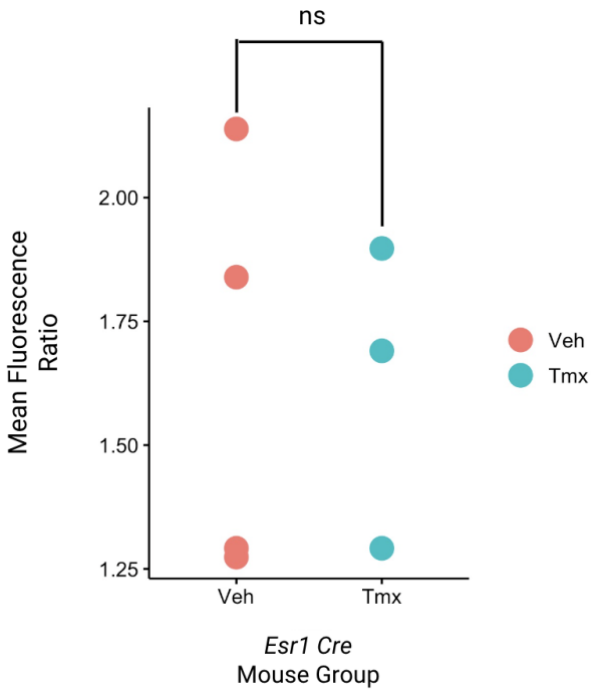

Figure 3.5: Quantifying GFP+ cells in the ARC of *Esr1 Cre* female mice that concurrently expressed RFP between Oil and Tmx-treated mice. This quantification compares the mean GFP/RFP fluorescence ratio per brain region of interest within each mouse, between Tmx and Vehicle (Oil)-treated *Esr1 Cre* female mice. Here, the fluorescent reporter was

stereotaxically injected into the caudal arcuate nucleus. There is no statistical significance in the mean fluorescence ratio in the ARC between Tmx and Oil-treated mice. Tmx, Tamoxifen. Veh, Vehicle. ns = not significant, P = 1 (Non-parametric test).

CHAPTER 3: Tmx does not induce statistically significant differences in ERE-dependent gene expression in thermoregulatory neurons in the medial preoptic area (MPA) compared to controls.

Introduction

The medial preoptic area (MPA) is a brain region containing neurons that express ERα, and E2 signaling through ERα has been shown to potently affect thermoregulation in mice. Recent advancements from our lab determined that activation of estrogen-sensitive (ERα-positive) neurons in the MPA were sufficient in reducing body temperature and metabolism in mice, thus inducing a state known as torpor [38]. Specifically, ERα-positive neurons in the MPA drive a rapid decrease in core body temperature in mice, which is one of the key classifications of a hot flash in humans. However, little is known about Tmx's role on this E2-ERα signaling in the MPA and whether Tmx binding to ERα in MPA neurons is sufficient to trigger ERE-dependent gene expression in these neurons. The critical experiment of Chapter 3 aims to determine if Tmx-treatment induces sufficient differences in ERE-dependent gene expression in thermoregulatory ERα+ neurons in the MPA compared to Oil-treated control mice.

Materials and Methods

Animals

Esr1 Cre female mice were maintained on a C57BL/6 genetic background and bred in our lab's colony at the University of California, Los Angeles (UCLA). All mice were maintained

under a 12:12 light/dark schedule and given food and water ad libitum. Mice were 2-3 weeks old at the start of all experiments.

Mouse Procedures

All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. UCLA is AALAS accredited, and the UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

Stereotaxic Surgery

The fluorescent reporter was delivered to the brain region of interest via stereotaxic surgery. For all stereotaxic procedures, *Esr1 Cre* female mice (n=3-4) were anesthetized with isoflurane and received two sets of bilateral injections of the fluorescent reporter to the rostral MPA and caudal ARC. 500 nL of the reporter were delivered in each injection at the following coordinates: AP -.2, ML +/- .3, DV -5.3 (Rostral MPA).

After surgery, mice recovered for 2-3 weeks before a 10-day treatment plan was implemented in which mice either received daily Tmx (n=3) or Vehicle/Oil (n=4) injection. Mice who received vehicle injection are denoted as 'Oil-treated'. Mice were sacrificed 6 hours after the final Oil or Tmx injection at the conclusion of the 10-day injection schedule.

Perfusion, Embedding, Cryosectioning

After the conclusion of the 10-day injection schedule, mouse brains were perfused, collected, embedded in OCT, and frozen at -80℃ after one overnight post-fixation in 4% PFA, and another overnight incubation in 30% sucrose to cryopreserve the tissue. Coronal mouse brain sections were obtained on a Leica CM1680 cryostat at 20 μm. The MPA was collected on slides for all *Esr1 Cre* female mice (n=3-4). The slides were stained with a 1:1000 dilution of stock

DAPI Hoechst (DAPI 1,000x) and PBS for 15 minutes, then washed with PBS and coverslipped with 180 µL of DAPI-free Fluoromount-G. Slides were housed in a 4℃ fridge.

Microscopy

Images were taken with a Nikon Eclipse Ti2 Series Inverted Epifluorescence microscope. All images were taken utilizing the Large Image feature on the accompanying NIS-Elements AR 5.30.04 64-bit software. This software takes images at 20x magnification and stitches together all images across a designated area displayed at 4x magnification. All images were taken using a consistent set of exposures across all channels (UV for DAPI, GFP, and RFP). Images were exported appropriately for quantification. Quantification was performed via a custom CellProfiler pipeline using CellProfiler software for macOS 4.2.1.

Statistics

All quantification and statistics were performed as in Chapter 1, similar to the first cohort of *Esr1 Cre* female mice, but with images for analysis taken from the MPA of the second cohort of *Esr1 Cre* female mice.

Results

Figure 4.1 shows unilateral microscopy analysis of RFP and GFP fluorescence, which corresponds to ERα presence and ERE-dependent gene expression, respectively, in the MPA neurons of *Esr1 Cre* female mice given Vehicle (Oil) and Tamoxifen (Tmx) treatment. Unilateral sections are shown as the viral delivery appeared to most efficiently permeate the left side of the mouse brain.

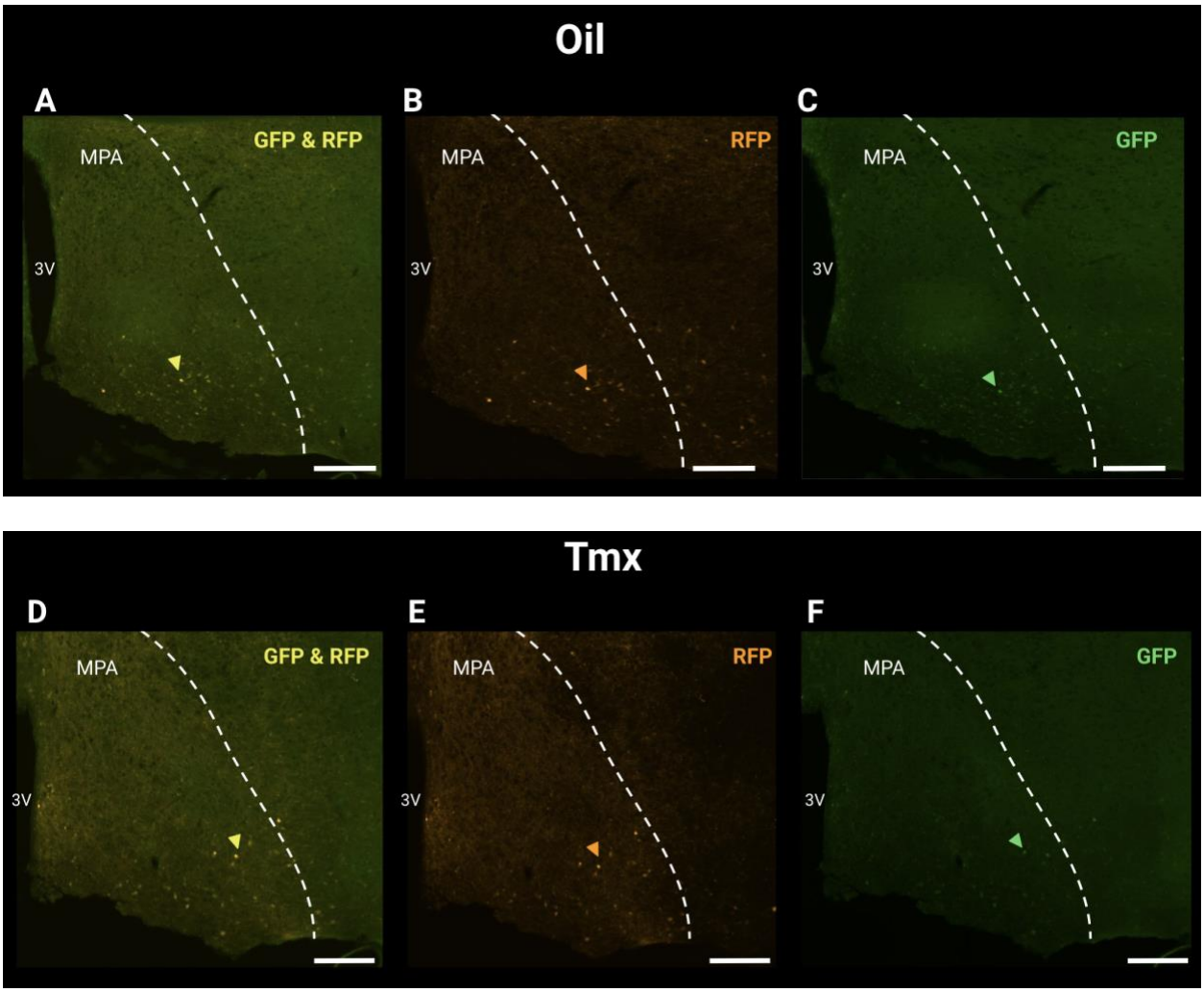

Figure 4.1: Microscopy analysis of ERα presence and ERE-dependent gene expression in the MPA of *Esr1 Cre* female mice given Oil and Tmx-treatment. A. Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Oil-treated mouse at Bregma 0.26 mm. Cells in the MPA that fluoresce yellow express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). MPA, medial preoptic area. **B.** RFP-positive (RFP+) cells in the MPA express ERα. **C.** GFP-positive (GFP+) cells in the MPA did not display significant ERE-dependent gene expression at the time of sacrifice due to a lack of GFP+ cells. **D.** Merged image of the RFP and GFP channel from a DAPI stain at 20x magnification in the Tmx-treated mouse at Bregma 0.26 mm. Cells in the MPA that fluoresce yellow express ERα (RFP-positive) and display ERE-dependent gene expression (GFP-positive). **E.** RFP-positive (RFP+) cells in the MPA express ERα. **F.** Cells in the MPA did not visibly display significant ERE-dependent gene expression at the time of sacrifice due to a lack of GFP+ cells. Scale bar: 400 μm.

Figure 4.1 A, D shows merged GFP/RFP images of the MPA in Oil and Tmx-treated mice. Across both groups, there appear to be few GFP/RFP-positive cells in the MPA. However, in the Oil-treated group, there appear to be slightly more GFP/RFP+ cells than what is observed in the Tmx group. Figure 4.1 B, E shows the RFP fluorescence (and therefore the presence of ERα-expressing neurons) in the MPA. The RFP fluorescence across both groups is present but not as high as expected since the MPA contains ERα+ neurons that regulate body temperature [38]. Figure 4.1 C, F shows little GFP fluorescence in the MPA, indicating little ERE-dependent gene expression in ERα+ neurons in the MPA of both Oil and Tmx-treated mice. In comparing both groups, there appear to be slightly more GFP+ cells in the MPA of the Oil-treated group than in the Tmx-treated group. To be certain of our results, we imported all of the images across the MPA into a custom CellProfiler pipeline to quantify the number of GFP+, RFP+, and GFP+/RFP+ cells in the MPA across both groups (n=3-4) and compare the mean fluorescence ratio of GFP+ and RFP+ cells in the MPA (Figure 4.2). We used a non-parametric Wilcoxon rank-sum test to determine if there were significant differences in GFP fluorescence between both groups.

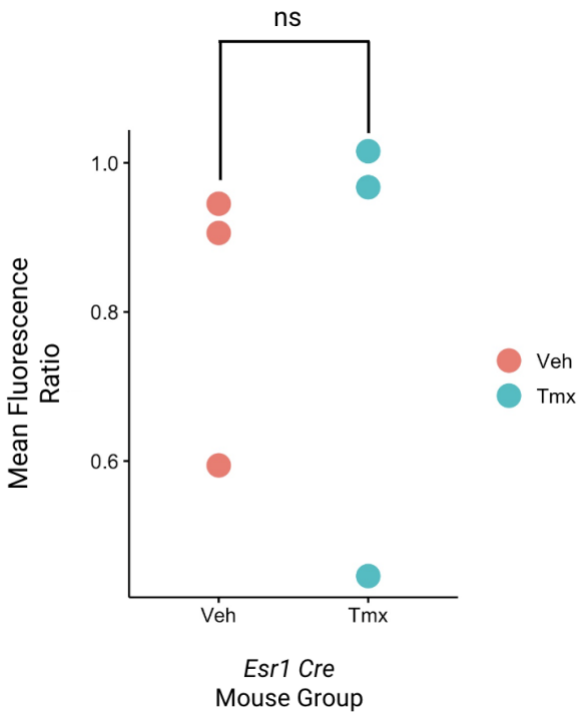

Figure 4.2: Quantifying GFP+ cells in the MPA of *Esr1 Cre* female mice that concurrently expressed RFP between Oil and Tmx-treated mice. This quantification compares the mean GFP/RFP fluorescence ratio per brain region of interest within each mouse, between Tmx and Vehicle (Oil)-treated *Esr1 Cre* female mice. Here, the fluorescent reporter was stereotaxically injected in the medial preoptic area. There is no statistical significance in GFP+ cells in the MPA between Tmx and Oil-treated mice. Tmx, Tamoxifen. Veh, Vehicle. ns = not significant, P = 0.7 (Non-parametric test).

The results of our quantification and non-parametric Wilcoxon rank-sum test on our quantified images determined that Tamoxifen treatment in our experimental design does not induce statistically significant differences in ERE-dependent gene expression in ERα+ cells in the MPA compared to controls (P = 0.7, Figure 4.2).

Discussion

Tamoxifen (Tmx)-induced hot flashes are a prominent inconvenience for those taking the drug to treat estrogen-sensitive breast cancer; therefore, understanding the mechanism by which Tmx induces hot flashes could lead to the development of new therapies that do not induce the negative side effects reported from Tmx therapy. To learn more about how Tmx affects thermodysregulation, recent work from our lab asked, "How does Tmx act on estrogen-sensitive nuclei in the hypothalamus? Is this binding sufficient to induce thermodysregulation similar to a hot flash in a mouse model?". We were able to successfully model thermodysregulation similar to what is observed during a human hot flash in mice, in which we observed a decrease in core body temperature and an increase in tail skin temperature in response to a 28-day course of Tmx treatment [5]. Results from our lab's most recent publication determined that Tmx signals through the estrogen-receptor alpha (ERα) in the hypothalamus. This interaction thus affects thermoregulation, bone density, and movement [5] in mice. We concluded that Tmx likely induces thermodysregulation in mice similar to what occurs during a human hot flash through interactions with estrogen-sensitive nuclei in the hypothalamus, specifically through ERα signaling [5].

Estrogen-sensitive nuclei that regulate core and peripheral temperature in the hypothalamus are likely acted on by Tmx, potentially initiating a hot flash: the three hypothalamic nuclei of interest are the medial-preoptic area (MPA), arcuate nucleus (ARC), and ventrolateral aspect of the ventromedial hypothalamus (VMHvl) as all three regions express ERα and are thermoregulatory [5]. To determine whether Tmx is affecting gene expression in estrogen-sensitive neurons in each nucleus, we employed a fluorescent reporter that dually reports the presence of ERα in neurons and whether there are changes in ERE-dependent gene

expression occurring in estrogen-sensitive neurons. This thesis aimed to delineate any changes in ERE-dependent gene expression in response to Tmx treatment in the estrogen-sensitive neurons of the MPA, ARC, and VMHvl separately.

The main findings of this thesis indicated that the results of our quantification and statistical analyses for each brain region of interest showed that Tamoxifen treatment does not induce statistically significant differences in ERE-dependent gene expression in ERα+ cells in the MPA, ARC, or VMHvl compared to control mice in our experimental design. These results suggest that Tmx may or may not exacerbate the mechanism of hot flashes through gene expression changes in estrogen-sensitive neurons in the hypothalamus. We did not observe significant differences in ERE-dependent changes in gene expression caused by Tamoxifen treatment compared to controls. This was true for both mouse models that we employed (*Esr1 Cre* and *Kiss1 Cr*e female mice). It may be that stereotaxic reporter injection coordinates, fluorescent reporter injection quantity, Tamoxifen/Oil injection duration, and the number of mouse subjects has limited our ability to see more subtle changes in gene expression. These limitations may explain the lack of statistical significance in our results.

Other potential limitations of our study include not collecting the estrus cycle stages of the first cohort of *Esr1 Cre* female mice (n=3-4) in Chapter 1 and the *Kiss1 Cre* female mice (n=2) in Chapter 2. Estrus staging was employed for the second batch of *Esr1 Cre* mice (n=3-4), and Tmx-treated mice appeared to be stuck in metestrus, which is a stage of the mouse estrous cycle characterized by low circulating levels of E2 [39]. Therefore, it may be difficult to compare intact, control-cycling mice across all stages of the estrous cycle to the Tmx mice stuck in metestrus.

Another limitation of this experimental approach is the inherent variability in stereotaxic delivery of the fluorescent reporter to both sides of the mouse brain for all mice. This may have affected the number of estrogen-sensitive neurons that were able to respond to Tmx treatment. Interestingly, upon comparing the expression of ERα in the MPA of our virally-treated *Esr1 Cre* female mice to previous work done in our lab [5], it is likely that we, in fact, did not fully target the MPA. The observed pattern of ERα expression (denoted by RFP fluorescence in Figure 4.1 B, E) in the cohort of *Esr1 Cre* female mice was rather low and ventrally located compared to what we would expect to see. Therefore, the lack of statistical significance in our results from Chapter 3 may be due to not fully targeting the MPA with the fluorescent reporter used in this thesis project.

Another potential limitation of the experimental design could be explained by the 10-day Tamoxifen/Oil injection schedule given to mice after recovering from stereotaxic surgery. A potential explanation for why we may not have seen significant changes in the extent of ERE-dependent gene expression within the MPA, ARC, and VMHvl is that a 10-day injection schedule may have been too short to observe any significant changes in estradiol-regulated gene expression. Notably, however, this injection schedule is sufficient to induce thermodysregulation in mice, indicating that if indeed gene expression is largely unchanged during this time period, gene expression changes likely do not account for all of the thermoregulatory effects of Tamoxifen administration.

Another potential explanation for the lack of statistical significance in the results may be due to the timing in which we sacrifice mice on the last day of the Oil/Tmx injection. Due to how novel the custom fluorescent reporter we used is, we are still learning about the effects of sacrificing mice at 6 hours post-injection and optimizing whether that time frame is an

appropriate representation of Tamoxifen's effects at the ERE. A potential explanation for our results from Chapter 2 could be that we were not allowing the ERα+ cells enough time to respond or exhibit significant changes in ERE-dependent changes in gene expression with a 10-day injection schedule or with the final perfusion occurring 6 hours after the final Oil/Tmx injection. Since the estrogen-sensitive neurons of the arcuate nucleus have been implicated in hot flashes in work by Naomi Rance [9], we would have expected to see significantly lower ERE-dependent gene expression (GFP fluorescence) in the Tmx-treated mice of both *Kiss1* and *Esr1 Cre* mice. However, our results did not show that.

A more intriguing possibility is that Tamoxifen can induce thermodysregulation independent of widespread changes in hypothalamic gene expression. Thermodysregulation could be the result of only a handful of important genes changing in response to Tamoxifen treatment. Alternatively, tamoxifen-induced thermodysregulation may be initiated by transcription independent mechanisms. I hypothesized that hot flashes in humans and equivalent thermodysregulation in mice are caused by changes in gene expression that is mediated by the estrogen-response element in ERα-expressing thermoregulatory neurons in the hypothalamus. Our results indicated that we do not see statistically significant differences in gene expression in the ARC and VMHvl. We may not be able to conclude with certainty that this is also true in the MPA, as we may not have appropriately targeted that region with the fluorescent reporter during stereotaxic surgery. We expected Tmx to act like an antagonist to the signaling mechanisms initiated by estradiol in these thermoregulatory nuclei to induce thermodysregulation, but perhaps there is an entirely different mechanism to induce hot flashes in humans and thermodysregulation in mice. Perhaps the mechanism of Tmx-induced hot flashes is not entirely transcriptional like we hypothesized. A plausible alternative hypothesis is that Tamoxifen

induces thermodysregulation in mice via membrane effects of ERα. Membrane-initiated changes in ERα signaling are known to be critical in dendritic spinogenesis in the arcuate nucleus of female rats to regulate female sexual behavior [40]. Membrane-initiated effects of ERα are also critical in estradiol-induced production of progesterone involved in the positive feedback loop of the hypothalamic-pituitary-gonadal axis, which gives rise to a surge of luteinizing hormone during ovulation [41]. Both physiological mechanisms require membrane ERα and type 1a metabotropic glutamate receptor signaling for the observed phenomena [40, 41]. Based on our negative data regarding tamoxifen action on ERE-dependent transcription, we propose that there could be non-genomic effects of ERα signaling causing thermodysregulation involved in hot flashes, which would mean that this process is not solely transcriptional. This may explain why we did not observe statistically significant differences in gene expression in thermoregulatory ERα+ cells in the MPA, VMHvl, and ARC in response to Tmx treatment.

The goal of this thesis was to determine which estrogen-sensitive thermoregulatory hypothalamic nucleus could be potentially responding to Tmx to activate the cellular mechanisms that lead to a hot flash in humans and thermodysregulation (a decrease in core body temperature and increase in tail skin temperature) in mice. Identifying the potential nucleus that is most readily responding to Tmx and observing the effects on gene expression would allow us to delve into the specific neuronal populations targeted and the subsequent genes expressed within that brain region in response to Tmx binding to ERα. By utilizing this multi-nucleus analysis to study changes in gene expression in the MPA, ARC, and VMHvl, our goal was to determine which of the three chosen thermoregulatory and estrogen-sensitive nuclei in the hypothalamus was responding to Tmx treatment via changes in gene expression mediated by the ERE. However, based on our experimental metrics, we are unable to confirm or report a

significant difference in changes in gene expression between Tmx and Oil-treated mice in estrogen-sensitive neurons of the MPA, ARC, or VMHvl. These negative results are very intriguing as they suggest the possibility of an important non-transcriptional mechanism contributing to Tamoxifen-induced thermodysregulation.

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