ELUDICATING TRIGGERS AND NEUROCHEMICAL CIRCUITS UNDERLYING HOT FLASHES IN AN OVARIECTOMY MODEL OF MENOPAUSE

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Menopausal symptoms, primarily hot flashes, are a pressing clinical problem for both naturally menopausal women and breast and ovarian cancer patients, with a high societal and personal cost. Hot flashes are poorly understood, and animal modeling has been scarce, which has substantially hindered the development of non-hormonal treatments. An emerging factor in the hot flash experience is the role of anxiety and stress-related stimuli, which have repeatedly been shown to influence the bother, frequency, and severity of hot flashes. Causal relationships are difficult to determine in a clinical setting, and the use of animal models offers the ability to elucidate causality and mechanisms. The first part of this work details the development and validation of novel animal models of hot flashes using clinically relevant triggers (i.e., compounds or stimuli that cause hot flashes in clinical settings), which also increase anxiety symptoms. These studies revealed that these triggers elicited strong (7-9 °C) and rapid hot flash-associated increases in tail skin temperature in rats. In a surgical ovariectomy rat model of menopause, which typically exhibit anxiety-like behavior, hot flash provocation revealed an ovariectomy-dependent vulnerability, which was attenuated by estrogen replacement in tested models. An examination of the neural circuitry in response to the most robust flushing compound revealed increased cellular activity in key thermoregulatory and emotionally relevant areas. The orexin neuropeptide system was hyperactive and presented as a novel target; pretreatment with selective and dual orexin receptor antagonists significantly diminished or eliminated, respectively, the response to a hot flash provocation in ovariectomized rats. The insertion/deletion polymorphism of the serotonin transporter has been linked to increased anxiety-associated traits in humans, and subsequent studies prolonged hot flashes in $SERT^{+/-}$ rats, which also caused hot flashes in highly symptomatic women. These studies indicate the orexin system may be a novel non-hormonal

treatment target, and future studies will determine the therapeutic importance of orexin receptor antagonists for menopausal symptoms.

Anantha Shekhar, M.D., Ph.D., Chair

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Abbreviations

3V	third ventricle
5-HT	5-hydroxytryptophan/serotonin
ACh	acetylcholine
AChE	acetylcholinesterase
AET	adjuvant endocrine therapies
AI	aromatase inhibitor
BNST	bed nucleus of the stria terminalis
BLA	basolateral amygdala
CBT	core body temperature
CeA	central amygdala
CO_2	carbon dioxide
DMN	dorsomedial hypothalamic nucleus
DORA	dual orexin receptor antagonist
DRN	dorsal raphe nucleus
DRVL-VLPAG	ventrolateral dorsal raphe nucleus, ventrolateral periaqueductal gray
DSI	Data Sciences International
DSM-V	*Diagnostic and Statistical Manual of Mental Disorders-V*
E	epinephrine
E_2	17-β estradiol
EIT	estrogen inhibition therapy
EPM	elevated plus maze
ETM	elevated T maze
ER	estrogen receptor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERT	estrogen replacement therapy
FMP	final menstrual period

FSH	follicle stimulating hormone
g	gram
GABA	γ-amino-butyric acid
GnRH	gonadotropin releasing hormone
HERS	Heart and Estrogen/Progestin Replacement Study
HRV	heart rate variability
IHC	immunohistochemistry
i.c.v.	intracerebroventricular
i.p.	intraperitoneal administration
kg	kilogram
LA	lateral amygdala
LC	locus ceruleus
L/D Box	light-dark box test
LH	luteinizing hormone
m-CPP	meta-chlorophenylpiperazine
MeA	medial amygdala
mg	milligram
MHPG	3-methoxy-4-hydroxyphenylglycol
mPOA	medial preoptic area
mRNA	messenger ribonucleic acid
NAMS	North American Menopause Society
NE	norepinephrine
NET	norepinephrine transporter
NRI	norepinephrine reuptake inhibitor
NRS	numeric rating scale
OFT	open field test
OX	orexin
OX-A	orexin-a
OX-B	orexin-b
OVEX	ovariectomized
p.o.	oral administration

PBN	parabrachial nucleus
PeF	perifornical hypothalamus
PVN	paraventricular hypothalamic nucleus
RPa	raphe pallidus
RVLM	rostroventrolateral medulla
s.c.	subcutaneous administration
SERM	selective estrogen receptor modulator
SERT	serotonin transporter
SHAM	sham-operated rat/condition
SNP	single nucleotide polymorphism
SNRI	selective norepinephrine reuptake inhibitor
SORA	single orexin receptor antagonist
SORA-1	single orexin receptor-1 antagonist
SORA-2	single orexin receptor-2 antagonist
SSRI	selective serotonin reuptake inhibtor
STAI	State-Trait Anxiety Inventory
SWAN	Study of Women's Health Across the Nation
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
TST	tail skin temperature
VMA	vanillylmandelic acid
VMS	vasomotor symptoms
WHI	Women's Health Initiative

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Part I: Overview of Menopause, Hot Flashes, Estrogen, and Therapeutics

1.1. Menopause and Hot Flashes

1.1.1. Naturally occurring menopause

Menopause occurs as a consequence of the natural course of aging in women, wherein the ovarian follicular cells degenerate and cease to produce sex steroid hormones, especially estradiol. With the extensive data garnered through the advent of large-scale studies that have followed thousands of women prospectively, menopause can be broken down into many stages based on menstrual bleeding patterns that were recently updated (as summarized by the Society of Reproductive Aging Workshop +10 [ten years after the initial meeting; STRAW+10)(Harlow et al., 2012)]. Generally, the perimenopause stage refers to the time period wherein menstrual cycles of a previously predictable length (the number of days between cycles) become subtly aberrant, and during this time a woman approaches the 'menopausal transition'. In the early perimenopausal stage, cycle lengths must be ≥7 days different for 10 consecutive months; the late perimenopause stage is marked by a period of amenorrhea that lasts for 60 or more days. The final menstrual period (FMP) is defined as the '0' point on the continuum, and at that point a woman enters the postmenopause, which is subdivided into early postmenopause and late postmenopause stages. Interestingly, the endocrine correlates are not the defining criterion; but rather supportive, as the bleeding pattern data has been verified across many cultures where reliable hormonal measurements were not always available. Throughout the reproductive years, follicle stimulating hormone (FSH), inhibin-B, and anti-Mullerian hormone (AMH) are low, and FSH begins to rise in the late reproductive stage until it surpasses 25 IU/L in the late menopausal transition (before stabilizing in the postmenopause years). Inhibin-B and AMH drop to very low levels in the late postmenopause stage.

The loss of ovarian function during menopause leads to symptoms associated with disrupted autonomic tone (e.g., hot flashes and racing heart),

sleep disruption, weight gain, and alterations in mood. Of these symptoms, hot flashes are the most problematic, and begin in the late perimenopause and early postmenopause stages. Thus menopause is perhaps every bit as emotionally and physically wrenching as puberty, which is the onset of reproductive viability. In the United States and other western cultures, the typical onset of menopause occurs at a median age of 51.3 (Dratva et al., 2009). This is a time of life already burgeoning with change and transitions such as children maturing and leaving the household and/or caring for elderly parents, and as discussed in section 2.3.2., this may contribute to anxiety and stress which are now becoming recognized as prominent risk factors for more severe and problematic hot flashes. Additionally, early onset of naturally occurring menopause is associated with more severe menopausal symptoms (Kuh et al., 1997).

1.1.2. Surgical and pharmacological menopause

For some women, menopause comes at a relatively young age and abruptly following surgical or pharmacological disruption of sex hormone activity. Surgically induced menopause occurs following ovarian removal (oophorectomy), which can be done to treat benign conditions, like cysts, or to treat or prevent ovarian cancer. In particularly high-risk women, like those with a family history of breast cancer and certain genetic mutations (e.g., *BRCA1, BRCA2*), oophorectomy is also done to treat or prevent estrogen receptor positive breast cancer. Pharmacologically induced menopause occurs following estrogen inhibition therapies (EIT) to treat estrogen receptor positive breast cancer. Selective estrogen receptor modulators (SERM; e.g., tamoxifen) are estrogen antagonists in breast tissue, and aromatase inhibitors (AI) prevent the conversion of androgens into estrogens. These compounds are commonly prescribed for cancer treatment, but also for prevention in high-risk women, similar to prophylactic oophorectomy. Additional health complications arising from surgical menopause (above and beyond natural menopause) are listed in **Table 1**, and include a diverse array of symptoms, such as increased risk of Parkinsonism, increased mood disruption, and many cardiovascular and metabolic problems.

Menopausal Symptoms Exacerbated After Oophorectomy, Relative to Natural Menopause

Specific Symptom	*References(s)*
Vasomotor	
Hot flashes, sweating	Berg et al., 1988 Gallichio et al., 2006 Ozdemir et al., 2009 Benshushan et al., 2009
Psychiatric	
Anxiety & Depression	Dokovic et al., 2015 Rocca et al., 2009
Decreased Memory	Ozdemir et al., 2009
Increased short-term cognitive impairment	Henderson & Sherwin, 2007
Decreased sexual desire	Ozdemir et al., 2009
Neurological	
Risk of Parkinsonism	Rocca et al., 2008 Ragonese et al., 2004
Long-term risk of dementia	Rocca et al., 2012
Neurological disorder	Soni et al., 2014
Increased migraine	Allais et al., 2015
Peripheral Organ Systems	
Increased body mass index/obesity	Gibson et al., 2013 Matthews et al., 2001
Increased osteoporosis	Ozdemir et al., 2009
Increased cardiovascular disease	Lobo et al., 2007
Increased LDL and total cholesterol	Pansini et al., 1993
Increased fasting glycemia	Lejskova et al., 2014
Metabolic Syndrome	Farahmand et al., 2015

Table 1. *Menopausal Symptoms Exacerbated by Oophorectomy*. Menopause induced through surgery (oophorectomy) leads to increased adverse health outcomes, of both short and long-term duration, relative to naturally-occurring menopause. The nature of the conditions is varied, and includes classical menopausal symptoms like hot flashes, but also neurological disease and disease affecting peripheral organ systems.

1.1.3. Menopause Symptoms

1.1.3a. Brief recapitulation of menopause symptoms

While there is no one defining universal menopausal experience, the constellation of symptoms is fairly consistent across cultures and includes a variety of physical and psychological/psychiatric perturbations. The cardinal symptom, hot flashes and night sweats, are discussed in greater detail in the following paragraph. Briefly, the symptoms that typically receive less attention include affective changes and mood disruption, such as anxiety and depression. This can include an increased number of anxiety and depressive symptoms (but not enough to warrant a clinical diagnosis) as well as meeting sufficient criteria for a diagnosis. Weight gain, sleep disturbances, fatigue, osteoporosis, and genitourinary syndrome (previously known as vulvovaginal atrophy) and sexual dysfunction are common complaints at the menopause. Cognitive loss, independent of aging, is also frequently reported.

1.1.3b. Hot flash sensation

In addition to irregular menstruation, the hallmark of the menopausal transition is the hot flash (more commonly hot flush in the U.K.), which consists of an intense sensation of heat sweeping across the body and is often, but not always, accompanied by sweating (see **Fig. 1**). It is often relatively restrained to the face and neck region, but can cover the entire body [though rarely the dorsal (backside) surface (Voda, 1981)]. **Figure 1** represents a body diagram where a woman has drawn where the hot flash occurs. Cultural or ethnic differences in physical localization of the experience of the flush have been described, such that Mexican women identify hot flashes on the neck and arms/hands whereas Caucasian women (historically, the major population of most menopausal studies) report sensations mostly on the face and chest (Sievert et al., 2002).

1.1.3c. Hot flashes are associated with cutaneous vasomotor responses

Physiologically, hot flashes consist of objectively measurable signs: increased

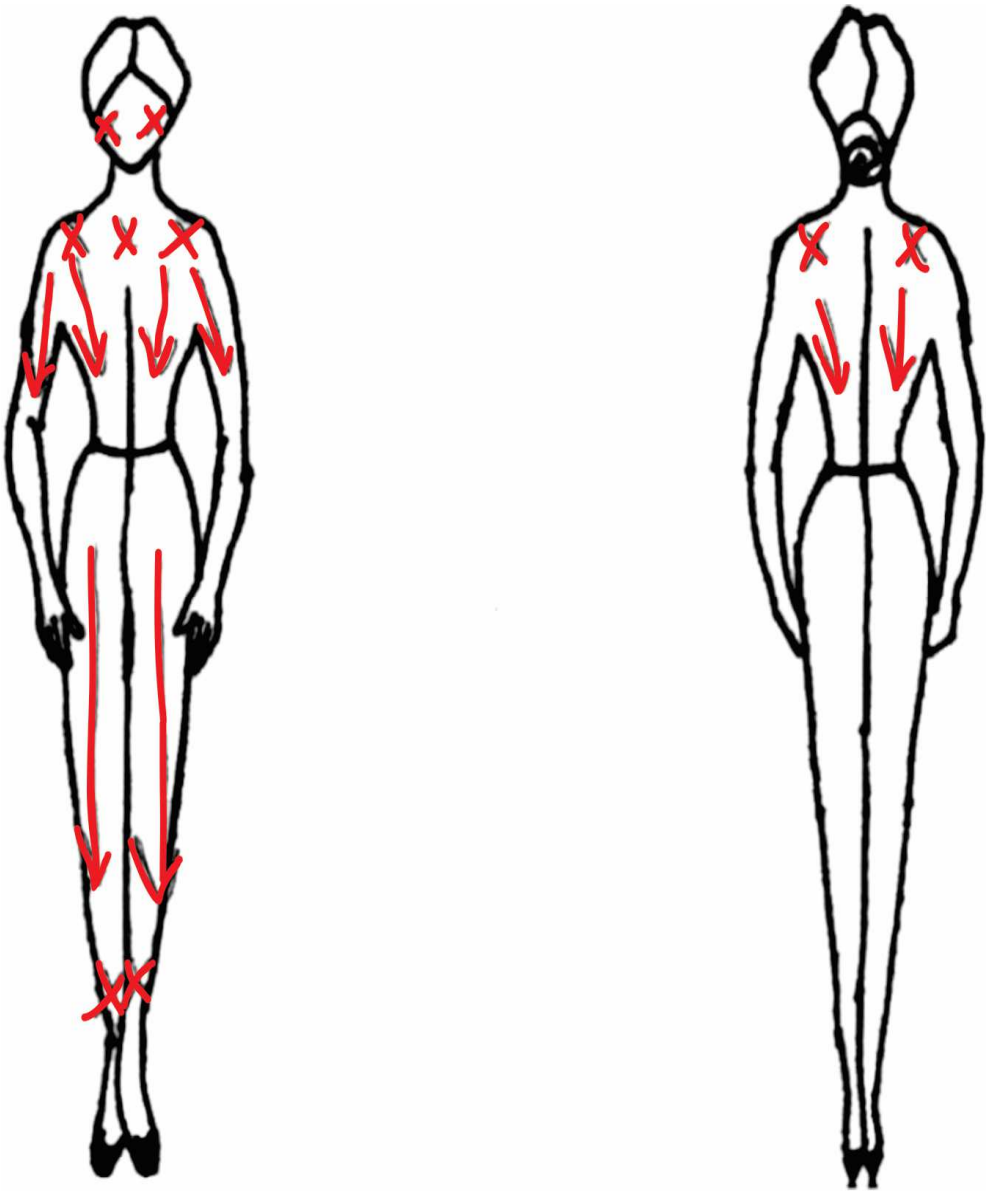


Figure 1. *Body Diagram of a Hot Flash*. 'X' indicates the origins of the hot flash, and arrows demonstrate the spread. Note the differences between the frontal/ventral and back/dorsal perspectives. Modified from (Sievert et al., 2007).

skin temperature, increased cutaneous blood flow, mild tachycardia, decreased core body temperature, and decreased skin resistance or increased skin conductance/sweating (Kronenberg, 1990; Low et al., 2008). In addition to all of the objectively measurable events, what usually defines a hot flash is the subjective labeling of these signs as a hot flash. Often, this is indicated by a button press (in laboratory studies) or in an electronic diary (for long-term observations). Despite this subjectivity, the physiology tends to correlate with subjective measures quite well, as illustrated in **Fig. 2.** Lastly, psychological symptoms at the time of the flush include feelings of anxiety and/or embarrassment, which may depend on the individual and social context in which the flash occurs, such as in the workplace versus at home. Anecdotally, some women describe severe anxiety during hot flashes that are consistent with an "escape" response, such as feeling the need to leave the store during grocery shopping.

1.2. Burden of Hot Flashes

1.2.1. Incidence and duration of symptoms

The incidence of hot flashes is striking; in both the U.S. and U.K., up to 90% of women report them (Williams et al., 2008; Hunter et al., 2012). While the long-held assumption was that hot flashes last only a short time around the menopausal transition, recent systematic studies demonstrated that hot flashes occur for much longer than a year or two. An earlier meta-analysis demonstrated that the *median* duration of hot flashes is four years (Politi et al., 2008); in 2011, Freeman reported a median duration of 10.2 years for *moderate-to-severe* hot flashes for women in the Penn Ovarian Aging Study (Freeman et al., 2011). The Study of Women's Health Across the Nation (SWAN) corroborated both studies, finding a median duration of 7.4 years for hot flashes overall, but persistence of up to 14 years (Avis et al., 2015). While hot flashes eventually subside for the

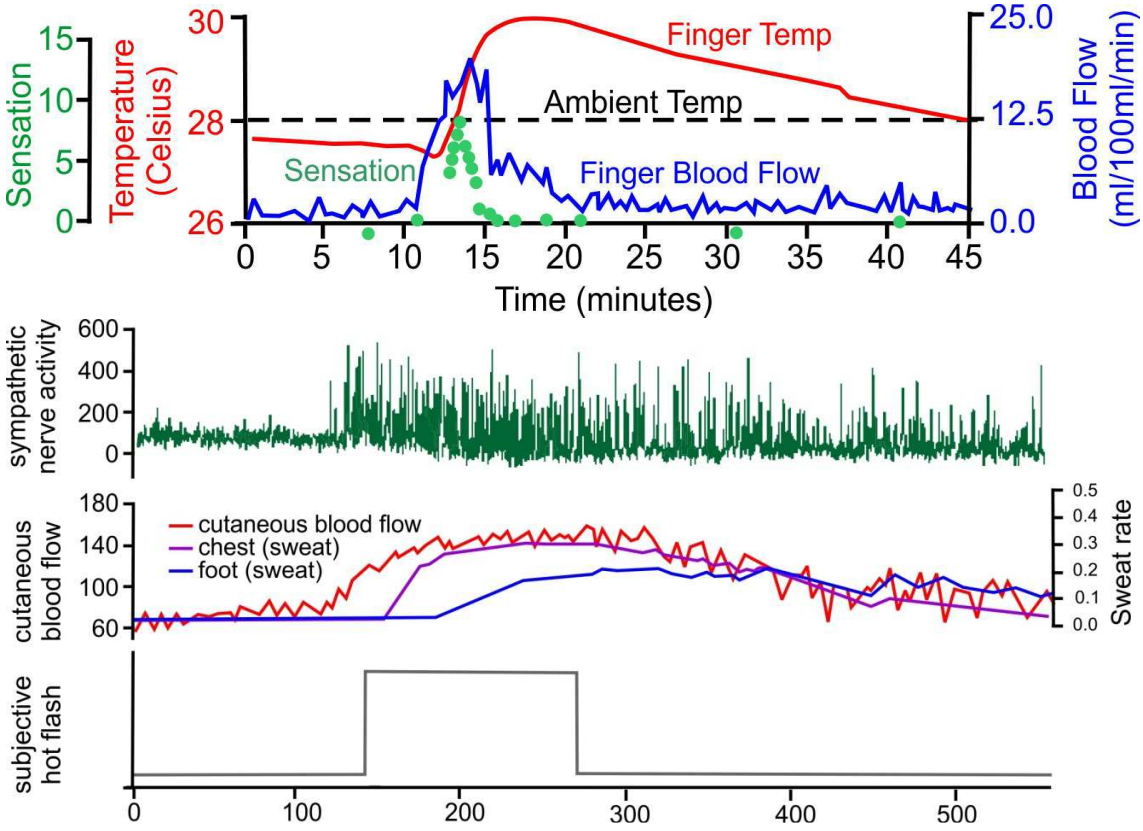


Figure 2. *Physiological Recordings of a Hot Flash*. The top panel illustrates the subjective sensation of a hot flash (as indicated with a button press) and its close temporal relationship with increased finger blood flow and finger temperature at the onset of the hot flash. Note the high ambient temperature; this was likely elevated to increase the probability of a hot flash. The bottom panel illustrates skin sympathetic nerve activity just precedes the onset of a hot flash (subjective sensation), which also correlates very well with sweat responses in the chest and foot. Upper panel adapted from Kronenberg, 1990, and lower panel adapted from Low et al., 2008.

majority of women, a small subset (10-15%) experience hot flashes for ten years or more or even for the remainder of their lives; a recent survey of 85-year old women reported 16% (still) experienced vasomotor symptoms (Vikstrom et al., 2013).

1.2.2. Healthcare and economic costs

Perhaps unsurprisingly, hot flashes are the primary menopausal symptom women seek medical treatment for, resulting in substantial direct and indirect healthcare costs. Until recently, the economic burden of vasomotor symptoms had been murky, though thought to be high, given that over 2 million women enter menopause annually (United States Department of Labor), and work ability has been reported as inversely related to climacteric symptoms (Geukes et al., 2012). In a retrospective study of over 35,000 women, Kleinman and colleagues found increased: direct medical and pharmacy costs, sick leave costs, and sick leave days among women with diagnosed menopausal symptoms (relative to control women matched on a number of parameters). Their estimates of hourly and annual productivity were 12.2 and 10.9% lower, respectively, for women with menopausal symptoms (Kleinman et al., 2013). In an analysis of 252,273 menopausal women (with symptoms but not utilizing treatment) using insurance database entries, Sarrel and colleagues estimated the cost of *untreated* vasomotor symptoms in excess of $375 million *annually* (Sarrel et al., 2015). Similarly, this figure includes both direct and indirect costs; specifically, these women had 82% more physician visits (all causes), 121% more VMS-related physician visits, and 57% more work productivity loss days. With regard to severity of symptoms and work, women with severe or moderate VMS experience greater presenteeism, or working while sick (which is associated with decreased productivity), than women with mild VMS (24.28 and 14.3%, respectively, for severe and moderate VMS compared to 4.33% for women with mild VMS). In terms of dollars, these percentages translate to an increased cost from $1100 (mild symptoms) to $6500 (severe symptoms) per woman [2009 US dollars (Whiteley et al., 2013)].

1.3. Relationship Between Hot Flashes and Estrogen

Although dramatic loss of estrogen during menopause is the major contributing factor leading to hot flashes and other menopausal symptoms (e.g., weight gain, sleep disruption, and changes in mood/anxiety and cognition), the exact mechanisms have yet to be fully understood. Estrogens are a complex family of molecules of several different types with varying affinities for their nuclear and membrane associated receptors. In this section, I will discuss the types of estrogens, estrogen receptors (types and localization with respect to function), and evidence of estrogen's critical role in hot flashes.

1.3.1. Types of estrogens

It is virtually impossible to discuss hot flashes without a mention of estrogens, whose replacement was the standard of care for VMS and is still the only known compound to substantively relieve hot flashes (albeit at high doses). These steroid hormones are synthesized from cholesterol precursors, and the enzyme aromatase converts androgens into estrogens. Estrogens are delineated among three major classes: estrone (E_1), estradiol (E_2), and estriol (E_3). Estrone is the primary circulating estrogen after menopause produced largely by the adrenal glands; it is the least potent agonist of the estrogen receptor. Estradiol, the "estrogen" that most colloquial and even scientific language refers to, is the primary circulating estrogen for non-pregnant, reproductive-age females and is the most potent of these molecules. Estriol, is exclusively produced during pregnancy, and has potency intermediate of estrone and estradiol. Throughout this document, estrogen refers to estradiol unless explicitly differentiated.

1.3.2. Types of estrogen receptors, locations, and function

Estrogen receptors (ER) fall into two classes: classical nuclear receptors and more recently discovered membranous receptors. The nuclear receptors, ERα and ERβ, are transcribed from genes on different chromosomes (in

humans; ERα on 6q25.1 and ERβ on 15a23.2) and have three and four known isoforms, respectively (Jia et al., 2015). Functionally, an ER consists of three domains: 1) the NH_2-terminal domain; 2) the DNA-binding domain; 3) the COOH-terminal ligand-binding domain. The DNA-binding domains are 97% conserved between the two receptors, and enter the nucleus to regulate gene transcription by binding to estrogen response elements (DNA sequences). Their temporal responsiveness is relatively slow, in comparison to a membrane receptor or ion channel, for example. ERs are widely distributed in the peripheral tissues of the rat, with high expression of ERα and ERβ in the ovary and uterus; high expression of ERα in the pituitary, kidney, and adrenals; moderate expression of ERβ in the uterus, bladder, and lung; low expression of ERβ in the pituitary, thymus, and spinal cord; and high expression of ERβ in the ovary (Kuiper et al., 1997).

Prior to the discovery of a second ER (ERβ), *in situ* hybridization studies of female rats revealed that ER mRNA transcripts were widely distributed throughout the brain, with dense expression in many amygdaloid and hypothalamic nuclei and less expression in the brainstem, septal areas, thalamus, and cerebellum (Simerly et al., 1990). Later studies revealed that ER expression varied over the estrus cycle, and that ovariectomy lead to increased ER expression (Shughrue et al., 1992). An initial characterization of ERβ compared to ERα revealed some restricted localization of the β receptor; specifically, it is expressed (virtually) exclusively in the supraoptic nucleus and paraventricular nucleus (Shughrue et al., 1996). Additionally, ERβ is expressed in the medial preoptic, anterior periventricular, arcuate, medial mammillary nucleus, and bed nucleus of the stria terminalis. Within the paraventricular nucleus, *in situ* hybridization combined with immunohistochemistry revealed that these neurons were largely (60-80%) positive for corticotrophin releasing factor (Laflamme et al., 1998). Osterlund and colleagues further found specific differences of expression of the two receptors within subnuclei of the amygdala (Osterlund et al., 1998). The expression of these receptors in hypothalamic nuclei strongly suggests a key role in regulating sexual, reproductive, stress-

responsive, and homeostatic physiological processes and behaviors, whereas the amygdaloid expression indicates an essential role in emotional regulation.

The other known estrogen receptor is a membranous receptor called GPER (G-protein coupled estrogen receptor 1, formerly GPR30) and has been found in breast tumors in women and is linked to invasive cancers (Filardo et al., 2006). As the name suggests, it is a 7-transmembrane, G_q-protein coupled receptor. It follows that GPER mediates fast, non-genomic effects of estradiol, and activation with a GPER-specific ligand increases intracellular calcium (Brailoiu et al., 2007). Distribution patterns of GPER parallel ERβ to some extent, with high expression in the supraoptic nucleus and paraventricular nucleus (Brailoiu et al., 2007; Hazell et al., 2009). GPER is also found in the forebrain, anterior pituitary, hippocampus, and brainstem nuclei that regulate the autonomic nervous system (area postrema, dorsal motor nucleus of the vagus, nucleus of the solitary tract, and nucleus ambiguous) and in some peripheral tissues (renal pelvis, adrenal medulla, and ovary) of the rat (Brailoiu et al., 2007; Hazell et al., 2009). As there is a specific antibody for this receptor, double immunohistochemical studies have identified that a significant proportion of GPER+ cells are also immunoreactive for either oxytocin or vasopressin in the hypothalamus (Brailoiu et al., 2007; Hazell et al., 2009). Similar to the classical receptors, GPER may also have a specific subcellular distribution, as one study demonstrated specific localization to the Golgi apparatus and endoplasmic reticulum (Matsuda et al., 2008). Again, the high expression of GPER in hypothalamic and autonomic nuclei suggests a prominent role for estrogen's regulation of homeostatic function.

Interestingly, more recent work has revealed that the classical 'nuclear' receptors can also mediate fast membranous effects. Currently, both ERα and ERβ have been shown to couple with metabotropic glutamate receptors (mGluRs) through scaffolding proteins called caveolins [see review by (Meitzen and Mermelstein, 2011)]. Caveolin 1 links ERα to Group I mGluRs (mGluR1 or mGluR5), which are presynaptically localized, excitatory, G_q-coupled receptors. G_q signaling initiates the cleavage of phosphatidylinositol 4,5-bisphosphonate

(PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) by Phospholipase C (PLC). IP_3 can stimulate intracellular calcium release from the endoplasmic reticulum, whereas DAG can increase cAMP production through the stimulation of adenylyl cyclase (via DAG-dependent protein kinase/PKG). cAMP can then bind to the cAMP response element (CRE) in the genome to modulate gene transcription. cAMP can also regulate ion channels (such as G-protein inward rectifying potassium [GIRK] channels, which control intracellular excitability) or modulate proteins [(review by (Kelly and Rønnekleiv, 2015)]. Conversely, caveolin 3 can link ERα and ERβ to Group II mGluRs (mGluR2 or mGluR3), which are inhibitory and both pre- and postsynaptically localized. These receptors are linked to G_i proteins, which inhibits adenylyl cyclase and cAMP production, consequently decreasing the activity of cAMP-dependent protein kinase (PKA). Through either membranous signaling pathway, estrogen can affect cellular excitability and gene transcription, even in the absence of an estrogen response element (ERE).

1.3.3. Estrogen inhibition

Estrogen disruption, either surgical or pharmacological, unequivocally leads to hot flashes. Women who have had an oöphorectomy experience severe menopausal symptoms, including depression and anxiety, and have greater moderate-to-severe and prolonged hot flashes when compared to naturally menopausal women (Gallicchio et al., 2006b; Rocca et al., 2008; Benshushan et al., 2009). Young, premenopausal women prescribed adjuvant endocrine therapies (AET) for cancer treatment (SERMs, AIs) frequently experience hot flashes [35.7-42.5% for AI, 69% for tamoxifen use (Carpenter et al., 1998)], and the majority of these women rate their hot flashes as severe (59%). In fact, studies have documented that the hot flashes resulting from SERM treatment is so severe that many patients discontinue taking these medications, which leads to increased mortality (Hershman et al., 2011; Murphy et al., 2012; Kemp et al., 2014).

1.3.4. Relationship between estrogen levels and hot flashes

Yet, the relationship between hot flashes and estrogen outside of an estrogen inhibition context is not as simple as it would appear. Estradiol levels in premenopausal women fluctuate across the menstrual cycle, and range from approximately 60 to 100 pg/ml (Rothman et al., 2011), as shown in **Fig. 3**. As a woman transitions into perimenopause, estradiol levels fluctuate around normal premenopausal values before dropping precipitously about one year before the final menstrual period (Burger et al., 1999). Estradiol levels then further decrease in the years following the final menstrual period, and ultimately are about 90% decreased from premenopausal values. The correlations between estradiol levels and the presence or absence of hot flashes have not always been the most straightforward, as numerous early studies failed to find consistent or even any positive relationships between these measures [for review, see (Whiteman et al., 2003)]. Notably, these were small, cross-sectional studies, and much larger (i.e. sufficiently powered) and longitudinal studies (typically 200 women or more) have revealed stronger and more consistent relationships between hot flashes and estrogen levels, such that low levels of estrogen were significantly associated with hot flashes (Gold et al., 2000; Avis et al., 2001a; Gallicchio et al., 2006b, 2015; Alexander et al., 2010). An additional consideration is the methodologies used to assess estrogen levels. Traditional methods have used immunoassays (i.e, radioimmunoassays), and are plagued by nonspecificity because the antibodies employed have difficulty in separating estradiol and estrone (Labrie et al., 2015). Furthermore, they are relatively insensitive, and the low levels of estradiol in the postmenopause stage are often around the limits of detection of some assays. More sophisticated methods (e.g., liquid chromatography tandem mass spectrometry; LC-MS/MS) are more suited to separate these compounds and accurately assess low levels. Despite the outstanding question regarding how estrogen loss contributes to hot

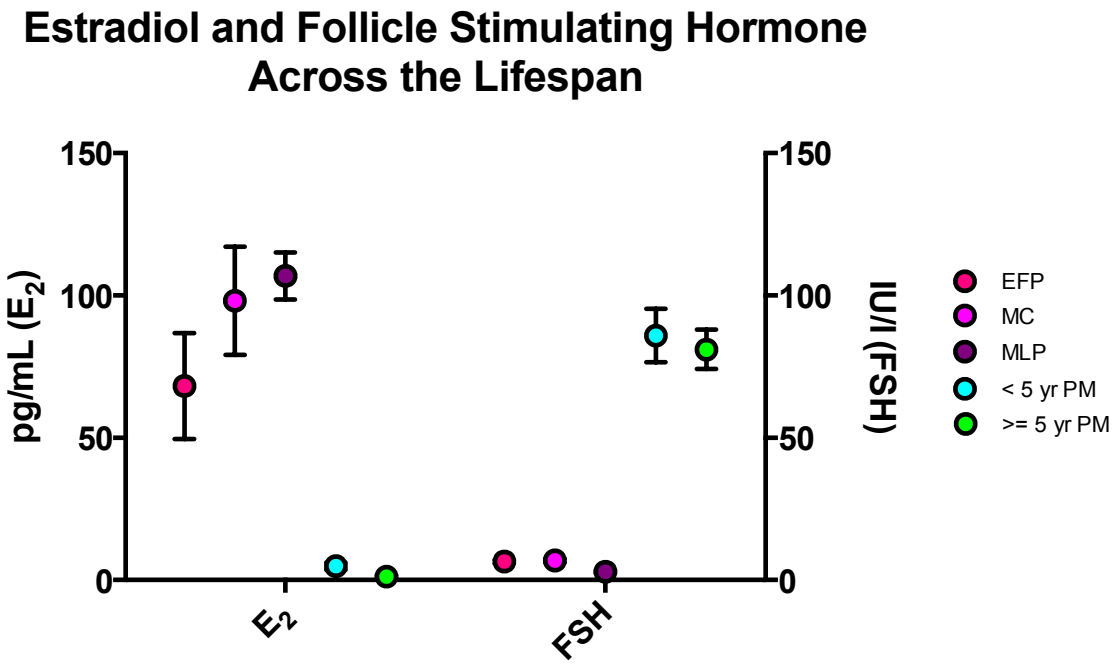


Figure 3. *Levels of Estradiol and Follicle Stimulating Hormone Across the Lifespan.* Estradiol (E_2) and Follicle Stimulating Hormone (FSH) are graphed according to measurements across a regular menstrual cycle in a cohort of young women [early follicular phase (EFP), mid-cycle (MC), and mid-luteal phase (MLP)] and in early menopausal (less than five years after the final menstrual period) and late menopausal women (five years or more after the final menstrual period). Data points represent group averages ± SEM; n=14,9, and 10, respectively for premenopausal, early and late menopausal participants. Overall, there is a 90% reduction in E_2 from premenopausal to post-menopause, and E_2 continues to decrease in the postmenopausal years. Graph is adapted from Rothman et al., 2011 *Steroids.*

flash generation and how its replacement ameliorates most hot flashes, estrogen clearly plays a very important role. Numerous studies of premenopausal (and therefore having largely normal endocrine profiles) cancer patients treated with estrogen inhibition therapies demonstrate that estrogen antagonism causes hot flashes. However, caution should be taken in interpreting studies that have quantified estrogens using immunoassays, and thus this represents another area for potential improvement in studying women's health.

1.3.5. Estrogen withdrawal is necessary

Despite more recent findings (with more sophisticated and sensitive assays), absolute levels of estrogen appear insufficient to cause or permit hot flashes or even prevent them. Seminal papers long reported that estrogen *withdrawal* is required for hot flashes, as pre-pubertal girls (with low levels of estrogen) do not experience hot flashes, and women with developmental defects that never go through puberty (i.e., those with hypothalamic amenorrhea) experience hot flashes only *after* withdrawing from estrogen therapy (Yen et al., 1972b). And while high doses of estrogen ameliorate hot flashes, after discontinuing ERT, most women will experience hot flashes again regardless of tapering or not (Haskell, 2004). In recent years, the advent of non-hormonal therapies has helped to provide a broader perspective on hot flashes that is less estrogen-centric.

1.3.6. Progesterone

Although much research in women's health has focused on estrogens, given their ability to reduce hot flashes, progesterone has shown promise as a therapy, both through correlational analyses and hormone replacement studies. A large-scale genetic study found low levels of progesterone were significantly associated with recent, severe, and frequent hot flashes in women who experienced hot flashes compared to women who have not experienced hot flashes (Schilling et al., 2007). Recently, Gallicchio reported that higher estradiol and progesterone levels significantly decreased the odds of all hot flash

measurements in a cohort of younger mid-life women (Gallicchio et al., 2015). Progesterone replacement was equally able to reduce hot flashes compared to conjugated equine estrogens in a cohort of premenopausal women that underwent oophorectomy for benign disease in an intervention trial (Prior et al., 2007), and significantly reduced vasomotor symptoms in postmenopausal women, including within a subset with severe symptoms (Hitchcock and Prior, 2012; Prior and Hitchcock, 2012).

1.4. Therapeutic Options for Hot Flash Amelioration

1.4.1. Decline of estrogen replacement therapy

In the last 15 years, estrogen has been the subject of much controversy for reasons that have little to do with its efficacy as a hot flash remedy, for which it is about 75% effective (reduction in the number of hot flashes) according to a detailed Cochrane analysis (Maclennan et al., 2004). The early landmark study in hormone replacement trials, the Women's Health Initiative [WHI; (Rossouw et al., 2002)], demonstrated increased risk for cardiovascular events, stroke, and breast cancer in women taking estrogen (with or without progesterone) replacement. Unfortunately, there were significant problems with the design of these studies, as more than half the WHI cohort had been post-menopausal for ten years or more before re-exposure to estrogen and/or progesterone. This is pertinent in the context of the critical window hypothesis of estrogen, which attempts to resolve some of the discrepant results between estrogen's protective and deleterious effects, see (Maki, 2013). This idea contends that while circulating estrogen levels are within a normal range, estrogen replacement may be beneficial. But once estrogen levels reach a nadir, replacement may have harmful effects (or at least failure to benefit). [There is some evidence of this in preclinical literature; following ovariectomy, estrogen treatment cannot induce an increase in long-term potentiation (long-lasting increases in synaptic strength) after a certain length of time before initiating estradiol treatment (Smith et al., 2010)]. A subsequent re-analysis of the WHI data stratified by both age and time

since menopause before starting hormone therapy revealed non-significant trends towards a protective effect of hormone therapy regarding coronary heart disease for women less than 10 years from menopause, though increased risk of stroke was unaffected by age since menopause (Rossouw et al., 2007). Consequently, many women who would have otherwise sought ERT did not. Strikingly, breast cancer incidence declined approximately 6.7-10% (Clarke et al., 2006; Ravdin et al., 2007) in the United States, and similar decrease was found in Germany, paralleling a decline in prescriptions for estrogen or hormone therapy (Katalinic and Rawal, 2008). These findings and the intense media attention they garnered precipitated a demand for non-hormonal alternative treatments for hot flashes. As a result, current guidelines for hormone therapy state that the lowest effective dose should be used for the shortest period of time (North American Menopause Society position statement). Lower doses of estrogen are still efficacious compared to placebo, but less so than higher doses (Utian et al., 2001).

1.4.2. Alternative treatment options

1.4.2a. Serotonin & norepinephrine reuptake inhibitors

To date, there are two commonly used non-hormonal treatments for VMS, selective serotonin and/or norepinephrine reuptake inhibitors (SSRI; SNRI, respectively) which increase the synaptic concentration of these neurotransmitters by inhibiting their respective transporter(s) and the reuptake processes. However, their effects take at least 1-3 weeks, and it is thought that they work through complex compensatory mechanisms to produce their therapeutic effect [and elucidating such mechanisms is still an outstanding research question (see review by (Stahl, 1998)]. There is only one non-hormonal FDA-approved compound with a hot flash indication; paroxetine mesylate (Paxil) at 7.5 mg/day, (marketed as Brisdelle®), a dose less than prescribed for any psychiatric indication (which typically start at 20 mg) (Simon et al., 2013). Other drugs in this class and related classes are often prescribed for vasomotor symptoms, but such use is “off label” (because that compound is not specifically

approved for hot flashes) and, while legal, may not be reimbursed by insurance companies. The response rates for all of these compounds are marginally greater than placebo (for a graphical representation, see **Fig. 9**); for example, paroxetine reduced hot flashes from 10 to 5 (median per day) whereas placebo reduced hot flashes from 10 to 5.9 per day (Simon et al., 2013). This pattern is typical amongst SS/NRIs, and hot flash trials in general have very high placebo response rates. Furthermore, these treatments present many side effects, like gastrointestinal distress and sexual dysfunction (Handley and Williams, 2015).

1.4.2b. GABA

Currently, there is one non-hormonal treatment strategy, gabapentin (Neurontin), for hot flashes that may target the non-monoamine neurotransmitter GABA (**ϒ**-aminobutyric acid, the predominant fast inhibitory neurotransmitter). This structural analogue of GABA is currently used for neuropathic pain and seizures, in addition to many off-label uses. A few groups reported that gabapentin (200-2400 mg) ameliorates hot flashes comparably to estrogen treatment with respect to both hot flash frequency and severity (Reddy et al., 2006; Aguirre et al., 2010; Allameh et al., 2013). Gabapentin seems to be safe and relatively effective in hot flashes induced by oöphorectomy or tamoxifen therapy for breast cancer (Loprinzi et al., 2002b; Pandya et al., 2005). GABA's receptors and gabapentin's pharmacology is discussed in greater detail in section 2.5.3.

1.4.2c. Non-pharmacological treatment options

A plethora of non-hormonal and alternative therapies have been evaluated for menopausal symptoms, including several plant-based estrogenic compounds (phytoestrogens) like soy (or isolated soy derivatives like S-equol), black cohosh, and red clover; overall, these interventions largely failed to have any effect on reducing VMS (Roberts and Lethaby, 2014). The modern panacea, exercise, has similarly failed to have overall demonstrated efficacy (Daley et al., 2015a, 2015b), and twelve weeks of yoga instruction and practice was no more effective

than usual care in reducing vasomotor symptoms frequency or bother, though did improve insomnia symptoms (Newton et al., 2014).

1.4.3. Importance of treating hot flashes

For cancer patients, non-hormonal therapies are the only viable option as ERT is virtually always contraindicated. However, SSRI administration in the context of tamoxifen treatment may additionally be contraindicated. Tamoxifen is a prodrug that requires enzymatic conversion into its active metabolite, endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen), which is responsible for most of the pharmacological activity of the molecule (Dickschen et al., 2012). Activity of the cytochrome P450 superfamily member CYP2D6 is required for this biotransformation, and, as shown in **Fig. 4**, several SSRIs, including paroxetine, are potent and irreversible inhibitors of this enzyme. Stearns and colleagues (2003) demonstrated that paroxetine can lower plasma levels of endoxifen by up to 64% (Stearns et al., 2003b). Additional studies have documented increased mortality with tamoxifen and SSRI co-therapy, and a longer duration of overlap was associated with increased mortality (Kelly et al., 2010). Despite these findings, a recent study in the Netherlands found that CYP2D6-inhibiting SSRIs were still over-prescribed to patients taking tamoxifen (Binkhorst et al., 2013). Since many women are poor or intermediate tamoxifen→endoxifen metabolizers (Goetz et al., 2007), clinical trials investigating the effectiveness of endoxifen hydrochloride as a treatment are currently underway (www.clinicaltrials.gov identifier NCT02311933). Clearly, the development of endoxifen would circumvent the problem in prescribing SSRIs to these patients. However, 'good' metabolizer status (of tamoxifen into endoxifen) and higher blood levels of endoxifen are positively related to hot flashes and have been shown to predict hot flashes and their severity (Goetz et al., 2005; Lorizio et al., 2012;). Therefore, even with the successful development of endoxifen therapy, it seems likely that this could lead to more hot flashes in the oncology population and increase demand for more effective, non-hormonal treatments for hot flashes.

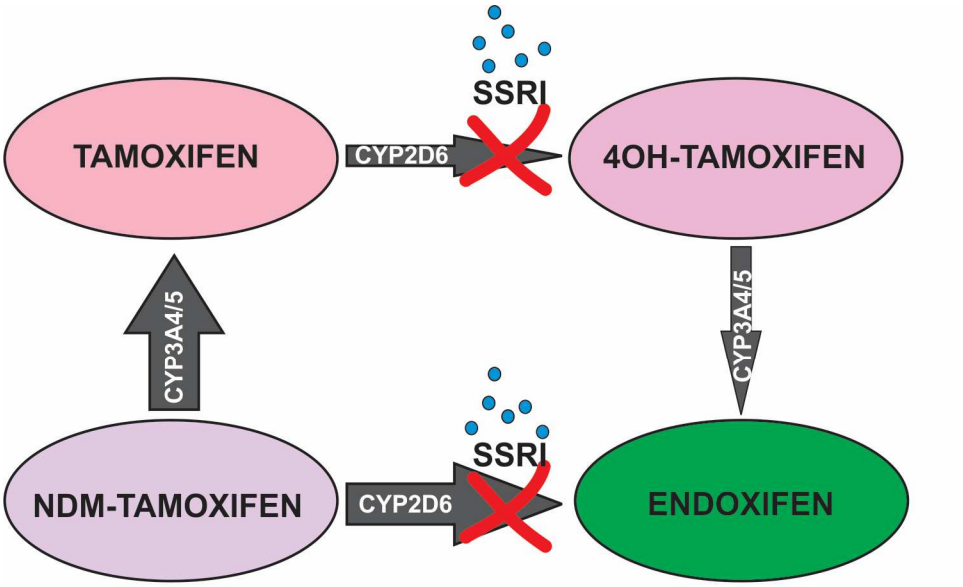


Figure 4. *Metabolic Pathway of Tamoxifen*. As indicated by the gray arrows, tamoxifen is metabolized into its active metabolite, endoxifen by the enzymes CYP3A4/5 and others and CYP2D6. CYP2D6 is required for the conversion of both NDM-tamoxifen and 4OH-tamoxifen into endoxifen, which is the metabolite responsible for tamoxifen's pharmacological activity. SSRIs, including paroxetine, are potent inhibitors of this enzyme, and can lower blood levels of endoxifen by up to 64%. Clinical trials are currently being conducted to determine if endoxifen hydrochloride is as effective as tamoxifen, and may circumvent the problem in prescribing SSRIs, yet may also precipitate an increased demand for effective hot flash treatments from this population of women.

Part II: Risk Factors for Hot Flashes

2.1. Classical Risk Factors

Risk factors for hot flashes are numerous, but have only been extensively characterized in large, diverse cohorts in more recent years. A careful delineation of factors that increase or decrease the probability and severity of hot flashes can reveal additional clues about their mechanisms that may lead to novel treatments. Early studies linked hot flashes most clearly to menopause stage; i.e., perimenopausal women are the most vulnerable. However, with the discoveries of several prospective, population-based, multi-ethnic, and multi-site studies, additional risk factors are becoming more apparent. This section will cover some of the key risk factors related to the experience of vasomotor symptoms, including smoking, obesity, race or ethnic background and also includes a discussion of emerging genetic factors, stress, and anxiety.

2.1.1. Cigarette smoking

Results from several studies have consistently indicated a role for cigarette smoking and the experience of vasomotor symptoms. In a sample of 233 peri- and postmenopausal women, smoking was positively associated with hot flashes (OR = 2.0), and smokers reported significant bother due to hot flashes in the 454 women enrolled in the Massachusetts Women's Health Study (Avis et al., 1997; Staropoli et al., 1998). Initial results from the SWAN confirmed these early findings (OR = 1.21-1.78) (Gold et al., 2000). A cross-sectional study of 1,087 women reported significantly increased odds of reporting both moderate-to-severe and daily hot flashes among current smokers compared to former smokers (Whiteman et al., 2003). While most reports have examined intensity and/or frequency of VMS, smoking had no effect on the *duration* of hot flashes in women followed in the Penn Ovarian Aging Study cohort (Freeman et al., 2011). Cross-culturally, the positive association between smoking and hot flashes has been relatively consistent as well in very large studies (Sabia et al.,

2008; Gjelsvik et al., 2011; Hunter et al., 2012). However, studies of Finnish and Portuguese women failed to find any association between smoking and VMS (Moilanen et al., 2010; Pimenta et al., 2011).

Furthermore, it is well-established that smokers reach menopause approximately 1.7 years before nonsmokers (McKinlay et al., 1985). Cigarette smoke may impact the ability of aromatase to produce estrogens, in addition to being potentially directly toxic to the ovaries (Mattison et al., 1983). Mechanistically, there are several ways in which cigarette smoking could be affecting (decreasing) hormone levels, and it is possible that this relationship mediates the observed effect of smoking on hot flash experience, though several reports, detailed below, do not support this idea. Gallicchio and coworkers reported a significant positive association between cigarette smoking and greater experience of any and more severe hot flashes in a case-control study of 653 women that retained significance even when controlling for estradiol and estrone levels, suggesting a mechanism independent from sex steroids (Gallicchio et al., 2005, 2006a). Later results from the same sample indicated that current smokers had higher androstenedione and progesterone levels and a higher androgen to estrogen ratio than “never smokers”, yet controlling for these hormone levels did not change the association between cigarette smoking and the probability of experiencing hot flashes (Cochran et al., 2008).

Recent work indicates the relationships observed between cigarette smoking and increased hot flashes may be mediated by interactions between gene variants (polymorphisms) and ethnic background. An investigation of the interaction of gene variants with smoking status on hot flash symptoms revealed European-American (EA) women homozygous for the COMT Val158Met variant had increased risk of hot flashes, and heavy smokers with such variant experienced more frequent moderate to severe hot flashes (Butts et al., 2012). EA smokers with the CYP1B1*3 variant also experienced more frequent moderate to severe hot flashes, and African-American smokers with the CYP1A2 variant were more likely to report hot flashes as well.

2.1.2. Obesity and body composition

Similar to smoking, obesity has shown strong associations with hot flashes, though its relationship appears complex and possibly menopausal stage-dependent. Initially, small studies observed that thinner women (Erlik et al., 1982) experienced more hot flashes, and thus the "thin hypothesis" was posited. This purported that body fat was protective against hot flashes based on the contention that thinner women have less body fat to be aromatized (peripherally) into estrogens (predominantly estrone). This idea directly competes with the "thermoregulatory hypothesis", which posits that women with more body fat would be at increased risk of hot flashes due to the heat-retaining/insulating properties of adipose tissue. It is still unclear exactly what may be correct, and both mechanisms may be contributing to hot flash pathology. In a study of Dutch women, those aged 40-44 in the highest quartile of body mass index (BMI; calculated by weight in kilograms divided by height in meters squared; normal is <25 kg/m^2, overweight 25.1-29.9, obese >30) and waist-to-hip ratio (measure of abdominal adiposity) report significantly more hot flashes, even when controlling for age than postmenopausal comparison women (den Tonkelaar et al., 1996). Additionally, Wilbur and Gold both demonstrated women with a higher BMI had more hot flashes (Wilbur et al., 1998; Gold et al., 2000, 2006). An increased risk of daily hot flashes was reported for women with high BMI only in *pre*- and *peri*menopausal women; BMI greater than 30 kg/m^2 impacted the risk of moderate to severe hot flashes in postmenopausal women (Whiteman et al., 2003). In 2004, Hyde Riley and coworkers showed that *peri*menopausal women with a BMI greater than 25 kg/m^2 had increased odds of reporting hot flashes (OR = 2.0) after controlling for multiple factors; yet in *post*menopausal women, BMI was not significantly associated with hot flashes (Hyde Riley et al., 2004). Gallicchio et al. (2005) report that severely obese women (BMI ≥ 35.0) had increased odds of any and more severe hot flashes as they transitioned through the menopause, but later found that controlling for estrogens, progesterone, and

sex hormone binding globulin negated that effect (Gallicchio et al., 2005; Schilling et al., 2007).

Importantly, BMI is a relatively crude measure, and does not distinguish between adipose tissue and lean mass, and so it is possible that persons with significant muscle mass may be classified as overweight (e.g., football players). Furthermore, self-reported measures of weight are particularly prone to inaccuracies and have been demonstrated specifically in this population (Lawlor et al., 2002; Engstrom et al., 2003). Therefore, objective verification of weight or body composition is highly desirable. The SWAN utilized bioelectrical impedance analysis to determine percentage of body fat in a multiethnic/racial sample, and the patterns uncovered have been illuminating. First, in 2008, Thurston and colleagues reported a significant positive association between percentage of body fat and hot flash experience (self-report), which were maintained even when controlling for FSH, estradiol, and free estradiol (estradiol not bound to sex hormone-binding globulin) (Thurston et al., 2008c). Using data gathered as part of the SWAN Heart Study, Thurston et al., (2008b) also reported that abdominal adiposity specifically was positively associated with hot flashes, and this effect was mediated by subcutaneous rather than visceral adiposity (fat distributed directly under the skin versus beneath the abdominal musculature). These associations were maintained after controlling for FSH and free and bound estradiol (Thurston et al., 2008d). Furthermore, increasing body fat over time was associated with greater odds of reporting hot flashes even when controlling for the aforementioned hormones (Thurston et al., 2011). Importantly, obesity is modifiable risk factor, and a pilot study of a behavioral weight loss intervention in women with hot flashes demonstrated a significant reduction in self-reported hot flashes, with trends for reduction on diary and physiological measures (the study was not designed or powered to determine the effects of weight loss on hot flashes *per se*) (Thurston et al., 2015).

2.1.3. Race and ethnic background

Several reports have consistently indicated racial/ethnic differences in the reported experiences of hot flashes. In the SWAN, women of Chinese and Japanese ancestry in the United States reported all menopausal symptoms less than Caucasians, who reported less symptoms than African-Americans (Gold et al., 2000). In the Hilo Women's Health Study of Japanese-Americans and European-Americans in Hilo, Hawaii, Japanese-American women were less likely to report both hot flashes and night sweats and were more likely to report symptoms as mild when they were experienced, even after controlling for soy consumption (which contains phytoestrogens that weakly activate estrogen receptors) (Sievert et al., 2007). However, later research using [objective] ambulatory and laboratory monitoring found no differences in subjective or objective hot flashes between Japanese-American and European-American women in Hilo (Brown et al., 2009). Over the duration of the SWAN, African-American women continued to report more hot flashes than the other ethnic and racial groups studied (Gold et al., 2006), consistent with Miller and colleagues' case-control study, which found that African-American women had increased risk of hot flashes with greater severity and longer duration than Caucasian women (Miller et al., 2006). In a study of 216 African-American and Caucasian women, race accounted for 10% of the variance in experience of vasomotor symptoms; again, African-Americans experienced more VMS and reported their symptoms as more bothersome (Appling et al., 2007). In a SWAN report of vasomotor symptom *bother*, perhaps a more nuanced view of hot flashes, African-American race was associated with significant bother from VMS (Thurston et al., 2008b). Importantly, above and beyond frequency and bother, VMS seemed to last longer overall for African-American women compared to other groups, with a median duration of 10.1 years (Avis et al., 2015), echoing Freeman's POAS conclusions regarding African-American race as a significant hot flash predictor (Freeman et al., 2011). The precise mechanisms that may be responsible for a racial discrepancy in hot flash experience have not been determined, but there may be genetic differences as outlined below.

2.2. Genetic contributions

Considering the discrepancies between estrogen levels and hot flash symptoms, several groups have proposed the idea that it may be how estrogens are metabolized or interact with certain polymorphisms/protein variants within tissues that lends itself to a meaningful clinical outcome. Subtle differences in receptors and/or synthetic and/or catabolic enzymes could affect this. The most obvious targets for consideration of this concept are the classical (nuclear) estrogen receptors, α and β (ERα, ERβ). Estrogen receptors are transcription factors that dimerize upon estrogen binding and then enter the nucleus to regulate gene expression at estrogen response elements. Polymorphisms within the genes that encode these receptors have been studied in the context of hot flash symptomatology, along with metabolic enzymes, are discussed in this section.

2.2.1. ESR1/ERα

The first report of a significant association with ERα comes from a study of 177 postmenopausal Mexican women that examined two single nucleotide polymorphisms (SNP) within ERα (Malacara et al., 2004). Significant associations were found only for hot flashes and vaginal dryness; hot flashes were lower for the Pp genotype (*PvuII* site of ERα or rs2234693). It is unclear how meaningful the measure of hot flashes were in this report, as the authors only state “hot flashes were measured by the presence of acute, localized short-lasting sensations of skin warming…symptoms were registered by yes = 1 or no = 0”. In the SWAN Genetics Study (examining African-American, Caucasian, and Chinese-American women), no significant associations of hot flashes within this SNP were found, but statistical significance for *reduced* hot flashes in the AG genotype for another ESR1 SNP, rs9340799 (*XbaI* site) was found in Caucasian women whose BMI was <25 (normal weight) (Crandall et al., 2006). For Caucasian women with the AG genotype and a BMI >= 30, this genotype

increased the odds ratio of VMS reporting by 2.6. Results from the Seattle Midlife Women's Health Study failed to find any association with hot flashes with either polymorphism (Woods et al., 2006b), consistent with a study of 290 Mexican women (Aguilar-Zavala et al., 2012). In premenopausal women, the *XbaI* GG and *PvuII* CC genotypes were associated with significantly more hot flashes before tamoxifen treatment; after tamoxifen initiation, the only group that showed a genotype-dependent change was postmenopausal women who were both *XbaI* GG *and PvuII* CC genotype (Jin et al., 2008).

2.2.2. ESR2/ERβ

Takeo and colleagues (2005) reported that CA dinucleotide repeats in intron 5 of *ESR2* were associated with increased risk of vasomotor symptoms and premenstrual symptoms in 51 postmenopausal Japanese women (Takeo et al., 2005). Women with the short/short genotype had a 7-fold increased risk of vasomotor symptoms, 13-fold increased risk of psychological symptoms and 7.6-fold increased risk of premenstrual symptoms, and women with the extremely short/long genotype had increased risk of both symptoms and depression, though the magnitude of the increased risk is unclear. In a cohort of 297 women taking tamoxifen for breast cancer, women with the *ESR2*-02 AA genotype were significantly *less* likely to develop hot flashes compared to GG or AG carriers (Jin et al., 2008).

2.2.3. Sex steroid metabolic enzymes

Additional areas of investigation include the anabolic and catabolic enzymes for sex steroid hormones, including several members of the cytochrome P450 (CYP) superfamily and catechol-o-methyl transferase (COMT), which degrades dopamine, norepinephrine, and epinephrine and converts estrogen into a hydroxy estrone.

Aromatase (CYP19) converts testosterone into estradiol. Results from the multi-ethnic Seattle Midlife Women's Health Study found a significant association between the 11r polymorphism in this gene and more frequent and severe hot

flashes in women in the middle and late menopause transition stages as well as experiencing hot flashes in post-menopause (Woods et al., 2006b). The sulfotransferase *SULT1A1* catalyzes the conversion of estradiol into a sulfated form; a polymorphism (Met223Val or rs1081030) in this gene was significantly associated with increased risk of hot flashes in European-American women in the Penn Ovarian Aging Study (POAS) (Rebbeck et al., 2010).

Estrogen elimination occurs largely through oxidation by attachment of hydroxyl groups. The enzyme CYP1B1 metabolizes estradiol and estrone into catechol estrogens (4-hydroxy estrogens), and a polymorphism at amino acid 432 exists (valine→leucine or rs1056836). Conflicting reports find both increased and decreased risk of hot flashes that may be race-dependent. In a community study of midlife women (total n=612), Visvanathan and coworkers found that women carrying this mutation were at a 30% increased risk of moderate to severe hot flashes and a 27% increased risk of having hot flashes for one year or more; risk ratios for experiencing any or weekly hot flashes were of borderline statistical significance (Visvanathan et al., 2005). Schilling and coworkers (2007) corroborated these findings. For African-American women in the SWAN Genetics Study, heterozygosity at this locus significantly *decreased* the odds of reporting hot flashes (Crandall et al., 2006), which agrees with the results of African-American women in the POAS, where again this genotype significantly decreased the odds ratio of hot flashes (Rebbeck et al., 2010). An additional pathway involving CYP1A1 mediates 2-hydroxylation of estrogens, and a polymorphism (rs2606345) AC genotype decreased the odds of Chinese women reporting hot flashes (Crandall et al., 2006). The 4- and 2-hydroxy versions of estradiol and estrone have different potencies, such that 4-OH estrogens still have significant biological activity whereas the 2-OH estrogens only weakly activate estrogen receptors.

A polymorphism in 3-β-hydroxysteroid dehydrogenase (3βHSD), which catalyzes several reactions (including the synthesis of progesterone from pregnenolone, 17-α-progesterone from 17-α-pregnenolone, DHEA to androstenedione, and androstrenediol to testosterone) was associated with a

significantly increased risk of moderate to severe hot flashes (Schilling et al., 2007). Three polymorphisms in 17β-hydroxysteroid dehydrogenase, which interconvert estradiol and estrone (rs615942, TG genotype; rs592389, TG genotype; rs2830, AG genotype) were associated with decreased odds of reporting hot flashes in Caucasian women in the SWAN (Crandall et al., 2006).

Overall, these studies have identified some additional genetic risk factors for hot flashes, but between-study or cohort effects are sometimes diametrically opposed. Several sources of variability could account for these discrepancies, including characteristics of the study sample (i.e. race/ethnic background, age, inclusion/exclusion criteria), and definition and methodology of assessing hot flashes and consistency between assays. Additionally, some studies are quite small, and extrapolation of meaning from such sizes is problematic. Despite these potential hindrances, the investigation into the genetics underlying hot flashes continues to point toward estrogen as a key hormone in hot flash pathology, though these findings are understandably biased by *a priori* hypotheses. Understanding the intricacies and mechanisms in these pathways will be essential to fully realizing effective non-hormonal treatment strategies.

2.2.4. Serotonin transporter

The serotonin transporter [SERT/5-HTT, encoded by *SLC6A4* gene] removes serotonin from the synaptic cleft and has a variable n-terminal domain repeat polymorphism (44 base-pair insertion/deletion) in the promoter region known as the *5-HTTLPR*, leading to a long 'l' and short 's' allele (Heils et al., 1996). The 's' allele leads to reduced transcriptional efficacy, and therefore, less SERT expression (Lesch et al., 1996). The s/s or s/l genotype has been associated with many neuropsychiatric conditions, including depression and anxiety (Lesch et al., 1996; Gonda et al., 2007, 2009). Increased anxiety has been linked to the presence and severity of vasomotor symptoms and is one of the strongest predictors of hot flashes and is discussed in great detail in section 2.4 (Freeman et al., 2005). Recently, a significant positive association between severity of climacteric symptoms and the 's' allele was found in a study of Polish

women using the Blatt-Kupperman Menopausal Index (Grochans et al., 2013). In a study of Mexican women, the 's' allele was correlated with increased cigarette smoking, a known risk factor for VMS (Aguilar-Zavala et al., 2012). The effectiveness of SSRIs for hot flashes in relation to SERT genotype has not been demonstrated yet; however, s/s carriers have been shown to have a poor response to SSRIs for other conditions (Stein et al., 2006). The effectiveness and tolerability of SSRIs for hot flashes may be related to *5-HTTLPR* genotype such that s/s carriers are at greater risk for treatment-emergent side effects (Perlis et al., 2003), and this genotype may mediate some of the discontinuation observed in SSRI trials for hot flashes.

2.3. Role of stress

In 1936, Hans Selye called stress the "non-specific response of the body to any demand for change." More recently, stress is conceptualized as a state of threatened homeostasis (Bonnavion et al., 2015). In the vernacular, stress is often incorrectly vilified to indicate a negative event or circumstance, like the stress brought on by an illness or death. Stressors can be both positive and/or negative (such as the birth of a child or a move) and can be qualitatively categorized as they relate to social roles, family, etc. A more appropriate view is that there exists an optimal level of stress, "eustress", and both too much or little stress is deleterious. The role of stress, as defined in many ways, in menopause has been examined primarily using self-report, survey instruments. There are a few laboratory studies that examined the effects of induced stressors on menopausal symptoms.

2.3.1. Stress in early life

Stress experienced in early childhood and adolescence has been linked to the later development of numerous psychiatric conditions and poorer health outcomes, including schizophrenia, anxiety, depression, and cardiovascular disease, to name but a few. High levels of stress can dysregulate the

hypothalamic-pituitary adrenal (HPA) axis, which ultimately releases glucocorticoids from the adrenal gland and suppresses energy-intensive processes in the immune system. Increased cortisol secretion has been linked to hot flash pathology, and 24 hour urinary cortisol levels explained 32.5% of the variance in scores on the Greene Climacteric Scale (Cagnacci et al., 2011), and both ACTH and cortisol levels have shown increases following hot flashes (Meldrum et al., 1984; Cignarelli et al., 1989). It seems plausible that stress that may alter the responsiveness of the HPA axis might have a role in the later experience of hot flashes, despite having occurred decades earlier. In the Mental Health sub-study of the SWAN, (n=332, conducted only at the Pittsburgh site, so participants were Caucasian and African-American only), Thurston and colleagues (2008) assessed the role of early life stress and vasomotor symptoms (Thurston et al., 2008a) (the first and only report in the literature to date). Childhood abuse and neglect were measured by the 28-item short form of the Childhood Trauma Questionnaire, which has five subscales (emotional abuse, physical abuse, emotional neglect, sexual abuse, and physical neglect). Approximately 38% of the sample reported one of the five dimensions of abuse. Childhood abuse (all five dimensions) was significantly positively correlated with VMS (both hot flashes and/or night sweats) reporting in multivariate models that controlled for age, race, BMI, smoking, educational attainment, menopause status, and depressive symptoms. Challenge tests (i.e., ACTH or CRF administration) can be used to assess the function of the HPA axis, and adult female survivors of childhood abuse have shown hypersensitivity to such measures (Heim et al., 2001). While not all women experience childhood abuse, the role such experiences may play in modifying the HPA axis could provide clues to the etiology of hot flashes, even if only in a particular subgroup.

2.3.2. Stress in mid-life

Many groups have found that stress increases across the menopause transition. In 1981, Cooke and Greene reported increased total life stress from pre-menopause (n=21, defined by age as 25-34) to early menopause (n=33,

defined by ages 35-44) that was due almost entirely to 'exit stress', or the stress one has when a person/persons leave his/her "social field"; in this study, due largely to deaths (Cooke and Greene, 1981). Gannon, Hansel, and Goodwin (1987) studied ten symptomatic women, mean age 55.5 (range 50-63) using a hot flash card to ascertain relationships between hot flash parameters and daily stress, using a version of The Hassles Scale (measure of 117 "microstressors") (Gannon et al., 1987). Overall, stress accounted for 55% of the variance in hot flash frequency. They also found statistically significant positive correlations (r=0.31—0.74) between daily stress and hot flash *frequency* in 5/10 women; significant correlations (0.37, 0.43) between daily stress and HF *intensity* for two subjects (one different from previous group); three subjects had significant correlations between hassles and hot flash *duration* (r=0.30—0.42). In a study of 19 symptomatic Japanese women and 44 matched controls, the severity of climacteric symptoms, as measured by the Simplified Menopausal Index, significantly positively correlated (0.51) with stress, as measured by the Life Event Inventory (Igarashi et al., 2000). They noted that the patient group was more likely to utilize avoidance-oriented coping for menopausal complaints, which was also positively correlated with the severity of climacteric symptoms. Binfa and colleagues (2004) found that perimenopausal women (n=46, 15.3% of the sample) had higher stress scores as measured by the Cooper Questionnaire, but this was not due to an increase in negative life events, family dysfunction, or poor social support (Binfa et al., 2004). Furthermore, they found that perimenopausal women also had increased climacteric symptoms as measured with the Greene scale. A critical limitation of the above studies is their relatively small numbers of participants. In 2004, results from the SWAN cohort found that perceived stress was significantly positively and independently associated with vasomotor symptoms (OR=1.4; 95% confidence interval 1.2—1.6, $p < 0.0001$) (Gold et al., 2004), and greater perceived stress significantly impacted the duration of experiencing vasomotor symptoms for SWAN participants (Avis et al., 2015).

2.3.3. Experimental stress

Similar to early studies of ambient temperature, initial studies of hot flashes and stress were correlational and self-reported, even with the use of validated surveys. Swartzman and coworkers (1990) performed the first laboratory study using physiological monitoring to determine if stressors had a causal role in hot flashes (Swartzman et al., 1990). After a habituation session, symptomatic postmenopausal women (n=21, age 37-71, 20/21 naturally menopausal, 1 surgically menopausal) were exposed to a variety of stressors at a rate of 2-3 per hour. These stressors included loud noises, physical exercises, paced arithmetic, watching a stressful film, recalling a stressful situation, etc.; a non-stress session (counterbalanced design) included watching films or reading magazines. During the stress session, 47% more objective and 57% more subjective flushes were recorded (with the same flush report bias in both conditions). Anxiety, as measured by the State-Trait Anxiety Inventory, was the same before both sessions, and did not correlate with either flush rate.

2.4. Role of Anxiety Symptoms

2.4.1. Prevalence of anxiety across the menopause transition

Increased symptoms of anxiety during the menopause transition have been reported by several large prospective studies. In the initial SWAN screening/eligibility interview, the majority of women interviewed endorsed feeling tense or nervous (51.9%) or irritable or grouchy (51.6%) in the last two weeks (yes or no) (Avis et al., 2001b). These symptoms were part of a 15-item list of symptoms used in SWAN measures (at all visits), and is important to distinguish that it is not a standardized measure of anxiety or a diagnosis of an anxiety disorder *per se*, but that these items reflect common symptoms of anxiety more broadly defined. Psychological distress, defined as self-reported feelings of tension/nervousness, irritability/grouchiness, *and* “feeling blue” during the past two weeks was similarly found to increase across the menopause transition in the SWAN cohort (Bromberger et al., 2001). Overall, 24.1% of women in the

study endorsed psychological distress, though 28.9% of early perimenopausal women reported these symptoms compared to only 20.9% of premenopausal women and 22% of post-menopausal women. "Persistent mood disturbance", operationally defined as indicating nervousness/tension or irritability/grouchiness on at least six days in the last two weeks, was found as a consequence of the menopausal transition (Bromberger et al., 2013). When controlling for typical confounding factors including vasomotor symptoms and sleep disturbance, 10% of the women studied (1473 + 1688) indicated "taking medication for nervous conditions", and 16.1% of perimenopausal women (n=1473) indicated feeling irritable compared to only 11.4% of premenopausal women (n=1688). A similar pattern for feelings of nervousness was found; 18.4% of perimenopausal women endorsed this symptom compared to only 12.0% of premenopausal women, while "feeling blue" was not affected by the transition in this study. Finally, in 2013 Bromberger and coworkers reported that anxiety, as measured by 1) irritability; 2) tension/nervousness; 3) feeling fearful for no reason; and 4) heart racing or pounding (the latter two being symptoms of anxiety or panic), was likely to increase for women with low baseline levels of anxiety as they transitioned into the perimenopause or postmenopause stages. For women with high baseline levels of anxiety, menopause stage was not associated with high levels of anxiety, and controlled for "upsetting life events, financial strain, fair/poor perceived health, and vasomotor symptoms".

2.4.2. Anxiety and menopause across cultures

Importantly, findings of increased anxiety at menopause appear to hold across cultures and ethnic groups and do not appear a consequence of reporting bias. Significantly higher anxiety and depression in both peri- and postmenopausal women, as assessed with the Hospital Anxiety and Depression Scale (HADS), which has seven anxiety items and seven depression items, were reported in women studied in Norway through The Health Survey of Nord-Trondelag County (HUNT-II) (Tangen and Mykletun, 2008). Both peri- and postmenopausal women had increased scores compared to both premenopausal

women (premenopausal women n=3632, score 4.54; perimenopausal women n=355, score 5.03; postmenopausal women n=2396, score 4.81). Importantly, it is possible that women with high levels of anxiety are more likely to report or even over-report hot flashes in studies that rely on self-report, potentially confounding results. While concordance between objective and subjective measures of hot flashes is relatively low (average 29%), in a systematic review (Mann and Hunter, 2011) a study of 140 peri- and post-menopausal women found that anxiety was *less* likely to cause a woman to under-report hot flashes but not more likely to cause over-reporting of hot flashes (Stefanopoulou and Hunter, 2014).

Part III: Physiology of Hot Flashes

3.1. Basic Concepts in Thermoregulation

3.1.1. The importance of being endothermic

Humans are endothermic, that is, we generate our own heat through metabolism and are not reliant on solar energy to maintain body temperature. This long-held state of affairs affords us our choice of environment, and comes with several mechanisms to defend against threats to maintaining an appropriate core temperature (37 °C). Without a relative constancy of internal temperature, enzymatic function suffers, and death can result from excessive internal temperature. To clarify, core body temperature/internal temperature refers to the temperature of the brain, spinal cord, internal organs, and large veins, whereas "shell" temperature is used to distinguish the temperature of the skin. Normally, in humans, skin temperature is 3-4 °C lower than core temperature. The maintenance of core body temperature comes from the balance between heat production and heat dissipation, each of which consist of multiple contributing processes affected by a wide variety of factors.

3.1.2. Thermodynamics: Basics of heat transfer

Heat transfer occurs in four major ways: conduction, convection, radiation, and evaporation. Conduction is the direct transfer of heat from one source to another, and always occurs down a thermal gradient (from hot to cold). This occurs frequently, as often a human body is the warmest object in a room (with notable exceptions, like kitchens or mechanical rooms) and touching an object will result in heat loss from the person to the object. Conversely, when the object is hotter than the person touching it, heat transfers from the object to the person. Convection is the transfer of heat through a current, in either a gaseous or liquid state, and a faster flow rate of the liquid or gas will move more heat. This is easily felt on a warm day with a slight breeze. Radiation is the transfer of heat from bodies that are not in contact through electromagnetic waves—i.e., the

sun. Evaporation is the loss of heat through the physical transformation of water from a liquid to gaseous state—i.e., sweating.

3.1.2a. Heat loss mechanisms

Humans possess two major ways of rapidly losing heat: cutaneous vasodilation and sweating. Cutaneous vasodilation expands the diameter of blood vessels near the surface of the body, which allows for more heat to pass through the skin and escape. In particular, specialized areas of the skin (acral skin), including the feet, hands, nose, and ears, possess ateriovenous anastomoses. These are shunting systems that mix arterial and venous blood in a large network, and the high surface area to volume ratio of these appendages facilitates heat loss. In the rat, cutaneous vasodilation is the major mechanism to defend against heat stress, and, as shown in the following experiment, surface warming through vasodilation prevents core temperature increases **(Fig. 5)**. This mechanism is only effective if the ambient temperature is less than the skin temperature—therefore, at ambient temperatures in excess of 37 °C, heat transfer through vasodilation cannot occur because the thermal gradient is in the wrong direction. Evaporative cooling produced through sweating works in conjunction with vasodilation to further cool the body. Again, there is an environmental limit; as relative humidity increases, evaporation is impeded, and cooling slows or is even physically impossible in this situation.

3.1.2b. Heat preserving and generating mechanisms

The primary mechanism to save heat from loss to the environment is to constrict surface cutaneous blood vessels and shunt the blood towards the body core, which results in an even greater difference between internal and skin temperature. The largest and most typical source of heat production comes as a by-product of overall metabolism, and other sources of heat production include shivering and non-shivering thermogenesis. Shivering thermogenesis occurs by non-voluntary contractions of skeletal muscle that do not produce movement. Non-shivering thermogenesis occurs in specialized adipose tissue called brown

adipose tissue (BAT). The relative importance of BAT in adult thermogenesis is contentious, though its importance for neonates is undisputed, as infants cannot shiver. Essentially, BAT is enriched in mitochondria, and themogenin/uncoupling protein-1 disconnects the mitochondria from generating ATP so that it just generates heat. Lastly, the contraction of muscles that innervate hair follicles in glabrous skin cause piloerection and increases the surface area of the skin. At best, this is a minor contributor/strategy to conserve heat, and is likely a vestige of humans' evolutionary past.

3.1.2c. Behavioral strategies

While humans can survive from episodes of severely decreased core body temperature, internal temperature can only increase a few degrees before becoming fatal, as enzymes only function within a certain temperature range (the upper limit of which is already close to normal internal temperature). For this reason, behavioral strategies are perhaps best understood in the context of the link between emotions and survival. It is not only logical but elegant to select for mechanisms that provide strong motivational salience to effect change to *prevent* heat stress or cold stress. Despite the existence of physiological mechanisms to cope with temperature change, behavioral strategies are at least as important and more frequently employed to defend against temperature change. This can easily be seen in heat or cold avoidance or warmth-seeking behavior depending on the state of the animal. Examples abound—keeping houses, workplaces, and vehicles at a comfortable temperature, drinking cold or hot drinks, wearing temperature-appropriate clothing and shoes, etc. Menopausal women are especially known for behavioral thermoregulation, such as the use of fans and air-conditioning to ameliorate the perception of heat that comes with hot flashes. Indeed, environmental cooling is just one source of indirect costs of menopausal care (Utian, 2005).

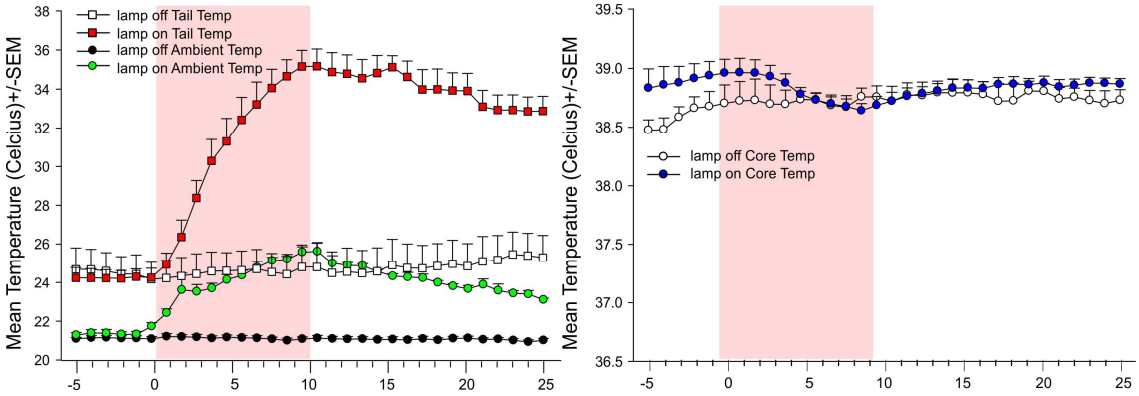


Figure 5. *Ambient Temperature-Induced Cutaneous Vasomotor Response Preserves Normothermia*. Adult male rats were placed in a box with a heat lamp above the surface, and tail skin temperature was measured with a thermistor. Over a period of 10 minutes, ambient temperature was increased approximately 5 °C from ~21 °C to 26 °C. **a)** Tail skin temperature increased significantly and rapidly from a baseline value of approximately 24 °C to 35 °C, while **b)** core temperature did not change (temperature changes as depicted in the right panel are not significant).

3.1.3. General thermosensory pathway

How then, does the detection of temperature occur, which is clearly a very basic and critical sensory survival function? Temperature sensation occurs when a receptor in the periphery is activated by thermal energy, which falls into four categories: cold, cool, warm, and hot. The 'cold' and 'hot' categories also have overlap with noxious/painful sensations, as these temperature ranges can signal the potential for acute tissue damage; i.e. freezing or burning. Thermal receptors are free nerve endings (several types to be described subsequently) that propagate signals along dorsal root ganglion neurons encoding thermal information along small diameter, relatively slowly conducting unmyelinated C fibers and lightly myelinated A_δ fibers to detect warmth and cold, respectively. Second order neurons in the dorsal horn decussate at the spinal cord level and form the anterolateral system, and have both direct and indirect projection pathways. A direct projection through the spinothalamic system relays information concerning the body position where the temperature information is being sensed, whereas branches emerge that relay thermal information to the hypothalamus through the spinoreticular pathway (via the reticular formation in the medulla and pons).

Specific receptors for thermal sensation largely include the transient receptor potential family (TRP) receptors, all of which are nonselective cation channels that are permeable to Na^+, K^+, and Ca^{2+} that consequently depolarize the neuron. Three subfamilies are relevant for thermoregulation and are known as "thermoTRPs": the vanilloid (TRPV), melastatin (TRPM), and ankyrin (TRPA) groups (Romanovsky, 2007). TRPV1-4 and TRPM2, 4, and 5 are warm sensors, whereas TRPM8 transmits cold signals. TRPV4 is activated by a range of approximately 20-35 °C, whereas TRPV3 is activated at a warmer range of temperatures, approximately 32-37 °C, and are probably the most relevant in terms of hot flashes; animals that lack TRPV4 have altered thermal sensation (Lee et al., 2005). These receptors rapidly desensitize to a constant temperature, and are more readily activated by *changes* in temperature. Importantly, while these are typically conceptualized as sensing external

temperature, such as the temperature of objects or hot or cold air, these receptors also mediate temperature sensation from changes within the body itself, which can result from activation of efferent heat loss pathways (i.e. cutaneous vasodilation during a hot flash or exercise or heat stress; see **Fig. 7**). Therefore, in the case of a hot flash, the sensation of heat on the face, chest, or elsewhere on the body, is relayed and transduced through these receptors. (As mentioned previously, the hot flash is not a phantom sensation—skin temperature objectively increases during the episode. However, this does not preclude the possibility that thermal receptors could be activated in the absence of thermal changes and produce a "thermal hallucination"). Though several of these receptors have been shown to be regulated by estradiol, including TRPV1 (Pohóczky et al., 2016), TRPM2 (Hiroi et al., 2013; Ahn et al., 2014), and TRMP8 (Chodon et al., 2010), there are no reports yet demonstrating any specific polymorphisms or gain or loss of function in menopause and resulting hot flashes. This represents another potentially illuminative area of investigation and gap in knowledge in hot flash research.

3.2. Triggers of Hot Flashes

As follows from the perspective of thermoregulation, the cutaneous vasodilation that occurs during hot flashes (and thus leads to the sensation of heat) is a heat loss mechanism. However, as this section will detail, thermal changes, whether in the environment or in the body itself, are only weakly linked to hot flashes, and why these heat dissipation responses are occurring remains enigmatic (at least from a purely thermoregulatory view). This section will describe the role of temperature in triggering hot flashes, including self-reported relationships between ambient temperature, and experimental studies of manipulating ambient temperature and direct heating paradigms. The potentially causal role of elevated core body temperature is also discussed.

3.2.1. Ambient temperature: Self-report

Perhaps due to the obvious presentation of hot flashes as a primarily thermosensory event, the most commonly assumed or accepted precipitant is "warm" ambient temperature. Some early studies provided correlational evidence that external/environmental temperature modulated hot flash frequency, intensity, or severity [hot flash parameters]. In a sample of 500 weather-sensitive women in Jerusalem, the arrival of the Sharav, or change to hot dry heat, increased hot flashes and chills (Sulman et al., 1975). Soon after, Coope (1978) studied 31 women (mean age 53; range 40-67) that experienced hot flashes (with or without heart palpitations) and insomnia (Coope et al., 1978). Using self-report hot flash cards, they determined the *number* of hot flashes per day significantly positively correlated with both the maximum and minimum daily temperatures (0.700 and 0.425, respectively). In 1981, 3 corroborated these findings in tracking his wife's hot flashes for 100 days over multiple seasons (Molnar, 1981), and found a strong positive correlation (0.68) for number of hot flashes per day and the mean daily temperature. However, a later group only found a significant positive correlation between hot flash parameters and daily temperature for *one of ten* women (Gannon et al., 1987). Similarly, Voda (1981) found no relationship between hot flash frequency and temperature variation in a study of 1041 self-reported hot flashes, despite reports from participants that they "thought" they had more hot flashes in warmer weather (Voda, 1981). Only one participant, whose hot flashes were analyzed for six months, demonstrated a relationship with ambient temperature; for her, hot flash *length* was directly related to temperature. A more recent study following 372 women in the United Arab Emirates (a hyper-arid climate) failed to find any association of seasonal variation with hot flash prevalence, frequency, or problem rating (Stefanopoulou et al., 2014a), mirroring a study of 717 women in India that also failed to find any association between hot flashes and seasonal temperature variation (Stefanopoulou et al., 2014b).

It is curious that the results of the previous studies are so discrepant, and perhaps these results were due to wide variability in the populations studied. In a

diary measure of 39 breast cancer survivors (mean age 52.6) experiencing hot flashes (mostly due to tamoxifen treatment), a group that likely consists of women with a more similar experience, Stubbs and coworkers (2008) investigated what participants thought as a proximal cause to the flush (Stubbs et al., 2008). This was a self-report measure in response to the question, "What events affected your symptoms?" Only a small proportion of the responses were temperature-related; of the 1,008 hot flashes reported, 2% indicated hot drinks, 6% indicated a hot environment, and 0.2% indicated a hot shower or bath. Of course, one cannot rule out the possibility that these response patterns were not specific to breast cancer treatment.

3.2.2. Ambient temperature: Systematic manipulations

As will become a consistent theme in hot flash research, self-report can be notoriously faulty, and, while difficult, controlled laboratory investigations can be highly informative. Kronenberg and Barnard (1992) systematically manipulated ambient temperature and used physiological monitoring to objectively measure hot flashes in six women experiencing ten or more hot flashes per day (ten per day is still the accepted criterion for "frequent" hot flashes as suggested by the FDA for hot flash intervention trials); five of the six participants were postmenopausal while one was perimenopausal (Kronenberg and Barnard, 1992). Their laboratory study utilized a cool, 19 °C ambient temperature or warm, 31 °C temperature; all subjects were exposed to an 8-hour session in both conditions. In the 12 experimental sessions, they recorded 93 hot flashes; 75 occurred in the warm condition while only 18 occurred in the cool condition. Hot flash frequency and intensity were significantly greater in the warm room, though duration did not reach statistical significance. The warm temperature in this study is quite warm (not very far from normal skin temperature values), and the lack of a control group of asymptomatic women precludes comparison between the experiences of these presumably different groups. It may have been that the same objective signs using physiological monitoring would be present, but the

symptomatic women label this experience as a hot flash, whereas the asymptomatic women merely report feeling warmer than usual.

Indeed, work in this laboratory (performed during an IBMG rotation by Jill Badin) revealed that ambient temperature-induced flushing does not differ between ovariectomized (OVEX) and intact female rats (shown in **Fig. 6**). A gradual warming protocol (using a shielded heat lamp to increase ambient temperature over a 30 minute period) caused an increase in tail skin temperature from a baseline of 25 °C to about 33 °C, yet there was no difference in the response between these groups. An OVEX group given chronic E_2 replacement appeared to have a baseline shift downward about 0.5 °C and TST increased about 2.5 °C less. This data is especially meaningful in light of work discussed in section 3.2.1, as it suggests that ambient temperature fluctuations may not be causally relevant to hot flashes.

3.2.3. Direct heating paradigms

In contrast to manipulating air temperature, a few groups have used direct heating of the body to provoke and study hot flashes. Sturdee and coworkers (1978) used a direct heating paradigm to evoke flushing in menopausal women and compared their physiological responses to premenopausal women (Sturdee et al., 1978). Using warm water bottles and blankets, they elicited 18 flushes in seven of eight menopausal women studied, and the flushes were accompanied by increased skin temperature (1 °C), a statistically significant tachycardia (increase from 71±3 beats per minute to 86±4 bpm) at the onset of the flush, and decreased skin resistance [increased sweating]. These parameters were concurrent with the subjective sensation of the flush. For the six premenopausal women studied, the increase in skin temperature and peripheral vasodilatation was greater than menopausal women, but not accompanied by tachycardia, and the decrease in skin resistance was much smaller, indicative of a lack of sympathetic drive in this group. Later workers closely followed this approach and used circulating water pads heated to 42 °C to evoke hot flashes (Germaine and Freedman, 1984). There were no significant differences in any of the

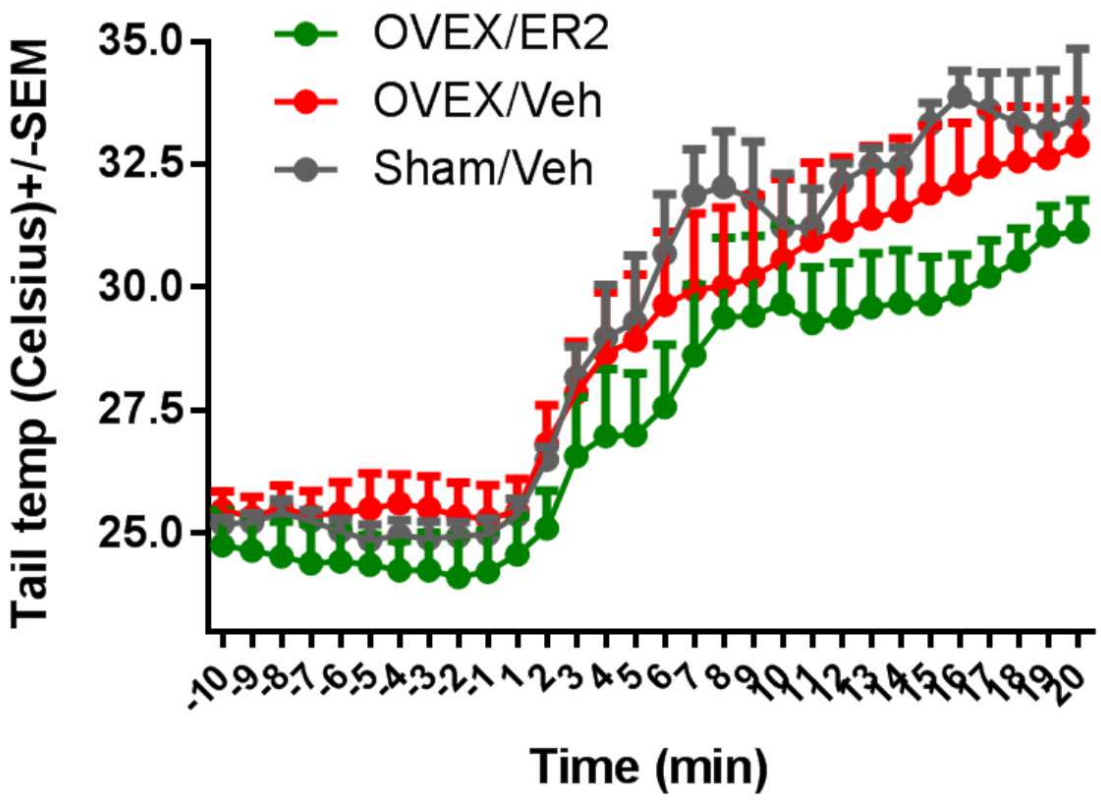


Figure 6. *Ambient Temperature-Induced Cutaneous Vasomotor Responses Do Not Differ Between Ovariectomized and Sham-Ovariectomized Rats.* Rats were exposed to an environmental heating protocol with a heat lamp (shielded) over an open experimental chamber. Ambient temperature was gradually increased over a 30 min period (following stable baseline). There were no statistically significant differences in treatment (p=0.21) between OVEX (n=6) and sham-OVEX rats (n=5) or OVEX rats treated with estradiol (n=5).

physiological parameters (heart rate, finger or cheek temperature, skin conductance, EMG, and respiration) between observed spontaneous hot flashes and hot flashes evoked with water pads in menopausal women. Moreover, no premenopausal women (n=12, mean age=31.6, range 22-45) had hot flashes (objectively defined as the start of tachycardia) whereas 12/14 and 13/14 of the symptomatic menopausal women (mean age 50.3, range 44-61) endorsed hot flashes in heat stress sessions one and two, respectively. Their results further replicated the tachycardia found by Sturdee; menopausal women exhibited a mean increase of 4.93 beats per minute while the premenopausal women's heart rate decreased 1.84 beats per minute to the heat stress procedure. Anecdotally, their participants reported that the evoked hot flashes were indistinguishable from spontaneous hot flashes.

3.2.4. Core body temperature

It is possible that ambient temperature affects only a subset of symptomatic women, and an unstable, elevated internal temperature may precipitate heat loss mechanisms in some cases. The thermoneutral zone refers to the "range of thermal comfort" for an individual (Hartgill et al., 2011), and can indicate 1) the range of *body* temperatures where there is no shivering or sweating or 2) the range of *ambient* temperatures where thermoregulation is achieved through "sensible heat loss", or through vasodilation and vasoconstriction to control blood flow. Beyond the upper threshold, sweating also occurs to dissipate heat by evaporative cooling, and below the lower threshold, shivering or non-shivering thermogenesis maintains body temperature. One leading hypothesis regarding the role of CBT in symptomatic peri- or postmenopausal women is that a narrowed-to-virtually negligible thermoneutral zone may play a key role in triggering hot flashes. Freedman and Krell (1999) measured the thermoneutral zone in symptomatic women to 0.0 ± 0.11 °C, while in asymptomatic women the zone was 0.4 ± 0.18 °C (12 symptomatic women and 8 asymptomatic women; CBT as assessed by ingested radiotelemetry pill)

(Freedman and Krell, 1999). Later work demonstrated that estrogen replacement widened the thermoneutral zone (Freedman and Blacker, 2002).

This deviation in the range of tolerable temperatures may play a role in the generation of hot flashes, as there are reports in the literature about very small, yet statistically significant, increases in core body temperature preceding the objectively measured hot flash (Freedman et al., 1995; Freedman and Woodward, 1996; Freedman, 1998). It is difficult to ascertain the precise increase in core temperature, but from the data presented in these papers, it appears to be about 0.05 °C. The graphs do not show error bars, and the variability observed is unclear. Interestingly, these increases in core temperature occurred in 46/77 (60%) and 24/37 (65%) of hot flashes observed in these studies, still leaving a significant minority of women with unchanged core body temperature. Furthermore, the relevance of these core temperature fluctuations are unclear, as further work has shown they are not specific to symptomatic women, as asymptomatic women have the same variation in temperature (Freedman, 2002). It is possible that these are causally related to hot flashes for some women, but a clear demonstration of such causality remains to be demonstrated.

Briefly, from an animal modeling perspective, I have found that core body temperature during pharmacological provocations does not increase prior to a tail skin temperature increase. The tail skin temperature increase occurs first, and then a core body temperature decrease follows. A similar series of events may occur for women that report that their hot flashes are followed by intense chills. However, in other paradigms that I have developed, such as a mild ambient temperature induced hot flash (see **Fig. 5**), core body temperature does not change. While it makes sense that hot flashes could be precipitated by core body temperature increases in some cases, this may be more applicable to certain circumstances or factors, such as during fever (although Kronenberg reports that during a febrile episode, hot flashes for one highly symptomatic woman stopped) or ingestion of hot (temperature) or spicy foods or beverages.

But as of now, research into delineating different “subtypes” of hot flashes is virtually nonexistent.

3.3. Neuroendocrine Contributions

3.3.1. *Gonadotropins: LH, FSH, GnRH*

Given the obvious role of hormonal decline in menopause, it is similarly intuitive that other (non-estrogen) reproductive related hormones may play an instrumental role in hot flash pathology. Luteinizing hormone (LH) received significant attention in the early hot flash literature as a putative causal factor. A neurohormone released from the anterior pituitary by Gonadotropin Releasing Hormone (GnRH), LH acts with Follicle Stimulating Hormone (FSH) in the ovaries to cause ovulation. LH also increases the production of androgens, the precursor to estrogens. Early work demonstrated a fairly tight temporal correlation between the onset of a hot flash and a pulsatile release of LH. The first report of neuroendocrine factors appeared to strongly implicate LH; in a study of 6 postmenopausal women, 55 hot flashes and 66 pulses of LH were recorded (Casper et al., 1979). The women with the most pulses of LH had the greatest number of hot flashes; while 11 pulses were recorded without a cognate hot flash, no hot flash was recorded without an LH pulse observed. FSH was also significantly associated with hot flashes but to a lesser degree. There was no change in prolactin or plasma catecholamines, including dopamine, norepinephrine, and epinephrine. The findings of Tataryn et al., (1979) largely corroborated Casper’s work; in a study of 6 symptomatic postmenopausal or oophorectomized women, they observed 31 pulses of LH in 32 objectively measured (finger temperature) hot flashes (Tataryn et al., 1979). In 26 of the 31 hot flashes, the pulse of LH “had a close temporal relationship” (range: 0.082-0.665 per patient; $p < 0.01$) with the onset of the flush. In this study, FSH had no relationship to hot flashes (range: -0.062-0.63; $p = 0.2$). Meldrum and colleagues (1984) studied 18 postmenopausal women (natural or surgical) that experienced severe hot flashes and assayed several neurohormones (Meldrum et al., 1984).

Again, LH was increased (15 min after the temperature increase) with no change in FSH or prolactin, though adrenocorticotropic hormone (ACTH) increased 5 min after the flush onset and an increase in growth hormone (GH) after 30 minutes was also found. Thyroid stimulating hormone was unchanged.

All of these studies are, of course, correlational and do not definitively indicate a causal role of LH. Pulses of LH were observed in both premenopausal and postmenopausal women (Yen et al., 1972a). Additionally, women whose pituitary glands were removed (hypophysectomized) had low or undetectable levels of LH and FSH but still had hot flashes (Mulley et al., 1977). Women with gonadal dysgenesis had intact gonadotropin profiles but low levels of estrogen (and never go through puberty) only had hot flashes *after* withdrawal from estrogen treatment (Yen et al., 1972b). However, these reports do suggest that the mechanisms that initiate release of LH may be involved in the neurochemical events that initiate hot flashes.

3.4. Role of the Autonomic Nervous System

Hot flashes are often accompanied by many other symptoms, including a pounding heart and blood pressure changes (e.g., lightheadedness), indicating broader autonomic changes. The additional evidence presented below demonstrates more globally heightened sympathetic tone that is present in symptomatic women, including cardiovascular perturbations. A discussion of autonomic neurochemistry as related to vasodilation and sweating is also included.

3.4.1. Heart rate, norepinephrine, and epinephrine

As mentioned earlier, tachycardia is often reported during hot flashes, providing some evidence that autonomic nervous system activation occurs during flashing episodes. Early laboratory studies of hot flashes using objective monitoring of highly symptomatic women confirmed this tachycardia, and even found that it slightly preceded the subjectively-defined hot flash sensation onset

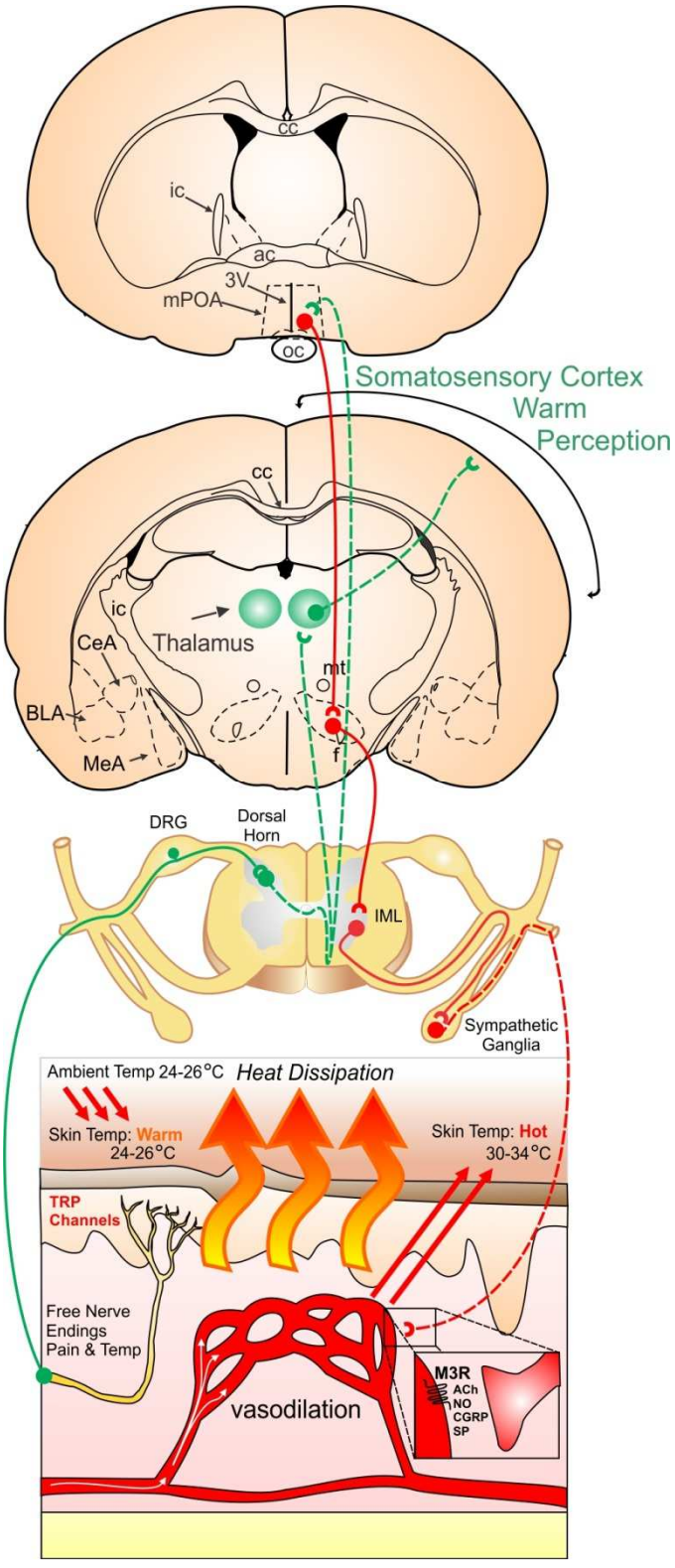


Figure 7. *Ambient Temperature-Induced Cutaneous Vasomotor Response*. Warm ambient temperature (i.e., 24-26 °C) increases skin temperature (sensed by transient receptor potential channels on the sensory nerve endings), and the signal propagates through the dorsal root ganglion and dorsal horn of the spinal cord. It decussates at the level of the spinal cord and travels up the anterolateral system to the thalamus or medial preoptic area to initiate a motor response. A third order neuron sends a collateral to the sensorimotor cortex for conscious perception of warmth while another collateral travels to the median preoptic area to initiate cutaneous vasomotor responses (traveling via the perifornical area of the hypothalamus). Cutaneous vasodilation makes the skin warmer (30-40 °C) which will increase temperature perception from warm to hot.

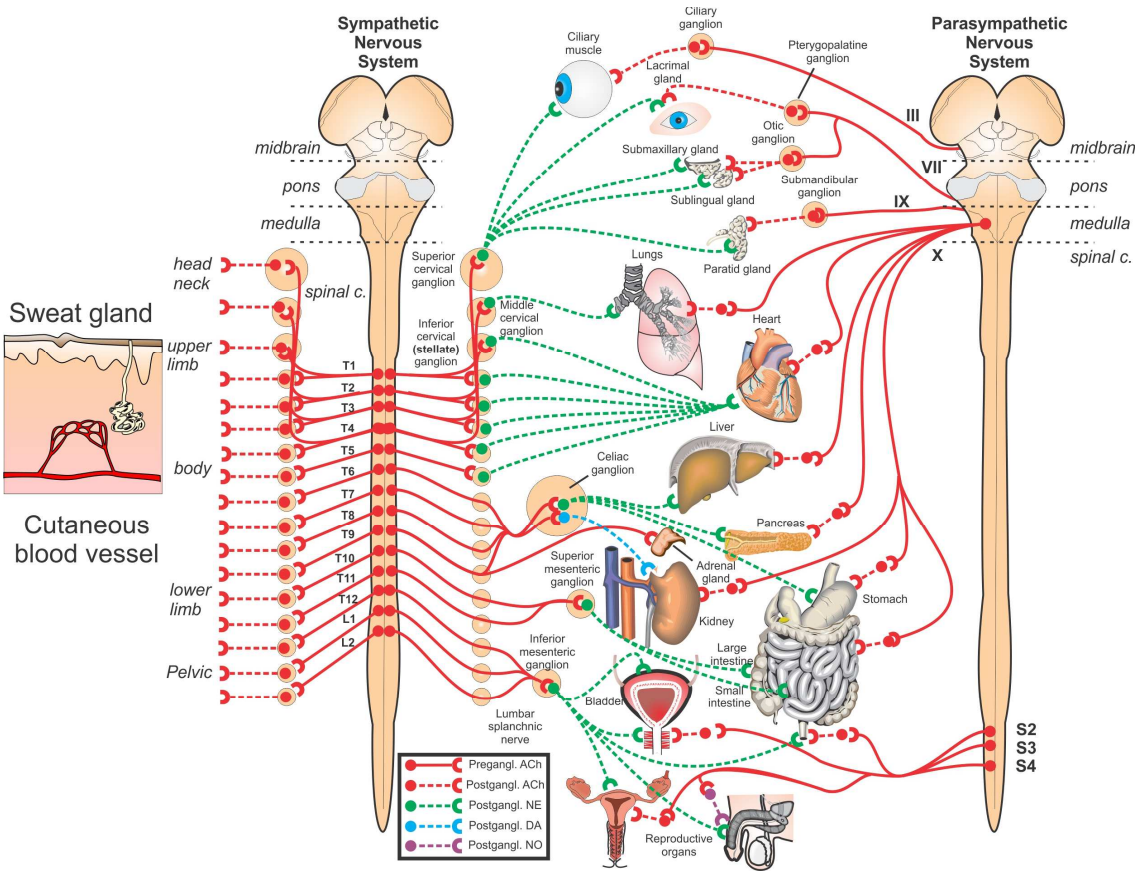


Figure 8. *Divisions and Neurochemicals of the Autonomic Nervous System*. The left side of the diagram indicates sympathetic innervation patterns and ganglia, which are predominantly preganglionic acetylcholine to postganglionic norepinephrine targets. An important exception to this pattern is observed in the cutaneous vasculature and sweat glands, where acetylcholine is also the postganglionic neurotransmitter and causes vasodilation and sweating (in concert with other co-transmitters and local transmitters, like nitric oxide). The parasympathetic nervous system also uses acetylcholine for preganglionic neurotransmission, but in this case, acetylcholine is also the predominant postganglionic neurotransmitter.

(Kronenberg et al., 1984). In this series of studies, assays of plasma norepinephrine (NE) and epinephrine (E) revealed increased E but decreased NE. Other groups (Casper et al., 1979; Lightman et al., 1981) measured these catecholamines during hot flashes and observed no changes, but may have missed changes due to less frequent sampling protocols. At odds with Kronenberg' findings, (Cignarelli et al., 1989) found a 100% increase in NE and unchanged E; however, according to Cutter et al., 1980 (as cited in Cignarelli), the levels of E obtained in Kronenberg's study were below physiological thresholds. Regardless of absolute physiological relevance, this does not account for the discrepancies observed in NE between several studies. Additional evidence of autonomic activation comes from assays of plasma levels of the NE metabolites 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid (VMA). MHPG is the brain metabolite of NE, and has been reported to increase significantly following hot flashes (Freedman, 1998). However, once MHPG is in the peripheral circulation, it can be converted to VMA, thus potentially making interpretation of the results unclear. Subsequent studies found that MHPG significantly increased following hot flashes, yet VMA levels were unchanged (Freedman, 1998). It is possible that the different patterns observed over many studies may be due to different 'subtypes' of hot flashes and/or individual variation that reflect differential activation of the autonomic nervous system. For example, Low and coworkers (2008) reported some women had decreases in blood pressure while others blood pressures remained constant, though all had tachycardia during hot flashes (Low et al., 2008).

3.4.2. Blood pressure

More global patterns of heightened autonomic activation in symptomatic women have been uncovered using ambulatory monitoring techniques. In 2004, James and coworkers studied 20 women for a 24-hour period using ambulatory blood pressure and hot flash monitors; women were stratified by current symptom status: symptomatic (hot flashes) during study, historically

symptomatic, and asymptomatic (James et al., 2004). The symptomatic during study group had significantly greater systolic blood pressures during work and sleep, and the heart rates of currently or previously symptomatic women were higher than the asymptomatic group. A larger 24-hour study of ambulatory blood pressure of women ages 18-65 found that those women endorsing hot flashes had significantly higher systolic awake and sleeping blood pressures (awake: 140.8 ± 2.5 vs 131.9 ± 1.7 mmHg for hot flashes vs no hot flashes; during sleep: 128.9 ± 2.8 vs 119.2 ± 2.0; $p = 0.004$ and $p = 0.007$, respectively). These findings maintained significance even when controlling for age, race/ethnicity, menopausal status, and conventional risk factors for hypertension, including smoking, alcohol, and caffeine consumption. A similar ambulatory study of 110 women with a range of hot flash experiences found that women with mild essential hypertension were more likely to have hot flashes, and there were no differences in age or menopausal status between the hypertensive and normotensive participants (Erkal et al., 2014).

3.4.3. Heart rate variability

Yet another autonomic-mediated parameter that has been studied in the context of hot flashes is heart rate variability (HRV). This measure reflects the beat-to-beat (R-R interval) or variability in instantaneous heart rate, and can be divided into two major components, low frequency and high frequency power. High-frequency bands, from 0.15-0.4 Hz, reflect parasympathetic control of the heart from the vagus nerve (cranial nerve X). Low-frequency power is reflected by bands from 0.04-0.15 Hz, and very low and ultra low frequencies are below that range. Low-frequency, very low, and ultra low frequencies reflect a mix of sympathetic and parasympathetic control of the heart and also the renin-angiotensin system; therefore, analyses regarding these components are not as clear. Several studies have documented changes in these components of heart rate variability in regards to hot flash symptomatology; in one study, women with more menopausal symptoms had increased low frequency to high frequency power, a measure that reflects the balance between the parasympathetic and

sympathetic arms of the ANS (Lee et al., 2011). Similarly, a study of 45 peri- and postmenopausal Japanese women found that those complaining of hot flashes and/or night sweats had decreased low frequency, high frequency, and total power (Akiyoshi et al., 2011).

In addition to generalized perturbations of HRV in symptomatic women, Hoikkala and Thurston and their respective colleagues both documented the change of HRV *during* hot flashes. The former group utilized ambulatory HRV monitoring and found very low and low frequency power increased while high frequency power decreased during hot flashes, indicating greater sympathetic activity and lessened parasympathetic activity (Hoikkala et al., 2010). Curiously, these changes did not differ by groups stratified by severity of hot flashes. Thurston's study found significantly decreased high frequency power during a hot flash (relative to pre- and post-flash periods) as well as an increase in the LF:HF ratio during both physiologically recorded and self-reported hot flashes (Thurston et al., 2010). A later ambulatory monitoring study confirmed this finding; additionally, they reported significant interactions with anxiety, age, and menopause status (Thurston et al., 2012). Collectively, these studies demonstrate that a perturbed autonomic nervous system (or the mechanisms that control the ANS) is likely essential to the mechanisms mediating hot flashes, and therefore understanding is a prerequisite to effective treatment strategies.

3.4.4. Autonomic neurochemistry: Cutaneous vasodilation and heat perception

Both cutaneous vasodilation and sweating (increased skin conductance) are objectively measurable indicators of hot flashes. However, their relative importance in producing the sensation of heat is differentiable, as cutaneous vasodilation will certainly produce a sensation of heat by activation of TRP receptors in the periphery. While sweating often accompanies hot flashes (the neurochemical mechanisms are similar, as elaborated below), sweating in the absence of vasodilation would produce a cooling sensation as long as the relative humidity is low. (In environments with low humidity and low ambient temperature, sweating *with* vasodilation could also cause a cooling sensation.)

The precise array of neurochemicals that mediate cutaneous vasodilation are still being elucidated. It is clear that human skin possesses an active vasodilator component; that is, vasodilation is achieved, in part, through activation of a separate acetylcholine system, not just withdrawal of a noradrenergic vasoconstriction system (Johnson et al., 1995). While cutaneous vasodilation is achieved by acetylcholine and unknown co-transmitters, including histamine, Calcitonin Gene Related Peptide (CGRP), NO, and Substance P, (for an excellent review, see [(Holowatz et al., 2010)], it was unclear if the menopausal hot flash possessed similar properties. As discussed already, hot flashes are autonomic events, and to address the sympathetic contribution to the flush, Low and coworkers (2011) performed a series of studies using botulinum toxin (Botox®) to selectively block cholinergic sympathetic nerves (Low et al., 2011). In these studies, botulinum toxin was used to block cholinergic neurotransmission in the forearm and forehead; hot flashes were induced in symptomatic women using mild heat stress (whole body warming using water-perfused suits). Both sites had identical attenuation of the hot flash as measured by cutaneous vascular conductance, a measure of blood flow that controls for blood pressure. A similar study demonstrated that blockade of NO synthesis using the compound L-arginine-N-methyl ester (L-NAME) attenuated the hot flash response (either spontaneous or evoked with mild heat stress in symptomatic women) (Hubing et al., 2010). Importantly, they demonstrated that skin sympathetic nerve activity (SSNA) increased four-fold immediately prior to the hot flash, and returned to baseline after its abatement (Low et al., 2011).

3.4.5. Autonomic Neurochemistry: Sweating and evaporative cooling

While not all hot flashes are accompanied by sweating, a brief overview of what is known about sweat physiology is covered here. As discussed earlier, sweating is essential to maintain a normal internal temperature, especially when ambient temperature is higher than skin temperature (and, therefore, thermoregulation cannot be achieved solely by releasing heat through vasodilation). Sweat, which is 99% water, contains various ions, including

sodium, chloride, calcium, potassium, and lactate, and some waste products (i.e. urea) and promotes cooling by the evaporation of water, releasing heat. Eccrine glands are the major type of sweat gland and are located across the entire body except for the lips (and penis in males) whereas apocrine glands are located in hairy areas, including axillary regions and the anogenital region. For humans, the apocrine glands are not a major contributor to thermoregulation, though they are important for some species. Lastly, there exists a mixed type of gland, the apoeccrine gland, which is also found in anogenital areas. It has long been recognized that vasodilation and sudomotor sweat responses often paralleled each other, suggesting similar molecules mediate both activities. Like vasodilation, sweating is achieved by cholinergic mechanisms and unknown co-transmitters that overlap with vasodilatory factors (including Substance P, NO, CGRP, and VIP) (Shibasaki and Crandall, 2010). Human sweat glands express several nicotinic and muscarinic receptors, including m1-m5, and α3, α7, α9, and β2 (Kurzen et al., 2004). Importantly, a variety of stimuli can elicit sweating, including increased ambient temperature, but also stress, anxiety, and other emotional states. There appears to be some dissociability between emotional and thermoregulatory sweating and this is discussed further in the commentary.

3.5. Neurochemical Involvement as indicated by non-hormonal treatments

Despite the relatively modest efficacy of extant non-hormonal therapies, these compounds have provided some evidence of key neurochemical systems that may be involved in the generation of hot flashes. Therefore, the following section discusses the neurochemical systems currently implicated in hot flash physiology, including norepinephrine, serotonin, and GABA. A graphical summary of selected hot flash drug intervention trials is presented in **Fig. 9**.

3.5.1. Norepinephrine

Norepinephrine, the classical sympathetic neurotransmitter underlying the fight or flight response, is perhaps the earliest and most well explored

neurochemical regarding menopausal flushing. Early studies demonstrated that gonadal removal in rodents (ovariectomy or orchidectomy) increased norepinephrine turnover in the brain, suggestive of increased catecholamine synthesis following gonadal steroid ablation (Anton-Tay and Wurtman, 1968). In the laboratory, clonidine, an α_2-adrenergic receptor agonist, has helped elucidate some clues as to the neurochemistry of hot flashes. Clonidine is thought to be relatively specific for presynaptic receptors in vasomotor centers of the brain; hence its use to lower blood pressure (for review, see (Slim et al., 2011)). Freedman and colleagues (1990) demonstrated that 1) yohimbine (an α_2-adrenergic *antagonist*) infusions precipitated hot flashes; 2) clonidine pretreatment increased the amount of time needed to provoke a hot flash in a warm room; and 3) clonidine blocked yohimbine-induced flushing in symptomatic menopausal women (Freedman et al., 1990). Furthermore, in a sample of symptomatic postmenopausal women, clonidine raised the sweating threshold, or the core body temperature set point in which sweating occurs (Freedman and Dinsay, 2000). While interventional studies have demonstrated significant reduction of hot flash frequency and severity, clonidine treatment presents many highly unpleasant side effects, including dry mouth, constipation, itchiness, and drowsiness, precluding it from a first-line non-hormonal vasomotor symptom therapy (Goldberg et al., 1994).

Alternatively, norepinephrine reuptake inhibitors are used off-label as a treatment for hot flashes. These molecules block the transport of norepinephrine across the plasma membrane back into the neuron, which allows for increased activity at their synaptic targets. Their efficacy, as typically measured by self-reported diaries, is significant, yet plagued by high placebo response rates (and often accompanied by underpowered sample sizes). As reviewed recently, both venlafaxine and desvenlafaxine have clinical efficacy, and many trial participants often elect to continue on the therapy after the trials end (Handley and Williams, 2015). A trial examining venlafaxine that used objective hot flash monitoring highlights the difference between objective and subjective monitoring in hot flash trials (which are almost entirely dependent on subjective instruments) (Carpenter

et al., 2007). Using ambulatory sternal skin conductance monitors, they observed a 22% decrease in hot flash frequency compared to a 0% decrease with placebo. Yet as gauged by diaries, hot flashes decreased 42% for the venlafaxine group, and for placebo, decreased 18%. Like clonidine, there are many side effects of NRIs, including dry mouth, decreased appetite, sleeplessness, nausea, and constipation. Additionally, NRIs may increase blood pressure, so their use must be carefully considered, and this is especially critical for menopausal women, for whom increases in blood pressure are common and associated with hot flashes (as described above).

3.5.2. Serotonin

3.5.2a. Selective Serotonin Reuptake Inhibitors

Several studies have suggested a prominent role for serotonin in relation to menopause. Serotonin is positively correlated with both plasma estrone and estradiol in healthy postmenopausal women (Guicheney et al., 1988) and probing the serotonergic system with the serotonin receptor agonist meta-cholorphenylpiperazine (m-CPP) in postmenopausal women revealed deficits that were ameliorated with estrogen treatment (Halbreich et al., 1995). Clinical studies have found significant decreases in serotonin levels in the blood in postmenopausal women, and treatment with estrogen restored these levels to values comparable to premenopausal women (Gonzales and Carrillo, 1993; Blum et al., 1996). Estrogen replacement also increased urinary 5-HIAA excretion, indicating increased serotonin turnover, following estrogen replacement in postmenopausal women (Lippert et al., 1996). Moreover, women with moderate or severe climacteric symptoms, as measured by the Kupperman Index, had significantly lower plasma 5-HT levels compared to women with only mild climacteric symptoms (Slopien et al., 2003).

Selective serotonin reuptake inhibitors (SSRIs) similarly block the reuptake process of serotonin at the synaptic cleft, thereby increasing the concentration of serotonin in the synapse and prolonging serotonin's effects. While initially used as a treatment for major depressive disorder, they have found

utility for a number of psychiatric conditions, including obsessive-compulsive disorder, panic disorder, and premenstrual dysphoric disorder (Stone et al., 2003). Initial research into SSRIs as a treatment strategy for hot flashes began with breast cancer patients unable to take hormone therapy but has since segued into the much larger population of women transitioning through menopause. Ultimately, this has led to the approval of low dose paroxetine as the only FDA-approved non-hormonal pharmacotherapy. Several trials have been conducted evaluating the efficacy of many drugs in this class, and, like norepinephrine reuptake inhibitors, the main outcome measures are typically reduction in hot flash frequency and severity, and sometimes a composite index of these measures. Handley & Williams (2013) reported that paroxetine, citalopram, and escitalopram are the most effective SSRIs for vasomotor symptoms. Two trials of fluoxetine (Loprinzi et al., 2002a; Suvanto-Luukkonen et al., 2005) had non-significant reductions in hot flash symptoms, and three trials describing the effectiveness of sertraline were underpowered (Gordon et al., 2006; Grady et al., 2007; Wu et al., 2009).

Paroxetine had three significant trials. Stearns and coworkers (2003) report 62% and 64% reductions in hot flashes compared to a 38% reduction with placebo (controlled release formulation), and, in a later report, a 40.6% and 51.7% reduction (10 mg and 20 mg, respectively) compared to a 13.7% and 51.7% reduction (Stearns et al., 2003a, 2005). While these data seem impressive, and are statistically significant, the use of percentages can be misleading, as the clinical benefit of the drug may not be impressive based on raw numbers. For example, Soares et al., (2008) found a statistically significant reduction in number of hot flashes per week, but in this trial, paroxetine reduced hot flashes by an average of 6.1 per week, while the placebo reduced hot flashes by 2.8 per week (Soares et al., 2008). In a systematic review and meta-analysis of trials of SSRIs for hot flashes, the overall reduction in hot flash frequency was approximately 10%, or about 1 hot flash a day in women experiencing ten or more hot flashes daily (Shams et al., 2014). In these types of trials, inclusion criteria are especially important, as some women may not be experiencing a

great enough and stable enough number of hot flashes to sufficiently gauge effectiveness of a medication. One approach to determining if a woman is significantly affected by hot flashes is to use lengthy (2-4 week) placebo-run in periods or diary measures prior to starting the test drug. Another consideration is the high attrition rates in many trials, which can be due to side effects of the treatment under study, ineffectiveness of the treatment, and other factors; in some trials reported in the Handley and Williams' analysis, attrition was in excess of 20%. This may represent an important source of variability that is lost in these studies.

3.5.2b. Tryptophan Manipulations

In addition to drug treatments, experimental studies have used tryptophan manipulations to study the role of serotonin in hot flashes. Tryptophan is the amino acid precursor of serotonin, and is biotransformed first by tryptophan hydroxylase (TPH) into 5-hydroxytryptophan (5-HTP), which is then decarboxylated by aromatic L-amino-acid decarboxylase into 5-hydroxytryptamine/serotonin (5-HT). TPH is unique in that it is not usually saturated, so increased consumption of tryptophan will increase serotonin synthesis (Young and Gauthier, 1981). To that end, two studies have explored whether dietary alterations of tryptophan can influence hot flashes. In a study of 27 breast cancer survivors, ingesting a concentrated amino acid beverage to *deplete* (outcompete at the amino acid transporters into the brain) tryptophan did not change objective or subjective hot flash frequency or self-reported intensity or bother (Carpenter et al., 2009). It could be that these women already had a low level of serotonin that this depletion paradigm was unable to further aggravate the pathology. The authors also suggest that there may not be any differences at baseline, non-stressed conditions or without any type of challenge. Another small study utilized the opposite approach, tryptophan loading, to study objectively measured hot flashes in 24 symptomatic postmenopausal women (Freedman, 2010). Study participants took a 50 mg capsule of 5-HTP three times a day, and there were no significant differences in hot flash frequency at

the end of the three-week study period. This study should be interpreted cautiously, as the sample was small and the doses used were relatively low, and no data is shown regarding the effect of the loading on overall 5-HT tissue content (i.e. blood levels). It could be that much higher doses are required observe an effect. Unfortunately, with the relatively poor quality of such studies, making concrete conclusions is troublesome, and these should not be interpreted as a lack of a role for serotonin.

3.5.3. GABA

γ-Aminobutyric acid (GABA) is the dominant fast inhibitory neurotransmitter in the central nervous system; yet regarding hot flashes, it has not received much attention. GABA mediates its cellular effects through a variety of receptors, including heteropentameric, ligand-gated chloride channels ($GABA_A$ receptors) and G-protein coupled receptors ($GABA_B$ receptors). The particular subunit composition of a $GABA_A$ receptor determines its affinity for GABA, and estrogen levels affect the transcription of $GABA_A$ receptor subunits (Herbison and Fénelon, 1995). Specifically, these changes were demonstrated in the BNST, an anxiety- and panic-associated brain region. Adding further complication to the relationship between hormones and GABA is that $GABA_A$ receptors have been shown to be sensitive to changes in progesterone or its metabolite, alloprenanolone [as reviewed by (Lovick, 2006)]. In animals, this leads to hyperexcitability in the periaqueductal gray, which is a key site in the generation of severe anxiety (e.g. panic). This link between altered GABAergic inhibition around the time of decreasing hormone levels may provide a clue as to the origins of certain premenstrual symptoms [which can include hot flashes (Hahn et al., 1998)].

As mentioned earlier, gabapentin, a structural analog of GABA, is used with some efficacy for hot flashes. However, its mechanism of action has been elusive and does not simply increase GABA concentration by virtue of its structure. It is thought to involve regulation of calcium channels and neurotransmitter release, as it binds to the $\alpha_2\delta$ subunit of voltage-dependent/L-

type calcium channels (Gee et al., 1996) and inhibits calcium currents in rat neurons from the neocortex, striatum, and globus pallidus (Stefani et al., 1998). Interestingly, Cai and coworkers (2012) report using 7-Tesla Magnetic Resonance Spectroscopy (MRS) to quantify glutamate and GABA levels in the brain before and after a 900 mg oral gabapentin [a common dose for use in treating hot flashes] challenge in 11 healthy men; GABA levels were significantly increased while glutamate levels were unchanged following the challenge (Cai et al., 2012). However, it is unclear how this is occurring mechanistically. It is possible that some of the effectiveness of gabapentin as a hot flash therapy may be due to its anxiolytic effects. In a study of 420 breast cancer patients, 300 and 900 mg doses of gabapentin were more effective than placebo at reducing state anxiety scores at 4 and 8 weeks (Lavigne JE et al., 2012).

Despite the aforementioned clinical studies demonstrating some efficacy a potential GABA-enhancing drug, more extensive investigation into GABA-centered therapies, such as benzodiazepines, is almost nonexistent. A search for “benzodiazepine hot flash(es)” or “benzodiazepine hot flush” yielded 0 directly relevant results in PubMed on 2/15/2016. These compounds are widely known to allosterically modulate the $GABA_A$ receptor to increase the flow of chloride into the channel when GABA binds, thus hyperpolarizing the membrane. A sole report in the literature demonstrated some efficacy in reducing climacteric symptoms following oöphorectomy in 30 women using the benzodiazepine oxazepam compared to the β-adrenergic antagonist propranolol (double-blind, cross-over design) (Erkkola et al., 1973). It is unclear if any newer drugs in this class, such as including alprazolam or lorazepam, have any effect on hot flashes, but considering their use as anxiolytics, and the afore-described relationship between anxiety symptoms and hot flashes, it seems likely that they may have some efficacy.

Overall, the physiological understanding of hot flashes is relatively limited. Major clues to the pathology and neurochemical systems mediating hot flashes come from non-hormonal treatments and implicate norepinephrine, serotonin, and GABA (potentially). These treatments are quite limited in efficacy, however,

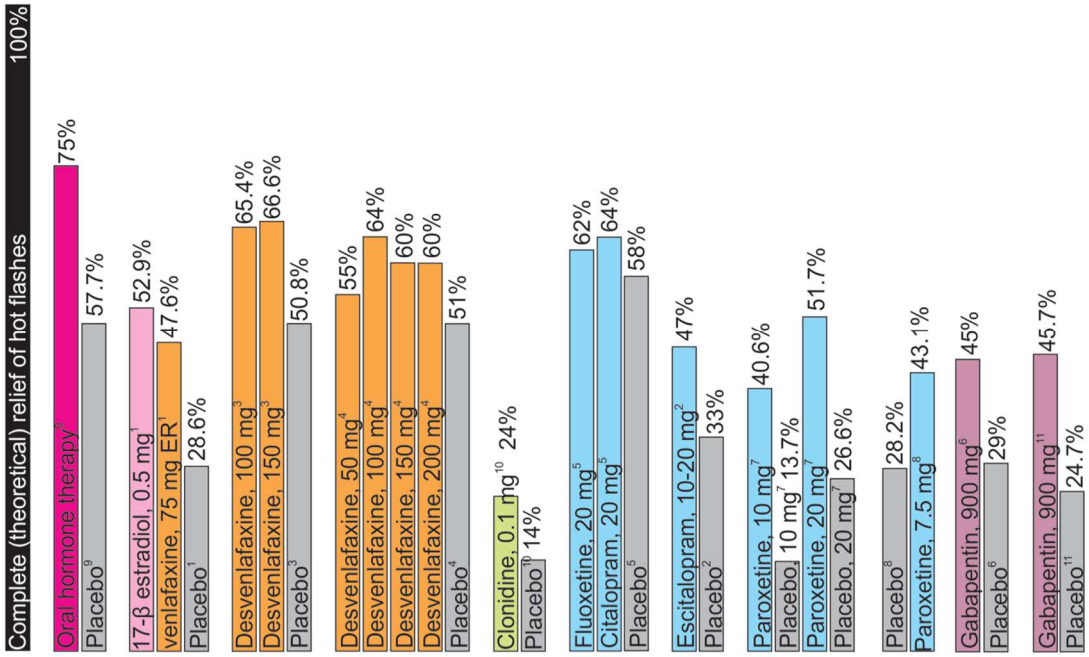


Figure 9. *Reduction in Hot Flash Frequency by Study*. Black bar represents theoretical maximal hot flash reduction compared to data from individual studies. The oral hormone therapy trial (bright pink bar) represents data from a meta-analysis of hormone therapy, whereas other groupings are individual clinical trials. Pink bars represent hormone trials, orange bars represent serotonin-norepinephrine reuptake inhibitors, green bar represents clonidine, blue bars represent selective serotonin reuptake inhibitors, purple bars represent gabapentin, and gray bars represent placebo results for each trial. Percentages at the top of the bar indicate reduction in hot flash frequency (diary measure). Note the strong placebo response and wide variability between studies.

[1]Joffe et al., 2014 *JAMA Internal Medicine*; [2]Freeman et al., 2011 *JAMA*; [3]Archer et al., 2009 *American Journal of Obstetrics & Gynecology*; [4]Speroff et al., 2008 *Obstetrics & Gynecology*; [5]Suvanto-Luukonen et al., 2005 *Menopause*; [6]Guttuso et al., 2003 *Obstetrics & Gynecology*; [7]Stearns et al., 2005 *Journal of Clinical Oncology*; [8]Simon et al., 2013 *Menopause*; [9]MacLennan et al., 2004 *Cochrane Database*; [10]Pandya et al., 2000 *Annals of Internal Medicine*; [11]Butt et al., 2008 *Menopause*.

so building hypotheses concerning the mechanisms of hot flashes around them should be undertaken with caution.

Part IV: Animal Modeling

4.1. Animal Modeling of Hot Flashes

Hot flashes are undeniably complex phenomena that arise in the wake of dramatic reduction of the ubiquitous hormone, estrogen. While clinical investigation has attempted to elucidate processes and mechanisms that lead to them, preclinical investigation can also contribute. Now that the features of a hot flash, associated neurochemical events, and risk factors have been described, this section will focus on the development of a preclinical line of research that may contribute to the understanding of hot flash generation and treatment strategies.

4.1.1. Establishing the validity of an animal model

4.1.1a. Traditional criteria

A full validation of an animal model is a difficult accomplishment, but is critical to maximizing its translational potential. In psychiatry, the most oft-cited criteria for validity are the tenets set forth by Willner: face, construct, and predictive validity (Willner, 1984). He enumerated five subcriteria within each category to evaluate an animal model. Generally, face validity refers to the extent that a model recapitulates ("looks like") the phenotype of the condition/disorder being studied, and is often the easiest (and sometimes only) accomplishment of a model. Secondly, construct validity is concerned with the extent that the underlying mechanisms of the animal model replicate the biology of the human condition; that is, do the same processes (i.e. oophorectomy or removal of the ovaries) result in the same or at least similar disease in both the model and human. Lastly, predictive validity is the model's capacity to accurately indicate treatment or progression as the result of a *novel* intervention that is mirrored in the clinical condition (i.e., can this model be used to find new treatments for the disease state or condition it represents).

4.1.1b. Updated criteria

The current emphasis on translational research, either beside-to-bench or bench-to-bedside, spurred an elaboration and update to Willner's criteria (Belzung and Lemoine, 2011). They propose five criteria: 1) homological; 2) pathogenic; 3) mechanistic; 4) face; and 5) predictive validity. Essentially, homologic validity reflects the appropriateness of the species and strain for the condition under consideration; pathogenic validity considers the role of ontological or vulnerability mechanisms (for example, early life stress or environmental insults) and "triggers"; and mechanistic validity mirrors construct validity. Face validity is relatively unchanged but incorporates both ethological relevance and biomarkers. Ethological relevance is an important consideration in anxiety testing and is elaborated upon below. Lastly, predictive validity includes both induction and remission validity, or the relationship between the precipitating factor and treatment, respectively, in both species. In addition to predictive validity, postdictive validity is another feature to consider (Griebel and Holmes, 2013). In contrast with Willner's predictive validity concept, which in the strictest interpretation is not immediately useful until a treatment is tested in a clinically relevant population, postdictive validity can more readily confirm the validity of a model, as it considers how *established* treatments for a condition (i.e. benzodiazepines for anxiety or estrogen replacement for hot flashes) impact the behavior or biology of the animal model. It should be noted that predictive validity has often been used the way postdictive validity has been defined, so caution is warranted in using these terms.

As for the validity of certain tests to assess physiology or behavior, similar rules apply. They should reflect relevant behaviors of the species (i.e. thigmotaxis, or wall-hugging behavior exhibited by rodents) and sensitivity to known influential factors, such as stress, or developmental abilities of the animal.

4.2. Induction of a Menopausal State

4.2.1. Ovariectomy

With validity in mind, then, how is the best way to study menopause in a preclinical species? The most obvious approach could be to mimic what causes menopause in most women—aging. However, even without respect to laboratory constraints (i.e., time and costs involved in aging animals) this is not the most feasible option. Rats do not go through a menopause *per se*; they may become acyclic or in constant estrus, but their estrogen levels never reach those as equivalently low to a postmenopausal woman; therefore, naturalistic models are extremely limited and could result in a relatively mild or inconsistent phenotype. Rapidly inducing a surgical menopausal state in a rodent is a straightforward procedure by bilaterally removing the ovaries. This model has been used for many decades in a variety of fields studying many aspects of menopause, and is the preferred model of choice in bone research studying osteoporosis, for example (Lelovas et al., 2008). Certainly, ovariectomy models a small percentage of menopausal women and cannot recapitulate every menopausal experience; the precipitous loss of ovarian function in this case also comes with loss of other hormones. With regards to oöphorectomized women, their experiences are often more dramatic and severe, especially with respect to vasomotor symptoms and anxiety (Taylor, 2001; Rocca et al., 2008). Almost all women who undergo oöphorectomy experience acute vasomotor symptoms, even those that had no vasomotor symptoms prior to the oophorectomy (Erkkola et al., 1973; Kronenberg, 1990). Therefore, inducing a robust phenotype may provide more consistent abnormalities, which could be desirable for treatment studies (with the assumption that the phenotype produced by ovariectomy is not excessively severe).

4.2.2. Chemical approaches

Another approach that has been developed more recently is the use of 4-vinyl cyclohexane diepoxide (VCD) to model the perimenopausal stage

specifically. This compound is an ovotoxin that selectively kills follicular cells over a period of weeks (for review, see (Kappeler and Hoyer, 2012)). Unlike ovariectomy, it recapitulates the fluctuating hormones observed in this transition period rather than abruptly removing all endocrine factors in the ovarian tissue. Follicle stimulating hormone levels rise, like naturally aging women, but high doses create significant mortality (Frye et al., 2012). Use of this compound requires additional regulatory approval, as the bedding is considered a biohazard. Tamoxifen or other estrogen receptor modulators or aromatase inhibitors can also be used in healthy animals to induce a pharmacological menopause. Yet, these methods may also be less reliable than ovariectomy insofar as producing a dramatic phenotype (see section 1.3.2.).

4.3. Modeling of the Hot Flash in Rodents

4.3.1. Role of the tail as a heat loss organ

Due to the obviously furred (and therefore highly insulated) nature of the rat, heat dissipation occurs in a restricted area—the tail. Owing to this difference in furriness, the tail is the major organ of heat loss in the rat, and while it takes up only a small fraction of the rat's volume, it accounts for approximately 15% of the total surface area. Its highly vascularized skin has the same specialized vessel structures called arteriovenous anastomoses (AVA) as human skin. These non-nutritive capillary beds formed at the interface of arteries and veins permit a rapid flow of blood, and heat is lost to the surroundings through convection. Rats without tails are unable to thermoregulate properly and have prolonged responses to pharmacological manipulations that increase core body temperature (e.g. injection of β-adrenergic receptor agonist isoproterenol) (Spiers et al., 1981). The following section will summarize the current knowledge of neurochemicals that regulate blood flow and tail skin temperature, discuss the relevance of cycling hormones in the rat, and existing models of hot flashes in rats.

4.3.2. Neurochemical control of tail skin temperature

4.3.2a. Acetylcholine

Despite the anatomical differences between heat exchange appendages in rats and humans, there is some evidence that the underlying mechanisms are conserved. The neurochemicals that control the flow of blood and concomitant temperature of the tail skin include the classical autonomic neurotransmitters acetylcholine (ACh) and norepinephrine, though the former's involvement has been controversial. In 1978, Cox and Lee reported dose-dependently increased tail skin temperature (1-2 °C) and decreased core body temperature (~1-1.5 °C) in rats by systemic intraperitoneal (i.p.) injections of oxotremorine, a muscarinic cholinergic agonist (Cox and Lee, 1978). These changes are similar to the changes observed by those who have attempted to model hot flashes (see below). However, these manipulations could ultimately be indirectly stimulating norepinephrine via the preganglionic acetylcholinergic innervation of postganglionic norepinephrine mechanisms controlling vasodilation. Reports of studies utilizing isolated segments of the tail artery are telling. In isolated rat arteries pre-constricted with phenylephrine (which stimulates arterial contraction through noradrenergic (α_1) mechanisms), acetylcholine and the muscarinic receptor agonist pilocarpine both induced endothelium-dependent, dose-dependent relaxation of isolated rat artery, which was subsequently confirmed by another group (Tonta et al., 1994; Geary et al., 1998). In 2006, Peuler & Phelps found that acetycholinesterase inhibitors (neostigmine and metrifonate) enhanced the relaxation response of isolated tail artery segments to acetylcholine (Peuler and Phelps, 2006). Isolated tail artery segments from ovariectomized rats had significantly impaired relaxation compared to sham controls, at the highest dose of acetylcholine (Shuto et al., 2011).

4.3.2b. Inhibiting presynaptic norepinephrine autoregulation

Not surprisingly, pharmacological stimulation of noradrenergic targets has also indicated an essential role for this neurotransmitter in the regulation of tail skin temperature and blood flow. Redfern and coworkers (1995) performed a

series of studies to delineate the roles of various adrenergic receptors. The α_2 receptors are primarily presynaptically localized, and when stimulated, inhibit release of norepinephrine; therefore, antagonizing these receptors would increase norepinephrine release (Redfern et al., 1995). Several α_2 antagonists caused profound increases in tail skin temperature, including delequamine (RS-15385-197), yohimbine, and idazoxan, all given at doses of 1 mg/kg. Delequamine and yohimbine elicited approximately 9 °C increases while idazoxan had a milder effect, increasing TST about 4°C. The α_2 agonist clonidine caused a small but significant increase in TST yet also a profound drop in CBT (which seems at odds with its potential therapeutic value, though that is quite low). These results parallel induction of hot flashes in symptomatic women with yohimbine and its ability to be blocked by clonidine (Freedman et al., 1990; Freedman, 1998). This should be interpreted cautiously, as these drugs are capable of having effects at both central and peripheral receptors, and it is currently unknown which of these are necessary for inducing hot flashes.

4.3.3. Role of steroid hormones in temperature regulation in the rat

4.3.3a. Relationship of temperature and estrogen status

Rats tail skin temperature varies across the estrous cycle. Williams and coworkers (2010) demonstrated a distinct diurnal pattern of TST in freely-moving animals that it was lower (~6 °C) in the active/dark phase and higher in the inactive/light phase (Williams et al., 2010). Across the estrus cycle, it was only significantly changed (decreased compared to other nights) on the night of proestrus, when the rat would be ovulating, and both estrogen and progesterone levels are high. A second study demonstrated that ovariectomy abolished this phase-dependent decrease in TST, and consequently TST was elevated during the active phase. The ovariectomy-induced increase in TST is consistent with many other groups reports (Kobayashi et al., 2000; Berendsen et al., 2001; Pan et al., 2001; Opas et al., 2004). The increase in TST appears to be fairly acute to the removal of the ovaries; Berendsen and colleagues observed a 6 °C increase three days after surgery and Kobayashi and coworkers found elevated TST

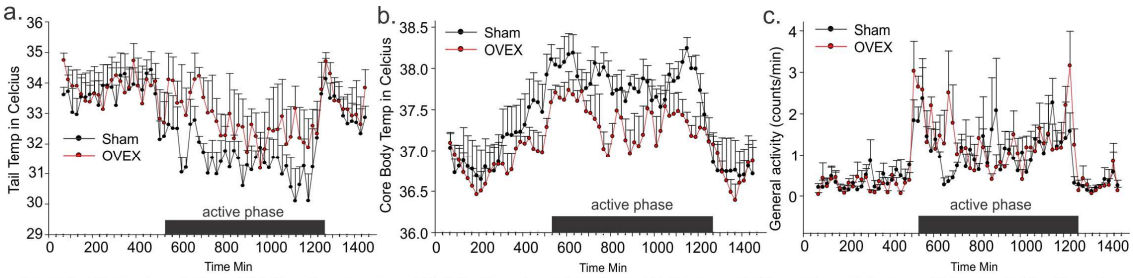


Figure 2 - Effects of ovariectomy (OVEX, n=4) versus sham-OVEX (n=4) on a) core body temp; b) tail temp; and c) locomotor activity over a 24 hour period (n=4/group). Lines represent means +/- SEM. Dark bar on x-axis represents dark cycle (active phase).

Figure 10. *Diurnal Variation in Temperature Between Ovariectomized (OVEX) and Intact (Sham) Controls.* Line graphs with error bars (SEM) depicts **a)** differences in tail skin temperature between OVEX and sham rats that occur in the active phase only; **b)** Core body temperature between OVEX and sham rats; **c)** Activity as a function of phase. Increased tail skin temperature of OVEX rats is not due to increased activity during the active phase.

within two weeks. It is also relatively long-lasting, as Kobayashi observed elevated TST from 2--7 weeks after surgery, yet not permanent, as they also found that it was not different from sham-ovariectomized rats from 8--20 weeks after surgery. Intriguingly, despite the increase in TST, in most cases, core body temperature (CBT) was unaffected; Kobayashi found elevated rectal temperature from 8--20 weeks. I also evaluated core and tail skin temperature alongside activity levels using implanted radiotelemetry probes and found similar results. Over a several day period of continuous monitoring, I found an active-phase dependent elevation in tail skin temperature only in ovariectomized rats that was not due to increased activity (activity levels were the same in both groups), consistent with these investigators. This increase in TST after ovariectomy suggests that these rats may be vulnerable to stimuli or conditions that may produce a hot flash-associated increase in TST (heat dissipation response), but does not replicate the episodic nature of the hot flash as described by symptomatic women. This state-dependent elevation of TST has been used to investigate novel therapies by examining the ability of a compound or set of conditions to restore the active phase decrease in TST. While studying this marker may be valuable, it is perhaps not the ideal model of a “spontaneous” hot flash.

4.3.3b. Utility of variation in tail skin temperature as a hot flash model

With respect to the aforedescribed approach to modeling, there is only one report of any “spontaneous” fluctuations in TST that mimic the episodic nature of hot flashes. Simpkins observed “pulses” of TST increases in a subgroup (9 of 20) intact but anestrus or constant estrus aged rats, yet these fluctuations were abolished by ovariectomy (Simpkins, 1984). The implications of this particular study are at odds with the larger literature, and is likely an unsuitable modeling approach.

4.4. Existing Neurochemical Modeling of Hot Flashes

An alternative approach to modeling hot flash-related increases in tail skin temperature could include administering an agent that reliably provokes a hot flash in a vulnerable (i.e. ovariectomized) rat rather than attempting to find restoration of normal TST. Such a probe would represent a threshold difference, which contrasts with the ambient temperature-induced flushing paradigm, wherein the responses were similar between surgical and estrogen treatments. Likewise, a model of this type should also be responsive to estrogen treatment, indicating postdictive validity. Similarly, these substances would also trigger hot flashes in symptomatic or menopausal women, but not asymptomatic or non-menopausal women, or at least to a lesser degree (providing postdictive validity). A provocation that reliably triggers a hot flash could then potentially be used in screening for novel therapies. The clinical research community has long recognized that a valid animal model of hot flashes is necessary for further mechanistic understanding of flushing to guide target development for therapeutics (see a summary of an NIH workshop on the state of the science of vasomotor symptoms (Miller and Li, 2004a)). As mentioned previously, inducing a menopausal state is relatively easy, but it is how to induce a flush in a valid manner that has proven elusive. To date, there are only two trigger-based models in the literature, and only one with relatively extensive publications. The first, and oldest, uses the opioid antagonist naloxone to trigger a hot flash, and the second manipulates norepinephrine with yohimbine.

4.4.1. Opioid antagonist model

In 1983, the first animal model of a menopause-associated hot flash was proposed (Simpkins et al., 1983). Based on observations that morphine withdrawal syndrome is similar to a menopausal hot flash (that is, persons complain of intense sensations of heat and chills and tachycardia), Simpkins and colleagues administered morphine (subcutaneous pellets) sufficient to produce dependence in female rats. At baseline, they observed a significant CBT

elevation of 0.81 °C. Following the addiction paradigm, they infused naloxone, an opioid receptor antagonist, and observed an increase in TST, from ~27 to 33 °C, and a resulting hypothermia from ~38.5 to 35 °C. Naloxone also caused a nine-fold increase in LH within 10 minutes, and a mild tachycardia—two additional signs frequently observed in hot flashes. Naloxone administration had no effect in animals implanted with placebo pellets. A second set of experiments used ovariectomized females following two weeks' recovery, and they report, "The response to NAL treatment was nearly identical, therefore all subsequent experiments employed OVX rats." The data is not compared directly or shown, but based on this statement; it seems that ovariectomy does not confer any *vulnerability* to naloxone-precipitated withdrawal (it does not add construct validity). Subsequent studies demonstrated that the response to this addiction-withdrawal paradigm is quite similar between male and female rats (Katovich et al., 1986). No differences in the response to naloxone withdrawal were evident between intact and castrated male rats. The only subtle differences between sexes were a less pronounced hypothermia in males (only ~1 °C). This set of experiments also measured foot temperature and found that it both increased and recovered faster that the tail.

Despite the evidence that gonadal steroids do not appear to be of any particular relevance to the withdrawal response generated by naloxone, chronic estrogen replacement (0.5 mg pellet of 17-β estradiol) in ovariectomized rats did attenuate the magnitude of the response to naloxone following morphine addiction by ~3 °C (Katovich and O'Meara, 1987), thus apparently providing the model with postdictive validity. No benefit of progesterone-only replacement was demonstrated, and progesterone did not augment estrogen-only treatment. A subsequent dose-response experiment utilized unopposed estrogen pellets (0.1, 0.5, 5, 15, and 50 mg) prior to morphine addiction. Basal rectal temperature was only significantly changed (decreased) in the two highest treatment groups. While the tail skin temperature increase to naloxone was again similarly attenuated, there was no dose-dependency. This may be due to the extremely high dose pellets used: 0.1, 0.5, 5, 15, and 50 mg. Post-mortem assessments of

the plasma estrogen levels in the rats revealed supraphysiological levels, ranging from 163.5 ± 28.8 pg/mL (0.1 mg pellet) to 36740 ± 10721.8 pg/mL (50 mg pellet). These levels are far in excess of what has been reported in the literature for rat levels of estradiol, which are typically somewhat elevated compared to the human; reports range from 36-88 pg/mL for an adult female rat in proestrus (the day of the estrus cycle when estradiol levels peak) (Overpeck et al., 1978).

Unfortunately, the evidence against this model is of the most damning type: clinical contradictions. If it truly replicates a menopause-associated hot flash, then administering naloxone to menopausal women should elicit flushing. DeFazio and colleagues (1984) tested this question by administering either saline or naloxone to 16 postmenopausal women with hot flashes in a crossover design and found naloxone "did not change the rate of objectively measured hot flashes, mean serum LH or FSH levels, or the frequencies or amplitudes of gonadotropin pulses" (DeFazio et al., 1984). Charney and Heninger (1986) found absolutely no effect of naloxone infusion (30 mg) in both healthy male and female subjects on hot *or* cold flushes (Charney and Heninger, 1986). Interestingly, these studies stand in contrast to Lightman's work wherein 6 menopausal women had *decreased* hot flashes and LH pulses during intravenous naloxone infusion (Lightman et al., 1981). In all cases, the effect of naloxone certainly does not elicit flushing, even when considering the small sample sizes of these studies.

4.4.2. Yohimbine model

The other animal model of hot flashes provokes them by administration of the α_2 adrenergic antagonist yohimbine. By blocking both presynaptic and postsynaptic norepinephrine receptors, yohimbine increases release of norepinephrine, and is a known anxiogenic drug in the clinic, and produces hot and cold flushes in patients with severe anxiety but not healthy controls (Charney et al., 1984; Charney and Heninger, 1986). Morimoto and colleagues (2011) demonstrated a differential flushing response and subsequent decrease in core body temperature between ovariectomized and sham-ovariectomized rats at a 3

mg/kg (s.c.) dose that was rescued with estradiol replacement (Morimoto et al., 2011). This flush was milder in magnitude (2 °C) but greater in duration than the flushing observed in the morphine-naloxone model, perhaps reflecting the route of administration. In an initial study, I replicated these results and found that ovariectomized rats have a greater response than sham-ovariectomized rats. While I used the same dose, I gave i.p. injections and observed a higher amplitude flush that occurred faster, likely resulting from faster and greater absorption of the drug.

While this model may be an improvement over naloxone-mediated opioid withdrawal, as menopausal women experience hot flashes in response to yohimbine (Freedman et al., 1990) [which is attenuated with estrogen replacement (Freedman and Blacker, 2002)], the mechanisms are not understood. Yohimbine increases norepinephrine, but increasing norepinephrine synaptically by using norepinephrine reuptake inhibitors provides relief from hot flashes (see **Fig. 9**). Therefore, in the major work presented here (**Ch. 2**), I used a provocation that was better understood neurochemically. Briefly, FG-7142 is a partial inverse agonist at the $GABA_A$ receptor, and existing literature has delineated the neurochemical sites in which it elicits cellular activity [based on c-fos responses; see (Singewald and Sharp, 2000; Singewald et al., 2003)]. Furthermore, our group has mapped where c-fos responses are able to be attenuated with pretreatment with an orexin-1 receptor antagonist (Johnson et al., 2012a), which proved helpful in analyses of neurochemical circuits in ovariectomized rats (**Ch. 2, Figs. 18 and 19**).

4.4.3. Emotional trigger of a hot flash?

As elaborated in Part III, clinically significant symptoms of anxiety are present as a consequence of the menopausal transition and appear to have a close relationship with vasomotor symptoms. Additionally, Freeman and coworkers (2005) found that anxiety was the best predictor of the frequency and severity of hot flashes (Freeman et al., 2005). Anxiety and hot flashes were directly related such that women with moderate anxiety were three times more

likely to report hot flashes, while women with high anxiety were five times more likely to report hot flashes. Interestingly, anxiety preceded the development of hot flashes in this cohort. However, as correlational studies, these findings naturally lead to the proposition: Can anxiety-provoking ("anxiogenic") stimuli elicit hot flashes in vulnerable species, such as ovariectomized rats? Would such a model be better suited to meet criteria for validity? Increased anxiety-like behavior following ovariectomy has been well-documented, in several different behavioral paradigms, such as the elevated plus maze and passive avoidance task (Mora et al., 1996; Frye and Walf, 2004; Koss et al., 2004). Prior work in this laboratory replicated these results, finding increased time spent in the 'safe' zones of an open field test (OFT) after ovariectomy (see **Ch. 2, Fig. 21**). Estrogen replacement was highly anxiolytic in this paradigm, suggesting that estrogen plays a key role in anxiety, again replicating prior work.

4.4.4. Similarities between menopausal symptoms and panic attacks

Previous work in this laboratory largely focused on the neural substrates of anxiety and panic-associated behavior and physiology using animal models. Clinically, it is well-established that the majority of patients with Panic Disorder (PD), but not healthy controls, will experience a panic attack to a mild interoceptive stimulus, such as an intravenous infusion of 0.5 M sodium lactate (NaLac) or brief inhalations of high concentrations of carbon dioxide (CO_2) (for review, please see (Johnson et al., 2014)). While fear of dying or losing control is the prototypical cognitive symptom of a panic attack, a wide range of physical symptoms can constitute a panic attack (4 needed of 9 possible symptoms, including the cognitive symptoms listed above, though a cognitive symptom is not a requirement). One of these symptoms is strong thermal sensations--hot flashes and chills. Interestingly, PD has a strong gender discrepancy such that women are at least twice as likely to develop PD as men. Anxiety plays a strong role in PD; anticipatory anxiety is one of the most consistent symptoms in clinical studies. Moreover, the treatment strategies (use of SSRIs and SNRIs) are identical in many cases, as are the neural circuits involved (amygdala,

a.

Brain Regions of interest	Estrogen receptors α	Estrogen receptors β	Thermo-Regulatory	Polysynaptic innervation of tail	Implicated in sleep regulation	Implicated in mood regulation
Hypothalamic nuclei						
PeF	●	●	●	●	●	●
PVN	●	●	●	●	○	○
mPOA	●	●	●	●	○	○
Limbic System						
MeA	●	●	○	○	○	●
BLA	●	○	○	○	○	●
CeA	○	○	○	○	○	●
BNST	○	○	○	●	○	●
Serotonergic Systems						
DRN/MRN	○	○	○	○	●	●
RPa/LPGi	○	○	●	●	○	○
Noradrenergic Systems						
LC	●	○	●	●	●	●

Table 2. *Key Regions of Interest with Respect to Menopausal Symptoms.* Rows represent an area of interest. Columns indicate criteria for relevance of a site to hot flashes—expression of estrogen receptors, involvement in thermoregulation, whether an area innervates the tail, if the area is implicated in sleep regulation, and if the area is implicated in mood regulation. Filled circles represent that the area fulfills that criteria whereas open circles indicate that it does not. The best candidates for areas involved in menopausal symptoms are the perifornical area of the hypothalamus (PeF) and locus ceruleus (LC). Abbreviations: perfornical area of the hypothalamus (PeF), paraventricular nucleus of the hypothalamus (PVN), median preoptic area (mPOA), medial amygdala (MeA), basolateral amydala (BLA), central amygdala (CeA), bed nucleus of the stria terminalis (BNST), dorsal raphe nucleus (DRN), median raphe nucleus (MRN), raphe pallidus (RPa), LPGi, and locus ceruleus (LC).

hypothalamus, insula), and associated neurochemical systems (serotonergic and noradrenergic). Key brain areas of interest and relevance to menopausal symptoms (that are also implicated in panic) are detailed in **Table 2**, and prominent panic-generating systems also meet many criteria for involvement in hot flashes and other menopausal symptoms. Comparisons between these conditions were well-described in a review article (Hanisch et al., 2008). However, direct, non-correlational evidence for the role of anxiety-provoking stimuli in hot flashes is lacking.

Our laboratory has a long history of using telemetric probes implanted in the abdominal cavity to measure the autonomic/physiological components of a panic-associated response to various anxiogenic or panicogenic stimuli. Aside from measuring heart rate, blood pressure, and locomotor activity, these probes also measure temperature. Some stimuli/provocations used to elicit panic responses in vulnerable rodents also caused a profound drop in core body temperature. Tail skin temperature was never measured in these studies, yet the weight of the evidence strongly suggested activation of a heat dissipation response. Considering the prominence of anxiety in women in the menopausal transition, similarities between hot flashes and panic attack symptoms, and the self-reported factors to hot flashes, it seemed that this might be a feasible method of inducing a hot flash-like response. Therefore, preliminary studies were conducted to verify that these stimuli were causing hot flash-associated increases in tail skin temperature (**Ch. 1**), and to explore the possibility that these stimuli may provide a useful and novel means of modeling hot flashes.

4.4.5. Criteria for a validated animal model of a hot flash

A fully validated animal model of hot flashes would meet the following criteria: 1a) rapid increase in tail skin temperature following clinically-relevant provocation agent and 1b) subsequent decrease in core temperature (fulfilling face validity); 2) vulnerability (differential or exaggerated response) to provocation following induction of a menopausal state (meeting construct validity); 3) amelioration of the tail skin temperature increase with systemic

estrogen replacement (post-dictive validity); and 4) ability to identify new therapies that are ultimately successful in preventing or reducing hot flashes in symptomatic women (predictive validity).

4.4.6. Goals of the Dissertation

The overarching goals of the dissertation are to develop a valid animal model of hot flashes—specifically, one that shows a vulnerability to clinically-relevant provocation agents following a treatment that causes or simulates menopause. The model will then be used to elucidate the neural circuitry and neurochemicals that mediate the hot flash-associated response, to 1) increase basic knowledge of the circuitry and associated neurochemicals that mediate the response and 2) potentially find novel, non-hormonal targets for treatments that are safe for not only naturally menopausal women but also breast cancer patients. Ultimately, if successful, the model will be used to screen for novel targets for treatment for hot flashes.

Materials and Methods

Animals and Housing Conditions

Adult male (300-350 g) or female (225-250 g) Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN USA) and housed individually in plastic cages under standard housing conditions (maintained at 22 °C) for at least 5 days prior to experimental procedures. Rats with heterozygous expression of the serotonin transporter ($SERT^{+/-}$) were purchased from Charles River Laboratories (Wilmington, MA USA) and bred in-house on a Wistar background. These rats possess a null mutation of the SERT generated by *N*-methyl-*N*-nitrosurea-mutation as described previously (Homberg et al., 2007). Female Wistar rats (225-250 g) were purchased (Harlan) as wildtype controls due to insufficient numbers of littermates in relevant experiments. Rats had *ad libitum* access to food and water and were maintained on a 12:12 light/dark cycle (lights on at 0700h). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011) and the guidelines of the Indiana University-Purdue University, Indianapolis Institutional Animal Care and Use Committee.

Surgical Procedures

Ovariectomy (OVEX) and Sham OVEX Surgeries

All surgical procedures were conducted under sterile conditions. Rats were anesthetized under isoflurane delivered through a nose cone (2-3% by volume MGX Research Machine, Vetamac, Rossvile, IN dissolved in medical air, Praxair, Indianapolis, IN). Animals were checked for corneal and paw-withdrawal reflexes to ensure adequate anesthesia before commencing surgical procedures. The skin was shaved on each side between the ribcage and hindlimbs and sterilized with iodine solution. A small (1-2 cm) incision was made to expose the lateral muscle wall, which was then opened and the ovaries were visualized. The ovaries are a prominent, spherical, reddish-pink structure that are readily seen

from the incision site. In the event that the ovaries were not readily visible, a cotton swab was used to carefully move overlying tissue to expose them. For OVEX rats, after visualization, the ovary was gently pulled up and the fallopian tube was clamped 3-5 mm under the ovary with a hemostat. A length of suture was used to tie off the tube under the clamp, and the ovary was removed with a scalpel. The tissue was inspected for bleeding and re-tied if necessary. Silk sutures were used to close the muscle wall and the skin was closed with surgical staples. The entire procedure was then repeated on the other side. For sham operated control rats, after ovary visualization, the muscle wall and skin were closed as for OVEX rats and procedure repeated on the other side. Following surgery, all rats were given pain medication (buprenorphine, Indiana University School of Medicine Laboratory Animal Resources) and monitored until consciousness was restored. Animals then recovered for the next 12 days in the animal housing facilities, and were monitored daily.

Radiotelemetry Probe Implantation

Rats were anesthetized under isoflurane (2-3% by volume, MGX Research Machine, Vetamac, Rossville, IN USA dissolved in medical air; Praxair Inc., Indianapolis, IN) under sterile conditions. The ventral skin was shaved and an incision made of approximately 2 cm. The muscle wall was opened and a radiotelemetry probe with an internal thermistor (model C50PXT or HD-S11, Data Sciences International, St. Paul, MN USA) was fitted into the peritoneal cavity. The probe contains a perorated rib to affix it in place, and non-dissolving silk suture was used to secure the probe. The skin was stapled, rats were given pain medication, and were monitored until consciousness was restored. Animals then recovered for at least 5 days in the animal housing facilities and were monitored daily.

Assessments of temperature

Methodology

Two methods of temperature assessment were performed. For measurement of core body temperature, data acquisition was accomplished using DSI DataQuest® software in continuous sampling mode. For assessments of tail skin temperature, a thermistor (Omega Precision Fine Wire Thermocouples, part no. 5SRTC-TT-K-30-36; Omega Engineering, Stamford, CT USA) was secured 1 cm from the tail base on the ventral side using 3M Durapore® medical tape. The thermistor was attached to a T-type pod and connected to a Powerlab data acquisition system running LabChart software (ADInstruments, Colorado Springs, CO USA) for continuous monitoring of temperature. As an additional verification of temperature change, infrared thermal images were acquired using a FLIR T440 thermal imaging camera (FLIR Systems, Boston, MA USA) with standard settings taken at a height of 1 meter. Analysis of images was performed using FLIR Tools to standardize scaling between images.

Calibration

Radiotelemetry probes from Data Sciences International are calibrated by the manufacturer prior to implantation. Thermistors were measured against air temperature (recorded on a digital thermometer) prior to commencing experimentation and readings were always within ±0.5 ºC. Thermistors were replaced with each series of experiments or more frequently if necessary. To determine with greater precision any differential between thermistors and changing temperature, a calibration experiment was performed. Thermistors were placed in a 700 mL water bath at 21 ºC and temperature was warmed to 40 ºC over a twenty minute period. Measurements were taken every 30 seconds with a glass thermometer at the same depth of the thermistors. Averages were computed and there was no difference between thermistor temperature and temperature measured with the thermometer; p=0.45 as shown in **Fig. 11.**

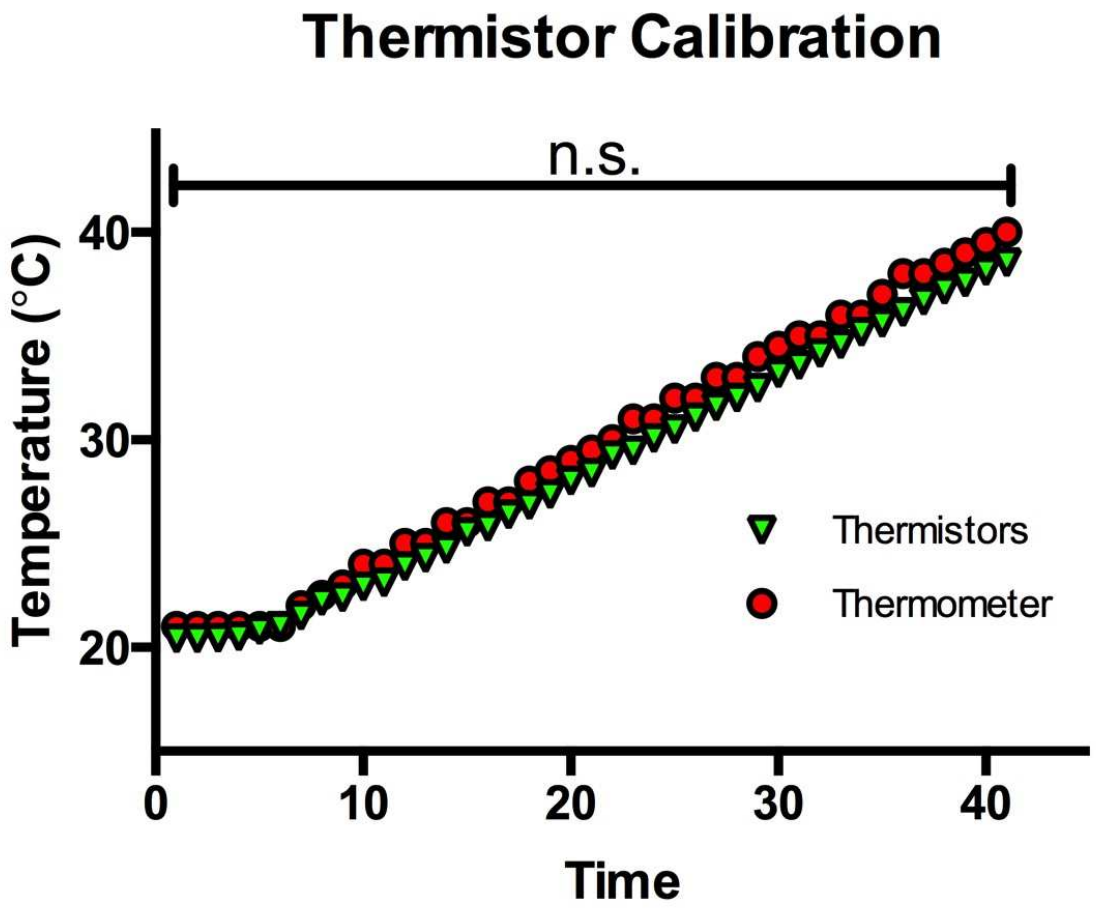


Figure 11. *Accuracy of Thermal Data Acquisition*. Calibration of thermistors reveals no difference between measured temperature and thermometer readings.

Provocations

General Protocol

All experiments were performed in a temperature-controlled laboratory at 20-22 °C. Animals were handled daily to reduce the novelty of the experimenter and environment. Four plastic cages were set up and cleaned with 10% Coverage Plus prior to testing for each rat. Cages were free of bedding and other debris. Rats were weighed and placed into the cages and then either thermistors were taped to the ventral part tail (using 3M Durapore® tape) and/or radiotelemetry probes were activated and recording commenced. Baselines had to be stable (less than 0.5 °C fluctuation) for a minimum of 10 minutes prior to injections (for both pre-treatments and hot flash provocations). Pre-treatment drug kinetics determined the waiting period between pretreatment and hot flash provocation, but in all cases a stable baseline had to be re-obtained if it changed after handling. After hot flash provocations, recordings continued for a minimum one hour (for all pharmacological provocations).

Pharmacological Challenges in Male Rats

Once a stable baseline was established for TST, rats were injected (intraperitoneally) with either vehicle or one the following compounds: FG-7142, a benzodiazepine receptor partial inverse agonist (7.5 mg/kg dissolved in 10% DMSO/90% ddH_2O); yohimbine, α_2 adrenoceptor antagonist (5 mg/kg dissolved in 10% DMSO/90% ddH_2O); or d-fenfluramine (10 mg/kg dissolved in 0.9% saline). Separate groups of animals were used for each drug treatment. A counterbalanced, crossover design was utilized such that all rats received both vehicle and panicogenic drug with 48h between treatments. All reagents were purchased from Sigma Aldrich, St. Louis, MO USA. A second experiment was performed identical to the first, except CBT was assessed in a separate cohort of rats. I took this approach to first determine what changes were occurring in the tail (i.e., to determine if we were successfully inducing a heat dissipation response) and then measured internal temperature.

CO_2 Challenge

Flow cages (12 in. width x 12 in. height x 24 in. length) were custom-built using Plexiglas®. When the lid of the cage was latched, gases could only enter the cage through an inlet connector (for the gas infusion) and could only exit the cage through an outlet connector. The gas flow into the cages was controlled using a 2-stage regulator (Praxair, Inc., Danbury, CT, USA) at a pressure of 0.6 Bar. We previously validated the consistency of the rate of CO_2 delivery using state-of-the-art infrared CO_2 ($ProCO_2$) and electrochemical O_2 ($ProO_2$) sensors (Johnson et al., 2005). Specifically, concentrations of O_2 remain at 21% throughout the gas infusion in the control and experimental cages [see (Johnson et al., 2005)]. The CO_2 concentration remains constant at < 1 % in the control cage during exposure of rats to atmospheric air (< 1% CO_2 / 21% O_2/ 79% N_2). Infusion of the premixed normoxic, hypercarbic gas (20% CO_2 / 21% O_2/ 59% N_2) results in a rapid increase in CO_2 concentration from < 1% CO_2 up to 20% CO_2 at the 5 min time point. After terminating gas infusion and opening the cages, the concentration of CO_2 rapidly decreases from 20% CO_2 to < 2.5% CO_2 during the following 5 min. Using a portable iSTAT gas analyzer (HESKA, Des Moines IA) we have also determined that this hypercapnic, normoxic challenge leads to arterial pCO_2/pH levels of ~130mmHg/7.01 during the challenge that are back to normal physiological range (~50mmHg/7.37) within 2 min post challenge (unpublished data).

Atmospheric air was infused from the start of the experimental session. After obtaining a stable baseline TST in atmospheric air, rats were exposed to hypercapnic or atmospheric gas for 5 min in a counter-balanced manner, and then atmospheric air was infused again for 10-15 min following the challenge (for male rats in **Ch. 1** and female rats in **Ch. 3**). A crossover design was utilized such that all rats received either 20% CO_2 or atmospheric air with 48h between treatments. A second experiment, using a separate cohort of rats, was performed identical to the first, except that CBT was measured (for male rats in **Ch. 1**).

Atmospheric Air Control Experiment

To determine if the experimental environment alone caused a change in tail skin temperature, the above experiment was performed, but only atmospheric air was infused for the duration of the experiment (**Ch. 3**). After obtaining a stable baseline, the air was turned off and immediately turned back on to mimic the gas change during a CO_2 infusion. Rats recovered in atmospheric air for 10 min following the challenge.

Thermal Imaging of the Tail

In order to determine if the increase in tail skin temperature was occurring along the entire length of the tail, hot flash provocations (pharmacological and CO_2) were repeated in male rats. In this case, thermal images were acquired at baseline and after hot flash provocation and closely followed the timeline of the results in **Ch. 1** (i.e., selecting when to image based on the peak response time). This experiment was done in a small number of animals and requires replication prior to publication.

Subthreshold Panicogenic Drug Effect on TST of OVEX Rats ± Estrogen

Twelve days post surgeries, OVEX rats received daily subcutaneous (s.c.) injections of either sesame oil vehicle (0.2 ml, sham-OVEX received this as well) or 17-β estradiol (0.25 mg/kg in 10% DMSO/90% sesame oil; Sigma Aldrich, St. Louis MO) for 5 days prior to testing. Once a baseline was established for TST, rats were injected with either vehicle or a low dose of the FG-7142 (3 mg/kg, i.p., dissolved in 10% DMSO/90% ultrapure H_2O). The experiment utilized a counterbalanced, crossover design such that all rats received either vehicle or panicogenic drug with 48 hours between treatments.

Benzodiazepine Pretreatment Effect on TST of OVEX Rats

OVEX rats were pretreated with either the benzodiazepine alprazolam (3 mg/kg, i.p., dissolved in 10% DMSO/90% ddH_2O) or vehicle. This is a dose we

have previously shown to be anxiolytic (Johnson et al., 2015). Once a baseline was established for TST, rats were injected with FG-7142 (3 mg/kg, i.p.).

Subthreshold Panicogenic Drug Effect on TST and CBT in OVEX Rats

Once baselines for TST and CBT were established, OVEX rats were injected with either vehicle or FG-7142 (3 mg/kg, i.p.). The experiment utilized a counterbalanced, crossover design such that all rats received both FG-7142 and vehicle with 48 hours between treatments.

Neurochemical System Cellular Responses in OVEX Rats treated with a Panicogenic Drug

OVEX and sham-OVEX rats were injected with either vehicle or a low dose of FG-7142 (1 mg/kg, i.p.). Ninety minutes post-injection, rats were anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). Brains were removed, post-fixed in the same fixative for 12 h, rinsed twice in PB (12 h), and placed in 0.1 M PB containing 30% sucrose for 12 h. Brains were blocked using a standard adult rat brain matrix (model RBM-4000C, ASI Instruments, Warren, MI) and frozen using liquid isopentane cooled on dry ice. Serial coronal sections (30 μm) were cut using a Leica freezing microtome and were immediately placed in cryoprotectant consisting of 27% ethylene glycol and 16% glycerol in 0.05 M PB to yield six alternative sets of sections. Sections were stored at -20 °C until immunohistochemical processing. All solutions used had pH level of 7.4.

Immunohistochemical staining for c-Fos, in the presence or absence of other neurochemical markers, was used to identify specific brain regions involved in responses to the anxiogenic drug. Immunostaining of nuclear c-Fos expression is a useful method of identifying functional cellular responses to anxiety-related stimuli (Johnson et al., 2012a). Four of the six alternate sets of 30 μm coronal brain sections were immunostained, one set each for c-Fos (full brain), or c-Fos (day 1) then on day 2 the tissue was immunostained with the cytoplasmically expressed *OXA* (hypothalamus); *tyrosine hydroxylase* (TH,

brainstem), or *tryptophan hydroxylase* (TPH, brainstem). Immunostaining of OXA, TH, and TPH were done to identify orexin, noradrenergic, and serotonergic neurons, respectively. Specifically, free-floating sections were washed in 0.05 M PBS for 30 min, then incubated in 1% H_2O_2 in PBS for 20 min. Sections were then washed 10 min in PBS and 20 min in PBS with 0.3% Triton X-100 (PBST). Sections were then incubated 12-16 hr in PBST with the primary antibody solution at room temperature [i.e., day 1: c-Fos (rabbit anti-c-Fos polyclonal antibody, cat. no. sc-52, Santa Cruz Biotech., Santa Cruz, CA; diluted 1:10,000); day 2: OXA (rabbit anti-OXA-polyclonal, affinity-purified antibody, cat. no. H-003-30, Phoenix Pharmaceuticals, Burlingame, CA; diluted 1:8,000); TH (rabbit anti-TH polyclonal affinity-purified antibody cat. no. AB152, Millipore, Billerica, MA; diluted 1:800; or TPH (sheep anti-TPH polyclonal affinity-purified antibody, cat. no. 9260-2505, AbD Serotec; diluted 1:1,000 for midbrain/pons and 1:2000 for medulla)]. After a 30 min wash in PBST, sections were incubated 2 hr in the appropriate secondary antibody: biotinylated goat anti-rabbit IgG (c-Fos, OXA, TH; cat no. BA-1000, Vector Laboratories, Burlingame, CA; diluted 1:200), biotinylated rabbit anti-sheep IgG (TPH; cat. no BA-6000; Vector Laboratories, 1:200 dilution). Sections were washed again for 30 min in PBST then incubated 1.5 hr in an avidin-biotin complex provided in a standard Vector Elite kit (cat no. PK-6100, Vector Laboratories; diluted 1:500). Substrates for chromagen reactions were SG (c-Fos; SK-4700, Vector Laboratories) or 0.01% 3,3'-diaminobenzidine tetrahydrochloride (DAB; OXA, TH; TPH) (D-5637, Sigma, St. Louis MO, USA) in PBS containing 0.003% H_2O_2, pH 7.4. Substrate reactions were run for ~10-15 min. All sections were mounted in 0.1% gelatin dissolved in ultrapure water on glass slides, dried overnight, dehydrated and mounted with coverslips using DPX mounting medium (BDH Laboratory Supplies, Poole, U.K.). All washes and incubations were done in 12 well polystyrene plates with low frequency shaking on an orbital shaker.

Orexin, Glutamate, GABA gene expression in the PeF of OVEX Rats

Ovariectomized and sham-ovariectomized rats were anaesthetized under isoflurane and rapidly decapitated. Brains were removed and flash frozen in isopentane precooled with dry ice then stored at -80 °C until processing. The PeF region was bilaterally dissected from two consecutive 300 µm coronal sections and tissue was processed for absolute qRT-PCR for Orexin mRNA using methods previously described (Johnson et al., 2010), or for glutamate and GABA-related genes using the custom-designed TaqMan Low Density Array (TLDA) as previously described (Truitt et al., 2015). Orexin gene expression analysis was normalized to beta-actin as previously described (Johnson et al., 2010). The glutamate and GABA-related gene expression panel was normalized using geNorm approach as previously described in detail (Truitt et al., 2015).

Behavioral Testing Following Ovariectomy

SB-334867

Following 12 days, OVEX and sham-OVEX rats were injected i.p. with a control vehicle [0.2 ml/100 g volume dimethyl sulfoxide (DMSO)] or a 30 mg/kg dose of the single OX1R antagonist (SORA1) SB334867 (cat. no. 1960, Tocris Bioscience, Bristol, UK, in 0.2 ml/100 g volume DMSO) which has selectivity of 50X for the OX1 receptor compared to the OX2 receptor; occupies 100% of central OX1Rs within 30 min post systemic injection (Bonaventure et al., 2015); and attenuates FG-7142 (7.5 mg/kg, i.p.) induced anxiety-like behavior and panic-associated cardioexcitatory responses without inducing somnolence (Johnson et al., 2012a). Therefore, we tested rats in an accepted test of anxiety-associated behaviors (i.e., open field test which was videorecorded and scored by Anymaze software, Stoelting, Woods Dale, IL) 60 min following injections. The experiment utilized a crossover design such that all rats received both vehicle and SORA1 with 48 hours between treatments.

Estradiol

Following 12 days, OVEX rats received daily subcutaneous injections of either sesame oil vehicle (0.2 ml, sham-OVEX received this as well) or 17-β estradiol (0.25 mg/kg) for 5 days prior to testing in the open field.

Effects of Orexin Receptor Antagonists on TST Responses to a Subthreshold Panicogenic Drug in OVEX Rats

Following 12 days, all OVEX rats were pretreated systemically with highly selective and centrally active OXR antagonists (details for each experiment follow) prior to receiving an injection of FG-7142 (3 mg/kg, i.p.). These experiments utilized a counterbalanced, crossover design such that all rats received both vehicle or an OXR antagonist with 48 hours between treatments. Five days prior to experiments, rats were handled for 5 min and trained with either i.p or s.c injections or mock gavages. Tail skin temperature was assessed as previously described.

Dual Orexin Receptor Antagonist (DORA; DORA-12)

On the experimental day, rats received an oral gavage of a control vehicle (0.2 ml/100 g volume 20% vitamin E/TPGS) or 30 mg/kg of a dual OXR antagonist, DORA-12 (Merck & Co.) with balanced potency for OX1R and OX2R; good brain exposure; 47% oral bioavailability; and a favorable brain to plasma ratio of 0.4-0.6. The 30 mg/kg p.o. dose of DORA-12 being used here achieves a plasma Cmax of 2.02 μM with CSF exposure of 66 nM and *ex vivo* occupancy of 97%, and has been shown to promote sleep in rats (Gotter et al., 2013).

Single Orexin-1 Receptor Antagonist (SORA1; SB-334867)

On the experimental day, rats received an i.p. injection of a control vehicle (0.2 ml/100 g volume DMSO) or a 30 mg/kg dose of SB-334867 i.p. Following this experiment, concerns about SB-334867 were raised, including hydrolytic instability (McElhinny et al., 2012) and target specificity. In a binding panel of

170 enzymes, transporters, and receptors, SB-334867 was found to have significant activity at monoaminergic targets, including the $5HT_{2B}$ and $5HT_{2C}$ receptors; adenosine A2A and A3 receptors; and monoamine, norepinephrine, and adenosine transporters (Winrow et al., 2012). Consequently, to ensure that the effects I observed were due to antagonizing the orexin-1 receptor, I repeated this experiment with a more selective drug, the SORA1 Compound 56 described below.

Single Orexin-1 Receptor Antagonist (SORA1; Compound 56).

All rats were injected s.c. with a control vehicle (0.2 ml/100 g volume DMSO) or the highly selective SORA1, Compound 56 (10 mg/kg, dissolved in 30% SBE-β-cyclodextrin/70% ddH_2O supplied by Janssen Research & Development, LLC., La Jolla, CA, which has 44X selectivity for the OX1R compared to the OX2R, and the 10 mg/kg systemic dose occupies ~90% of central OX1Rs within 30 min (Bonaventure et al., 2015). While SB-334867 has been shown to have off-target affinities for non-OXRs, in a binding assay panel of 50 receptors, ion channels, and transporters, Compound 56 did not exhibit a significant affinity to anything other than the OX1R (Bonaventure et al., 2015).

Single Orexin 2 Receptor Antagonist (SORA2; JNJ10397049)

All rats were injected s.c. with a control vehicle (0.2 ml of 10% pharmasolve, 5% solutol, and 85% dextrose in water) or a dose of JnJ10397049 (10 or 30 mg/kg, Tocris Bioscience, Bristol, UK, in 0.2 ml) which has selectivity of 630X for the OX2R compared to the OX1R and the 30 mg/kg systemic dose occupies ~80% of central OX2Rs within 30 min (Dugovic et al., 2009).

Single Orexin 2 Receptor Antagonist (SORA2, TCS OX2 29)

All rats were injected i.p. with a control vehicle (0.9% saline) or TCS OX2 29 (30 mg/kg, Tocris Bioscience, in 0.2 ml), a SORA2 that displays >250-fold selectivity for OX2Rs over OX1Rs (Bonaventure et al., 2015).

Statistical Analyses

Dependent variables for analyses of tail skin temperature (TST) were analyzed using a one-way ANOVA with repeated measures, using drug treatment or gas infusion as the between-subjects factor and time as a within-subjects factor. In the presence of significant main effects or main effect x time interactions, Fisher's Least Significant Difference (LSD) test was used for post hoc testing between-subjects comparisons. Analyses of behavior (SORA1), and TST responses in females were analyzed using an ANOVA with drug treatments and/or surgical treatments as main factors (i.e., independent variables) and time as a repeated measure. In the presence of a significant drug effect or drug x time interaction, an ANOVA was run at each time point in combination with a Fisher's LSD test post hoc test to detect within subject differences. Analyses of OXA gene expression and behavior (estrogen) were performed with an independent two-tailed t-test or a nonparametric Mann-Whitney Rank Sum Test U if unequal variance was detected with a Shapiro-Wilk normality test $p<0.05$. Expression of each glutamate/GABA-related gene was calculated relative to the sham treatment group using the delta Ct method. These values were then converted to $\log_{10}$ ($\log_{10}(\Delta\Delta Ct)$) for statistical analyses with an ANOVA and Fisher's LSD for post hoc testing. Analyses of cell counts were analyzed with an ANOVA with drug treatment and surgical treatments as main factors and a Tukey's post hoc test. Levene's Test of Equality of Error Variance was also done to determine equal variances in the groups to determine if nonparametric testing was needed. The alpha level was set at 0.05 in all cases. All statistical analyses were carried out using SPSS 21.0 (SPSS Inc., Chicago, IL, USA), and all graphs were generated using SigmaPlot 12.0 (SPSS Inc.) and an illustration program (CorelDraw X5, Ontario, Canada).

Chapter 1: Select Panicogenic Drugs and Stimuli Induce Increases in Tail Skin Temperature and Decreases in Core Body Temperature

Panic attacks (PA) are discrete episodes of intense fear or discomfort, and are accompanied by a variety of somatic and psychological sensations. Isolated PAs (without diagnosis of Panic Disorder and/or agoraphobia) occur relatively frequently, estimated at 22.7% [US National Comorbidity Survey Replication, a nationally representative sample of 9282 participants (Kessler et al., 2006)]. According to the *Diagnostic and Statistical Manual of Mental Disorders*, (DSM-IV), a PA must produce at least any 4 of 13 symptoms: racing or pounding heart/palpitations, chest pain, trembling/shaking, dizziness, sweating, dyspnea (feeling of suffocation), choking sensation, paresthesias (tingling), nausea, flushing or chills, fear of dying, fear of losing control, and derealization or depersonalization (Craske et al., 2010). While cardiac symptoms (e.g., racing heart) are the most commonly reported event in 86.1-97% of cases (Cox et al.; Brown et al., 1995), all of the symptoms are relatively common and overall endorsed in 36.4-96.6% of panic attacks (Ietsugu et al., 2007). As may be expected, some symptoms are regarded with more gravity than others; for example, patients presenting to emergency centers with (potentially life-threatening) chest pain are given priority. However, other symptoms, such as paresthesias or choking, are associated with more intense (self-reported) panic attack severity (Ietsugu et al., 2007). While thermal sensations (hot flashes or chills) are not commonly thought of as a symptom of a panic attack, they are a reliable marker of a panic attack of moderate (as compared to mild or intense) severity (Ietsugu et al., 2007) and are present in about 43% of panic attacks [study of 1276 panic attacks from 94 patients over a 6-12 week period (de Beurs et al., 1994)].

Animal models of panic-associated physiology and behavioral responses have been employed for decades in an attempt to understand neurochemical mechanisms underlying adaptive and maladaptive panic in order to identify new treatment strategies. Recapitulating the physiological signs associated with

somatic symptoms of panic attacks in animals is possible by administering clinically-relevant, panic-provoking pharmacological agents (i.e. yohimbine) or interoceptive challenges (such as carbon dioxide exposure) [for review, see (Johnson et al., 2014)]. Physiological assessments of panic in animals reveal cardiovascular activation (increased heart rate and pressor responses) and behavioral flight-associated increases in locomotion in response to these same challenges and are similarly sensitive to panicolytic treatments like benzodiazepines (Bhattacharya et al., 1997; Johnson et al., 2015b). Though some symptoms may be unmeasurable in rodents (i.e., nausea or tingling sensations), thermal changes are objectively measurable yet underexplored in the context of panic.

Panic is an adaptive response to cope with an imminent threat where cardiovascular and respiratory responses are mobilized to initiate an optimal fight or flight behavioral response, but heat dissipation is also an important adaptive aspect of panic. The rat tail serves in part to regulate internal temperature (Stricker and Hainsworth, 1971; Spiers et al., 1981), and increases in temperature dissipate heat to the surroundings due to its highly vascularized nature and lack of insulation. Therefore, increases in tail skin temperature (TST) may reflect a hot flush/flash or heat sensation. We hypothesized that threshold panicogenic challenges would cause strong heat dissipation responses and resulting decreases in internal temperature. Therefore, in the following series of studies, we test the ability of a diverse array of panicogenic challenges to elicit CBT and TST changes: FG-7142 (partial inverse $GABA_A$ receptor agonist), yohimbine (α_2 adrenoceptor antagonist), d-fenfluramine (serotonin agonist), and 20% CO_2 exposure (to induce acidosis). All of these challenges have previously been reported to elicit panic-associated behavioral and/or physiology at or below the doses we used; please see (File and Pellow, 1984; Baldwin et al., 1989; Johnson et al., 2012c, 2012d, 2015a).

Results

Pharmacological Challenges

Panicogenic Drugs Elicited Increases in Tail Skin Temperature

An ANOVA with drug as main factor and time as a repeated measures (also protected by post hoc testing) revealed that systemically injecting male rats with panicogenic doses of FG-7142 (n=5,6), yohimbine (n=8/group) and d-fenfluramine (n=5/group) produced a marked 7-9 °C increases in TST [**Fig. 12a-c, left panel**]; Representative thermal imaging; [**Fig. 14a-d**] - FG-7142: *treatment x time interaction* $F_{(69,821)}$=13.3, p<0.001; yohimbine: *treatment x time interaction* $F_{(65,900)}$=11.3, p<0.001; d-fenfluramine: *treatment x time interaction* $F_{(70,560)}$=12.5, p<0.001]. One vehicle-treated rat was removed from FG-7142 experiment due to a high baseline temperature.

Panicogenic Drugs Elicited Decreases in Core Body Temperature.

Systemically injecting male rats with panicogenic doses of FG-7142 (n=12/group), yohimbine (n=7,8) and d-fenfluramine (n=5/group) led to a decrease in CBT [**Fig. 12a-c, right panel,** - FG-7142: *treatment x time interaction* $F_{(69,1518)}$=10.1, p<0.001; yohimbine: *treatment x time interaction* $F_{(65,845)}$=11.3, p<0.001; d-fenfluramine: *treatment x time interaction* $F_{(70,560)}$=41.3, p<0.001]. One yohimbine-treated rat was removed from the experiment due to a faulty radiotelemetry probe.

Interoceptive Challenge

Panicogenic Hypercapnic Gas Elicited Increases in Tail Skin Temperature and Decreases in Core Body Temperature

An ANOVA with gas as main factor and time as a repeated measures (also protected by post hoc testing) revealed that exposing male rats to 20% CO_2 (normal O_2) compared to atmospheric air (n=8/group) produced a marked 7-9 °C increases in TST and ~1 °C decrease in core body temperature [**Fig. 13a-b**– 20% CO_2: *treatment x time interaction* $F_{(19,247)}$=13.7, p<0.001; **Fig. 13c** *treatment*

x time interaction $F_{(19,247)}$=22.2, p<0.001. Representative thermal imaging; **Fig. 14e**].

Discussion

In a series of studies using a neurochemically diverse array of panicogenic challenges (affecting serotonergic, GABAergic, and noradrenergic systems, among others), we demonstrate rapid, high amplitude (7-9˚C) increases in tail skin temperature followed by substantial (1-3 ˚C) decreases in core body temperature. These results provide face validity and support our hypothesis that thermal changes occur during panic-associated challenges in rodents to help dissipate heat during periods of intense arousal and activity and increased metabolism. To the best of our knowledge, these are novel findings, though previous reports had demonstrated temperature perturbations with d-fenfluramine at high doses or under non-thermoneutral conditions [see review by (Clark and Clark, 1980) and (Subramanian and Vollmer, 2004)]. Importantly, all of the provocations used herein--20% CO_2, d-fenfluramine, FG-7142, and yohimbine are known to induce severe anxiety or panic symptoms in healthy persons or precipitate panic attacks in persons with Panic Disorder [as reviewed by (Bourin et al., 1998) and see also (Dorow et al., 1983; Charney et al., 1984; Woods et al., 1988; Targum and Marshall, 1989; Kaye et al., 2004)], providing postdictive validity in this modeling approach. Furthermore, prior studies have demonstrated that these provocations also elicit strong self-reported thermal sensations in humans, providing additional face validity to the results that we observed (Dorow et al., 1983; Charney et al., 1984; Freedman et al., 1990; Kaye et al., 2004). Future studies will determine the efficacy of established panicolytic treatments on thermal changes (both core and tail) relative to other panic symptoms, such as cardiovascular profile or behavioral outputs.

Thermal imaging affords a noninvasive tool to quickly assess tail skin temperature and could augment existing experimental protocols relatively easily. Thermal imaging has recently been used as the exclusive measure of tail skin

temperature, and this method has the inherent advantage of capturing multiple sites along the tail at once (Luong and Carrive, 2012). This method could be useful in determining a greater range of panicogenic or panicolytic properties of novel compounds.

The diversity of neurochemical mechanisms that produced similar thermal changes implicates common underlying neural sites/circuits. Indeed, all of the panicogenic pharmacological or CO_2 challenges we administered have been shown to increase cellular activity (e.g., c-fos immunoreactivity) in critical panic-generating brain areas such as the perifornical/dorsomedial hypothalamic nuclei (PeF/DMN) and dorsal periaqueductal gray (DPAG) (Singewald and Sharp, 2000; Singewald et al., 2003; Johnson et al., 2011, 2012a). Specifically, stimulation of the posterior hypothalamus and adjacent areas (including the DMN/PeF) in humans evokes cognitive and somatic symptoms strongly associated with panic attacks (e.g., fear of dying, anxiety, panic, tachycardia, thermal sensations, nausea, and paresthesias) (Rasche et al., 2006; Wilent et al., 2010, 2011). Studies using electrical stimulation of homologous areas in rats and rabbits reported similar defensive responses (Hess, WR Brugger, 1943)(Markgraf et al., 1991; McCabe et al., 1994; Duan et al., 1996) though it should be noted that electrical stimulation can elicit its effects through a variety of pathways and targets due to its nonspecific stimulation of fibers of passage. Chemical stimulation (achieved through disinhibition with the $GABA_A$ antagonist bicuculline methiodide) of the posterior hypothalamus in rats also elicits escape behavior and cardiovascular responses (DiMicco et al., 1986; Shekhar and DiMicco, 1987). Electrical stimulation of the DPAG also leads to marked defensive and fear responses, including tachycardia and pressor responses, in both human and animal studies, but temperature was not assessed (Nashold et al., 1969; Young, 1989; Schenberg and Lovick, 1994; Bittencourt et al., 2004). Stimulation of the dorsal and ventral PAG in rats with the glutamate receptor agonist n-methyl-D-aspartate also increased heart rate and mean arterial pressure, but also increased core body temperature (de Menezes et al., 2009). Interestingly, prior activation of $GABA_A$ receptors with muscimol in the DMH

prevented all of these responses (de Menezes et al., 2009). Importantly, the DMN/PeF areas are also heavily implicated in thermoregulatory effector pathways, see review by (Morrison and Nakamura, 2011).

Conclusions

These studies support the idea that thermal sensations—hot flashes and chills--can be elicited by a variety of panic provocations in animals, and studying this sign can complement the existing panic literature.

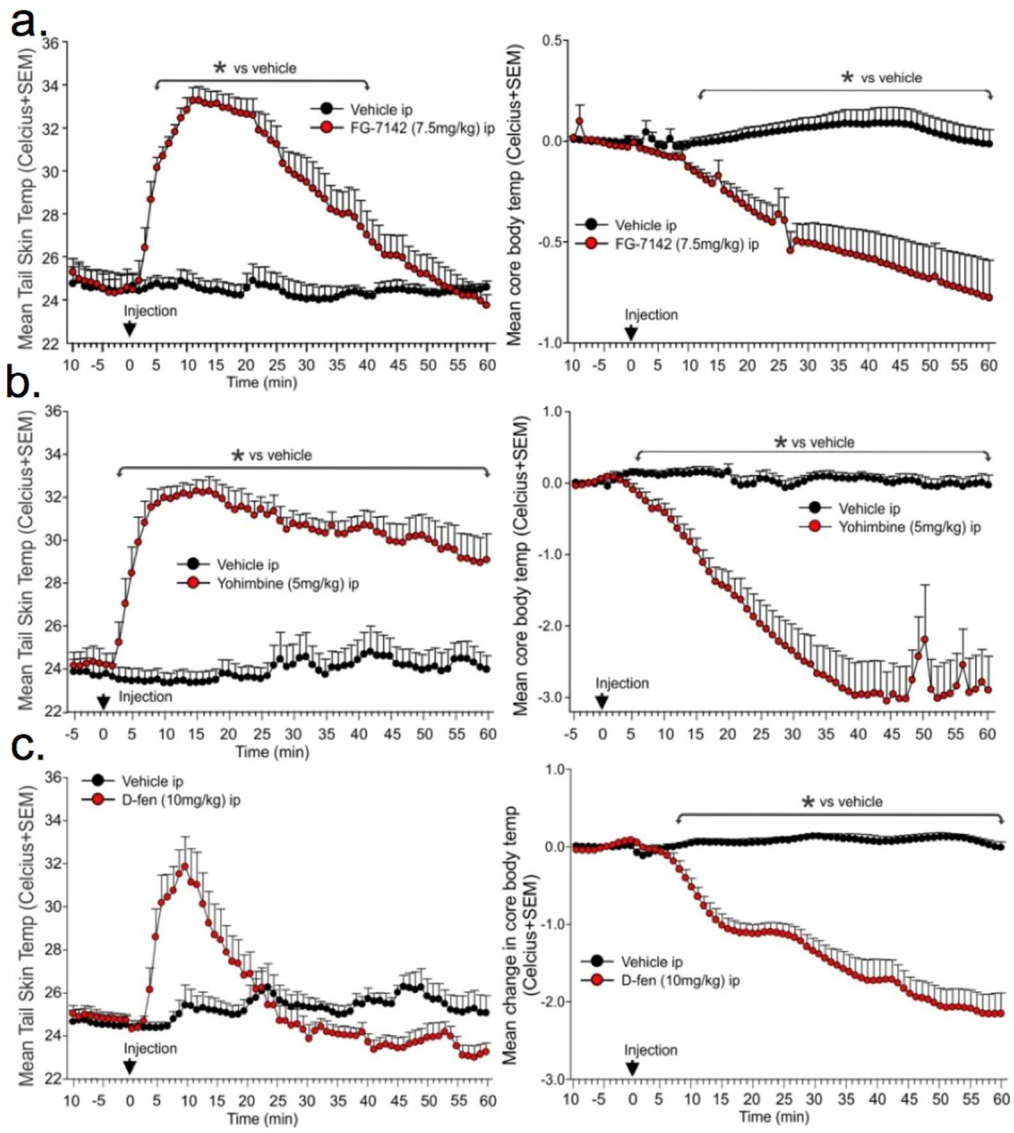


Figure 12. *Panicogenic Drugs Elicit Profound Hot Flash-Associated Cutaneous Vasomotor Responses in Male Rats.* Effects of panicogenic compounds on tail skin and core body temperature of male rats. **a)** indicates representative thermal images of rats respectively treated systemically (intraperitoneal) with vehicle; a benzodiazepine partial inverse agonist (beta carboline FG-7142, 7.5 mg/kg, n=12/group); an alpha 2 receptor antagonist (yohimbine, 5 mg/kg, n=8/group); or a serotonin reuptake inhibitor and vesicular storage disruptor (d-fenfluramine, 10 mg/kg, n=5/group). Line graph with error bars (SEM) in **b-d)** indicate mean tail and core body temperature activity in Celsius in freely moving male rats over a 10 min baseline and 60 min following treatment with **b)** FG-7142, **c)** yohimbine, or **d)** d-fenfluramine. Tail and core body temperature was respectively assessed with a tail thermistor taped to ventral surface of tail base and intraperitoneal implanted radiotelemetry probes, both of which were interfaced with data acquisition systems. * denotes indicates significance with a one-way ANOVA with drug as independent variable and time as a repeated measures $p<0.05$, and significant differences between groups using a Tukey's HSD post hoc test $p<0.05$, protected by an ANOVA at each time point.

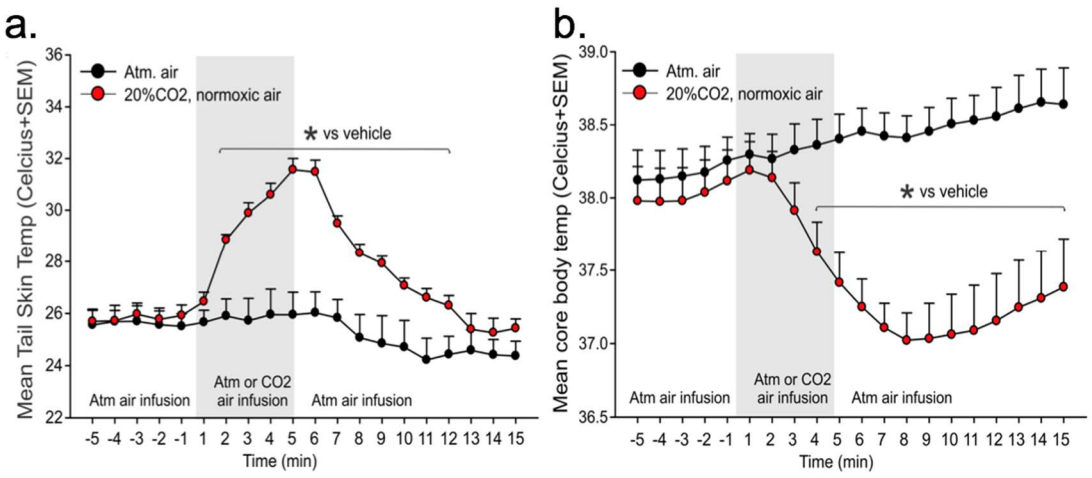


Figure 13. *Hypercapnic Gas Exposure Elicits Profound Hot Flash-Associated Cutaneous Vasomotor Response in Male Rats.* Effects of 20% CO_2 exposure (normal O_2) on tail skin and core body temperature of male rats. **a)** Line graph with error bars (SEM) indicates mean tail skin temperature (˚C) in freely-moving male rats over a 5 min baseline, 5 min atmospheric or hypercarbic gas exposure and 10 min recovery in atmospheric air. **b)** Line graph with error bars (SEM) indicates core body temperature (˚C) in freely moving male rats (n=8/group) over a 5 min baseline, 5 min atmospheric or hypercapnic gas exposure and 10 min recovery in atmospheric air. Tail skin and core body temperature were respectively assessed with a tail thermistor taped to ventral surface near the base of the tail and intraperitoneal implanted radiotelemetry probes, both of which were interfaced with data acquisition systems. *indicates significance with a one-way ANOVA with gas as independent variable and time as a repeated measures $p<0.05$, and significant differences between groups using a Tukey's HSD post hoc test $p<0.05$, protected by an ANOVA at each time point.

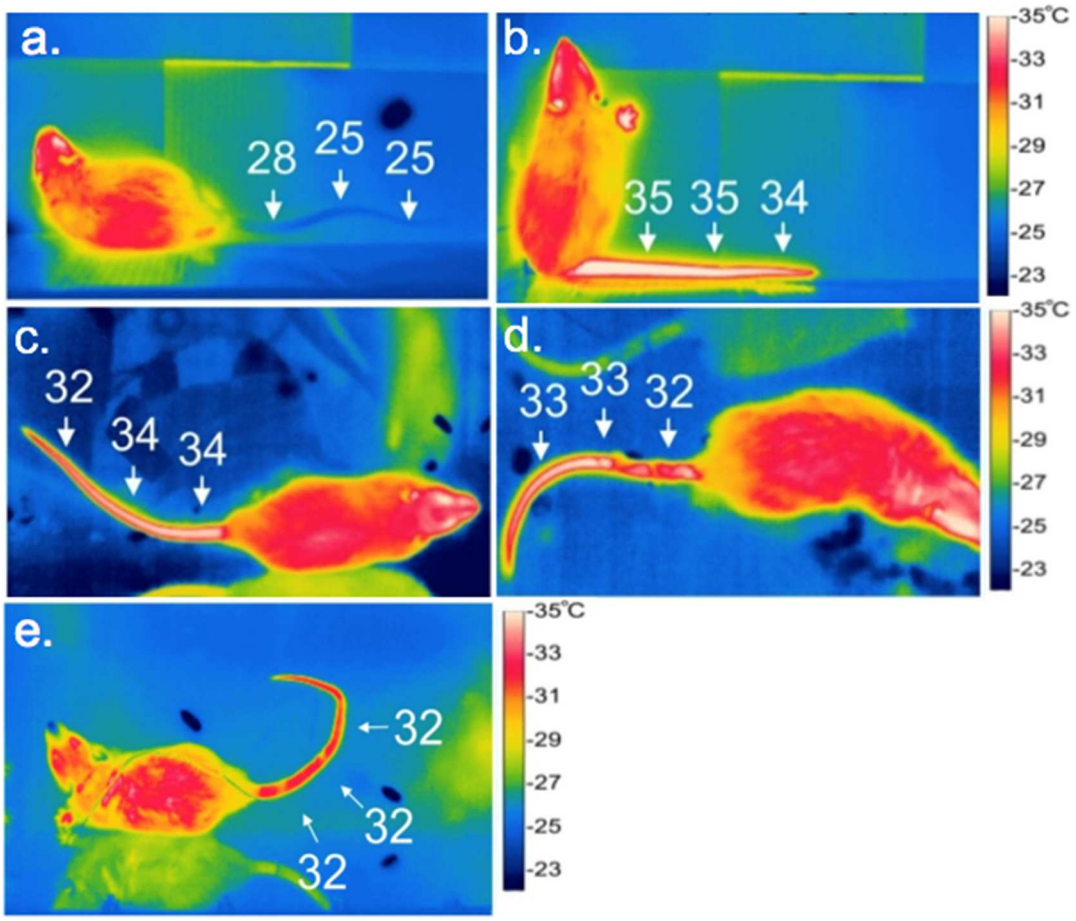


Figure 14. *Hot Flash Provocations Result in Increased Temperature Along the Entire Length of the Tail*. Thermal images were acquired at the peak height of the response (see **Figs. 12-13**). Images represent an animal from a) vehicle injection, b) FG-7142 (7.5. mg/kg, i.p.), c) yohimbine (5 mg/kg, i.p.), d) d-fenfluramine (10 mg/kg, i.p.), and e) 20% CO_2 exposure.

Chapter 2: Hypothalamic Orexin's Role in Exacerbated Cutaneous Vasodilation Responses to an Anxiogenic Stimulus in a Surgical Menopause Model

Menopause occurs following loss of ovarian function during natural aging, and a simulated menopausal state follows some breast and ovarian cancer treatments [e.g., surgical oophorectomy, or estrogen inhibition therapies (Carpenter and Andrykowski, 1999; Gallicchio et al., 2006b)]. Common symptoms during menopausal states include vasomotor symptoms (hot flashes and night sweats), anxiety and mood disruption, and sleep disturbances (Kronenberg, 1990). Hot flashes are the cardinal symptom, and 75% of postmenopausal women surveyed reported repeated hot flash episodes over an average of 4-7 years, but some over 10-13 years (Politi et al., 2008; Avis et al., 2015) following onset of menopause. Hot flash symptom presentations correlate well with objectively measurable, sympathetically-induced increases in cutaneous blood flow that raises skin temperature (Low et al., 2008).

Hormone replacement therapy [HRT, containing estradiol (E_2) in combination with estrone (E_1) and progesterone (P)] remains the most effective therapy for reducing hot flash symptomatology by ~75% treating in peri and post menopausal women [see systematic review (Maclennan et al., 2004)]. Yet, E_2 replacement therapy (ERT) alone is as effective as HRT at moderate doses (1-2 mg) (Baerug et al., 1998; Greendale et al., 1998) which restore plasma estrogen levels to 85-100% of pre-menopausal levels, respectively (Waaseth et al., 2008). Although ERT effectively alleviates hot flashes [even at lower doses of 0.5 mg (Joffe et al., 2014)], not all women are interested in taking ERT since initial use of E_2 is associated with side effects such as uterine bleeding and breast tenderness (Maclennan et al., 2004), and long-term ERT also increases the risk for estrogen positive breast cancer (Beral, 2003; Bolland et al., 2015). Additionally, ERT is contraindicated for postmenopausal women with estrogen receptor positive breast cancer, and hot flashes are the primary reason for noncompliance with estrogen inhibition therapies (Kemp et al., 2014), which increases mortality

(Hershman et al., 2011). Collectively these side effects and identified risks may explain a significant decline in HRT prescription and compliance (Zbuk and Anand, 2012) and an increase interest in non-hormonal therapies. Unfortunately, the few existing non-hormonal therapies are much less effective than ERT (Nelson et al., 2006). For instance, ERT reduces hot flash symptomatology by by ~75% [see systematic review (Maclennan et al., 2004)], whereas clonidine or serotonin/norepinephrine reuptake inhibitors (SSRI/NRI) are ~46% and 30% effective, respectively and are only marginally better than placebo responses that typically range from 20-50% (Nelson et al., 2006). These therapies are also commonly associated with side effects such as headaches, nausea, decreased sexual function, and insomnia, which can reduce adherence. The lack of effective, non-hormonal treatments is largely due to the limited understanding of the mechanisms that underlie menopausal symptoms. Menopausal symptoms are clearly induced by estrogen withdrawal; yet, how loss of estrogens leads to menopausal symptoms is largely unknown (Miller and Li, 2004a).

One mechanistic pathway that has not been explored is the orexin (OX and its two forms, OXA and OXB) neuropeptide system, which is unique in that OX-synthesizing neurons are restricted to the perifornical hypothalamic (PeF) region of rodents (Peyron et al., 1998) and humans (Thannickal et al., 2007). Estrogen receptors are expressed in the PeF (Laflamme et al., 1998), and stimulating the PeF in humans also produces symptoms associated with menopause [e.g., feelings of anxiety, racing heart, and hot flashes or chills (Wilent et al., 2011)]. Additionally, in female rats, E_2 administration decreases OXA content within the hypothalamus as well as at postsynaptic target CNS sites (Russell et al., 2001). More importantly, menopausal women have 300% higher plasma OX levels compared to reproductive controls, which is restored following Prempac, comprised primarily of equine estrones, and norgestrel, a form of progestin (El-Sedeek et al., 2010)]. This dramatic increase in OX activity during menopause is likely contributing to disrupted sleep since OX's most prominent role is to promote wakefulness (Sakurai, 2007), and higher OXA concentrations in cerebrospinal fluid have been demonstrated in individuals with poor sleep

quality (Allen et al., 2002). But there is also emerging evidence that a hyperactive OX system contributes to anxiety states and panic-associated sympathetic activity in rodents (Johnson et al., 2010, 2012a, 2015b), and that central OX levels are elevated in patients with increased anxiety symptoms (Johnson et al., 2010). These findings, combined with the building evidence that anxiety and stress are strongly associated with more severe and persistent hot flashes in large cohort studies (Avis et al., 2015), and that stressful stimuli can increase objective hot flashes (Swartzman et al., 1990) led to our current hypothesis that the OX system plays a critical role in menopause-related symptoms such as hot flashes, anxiety, and sleep disruption. We further posited that anxiety states and panicogenic-related stimuli may contribute to hot flash severity and frequency, and could help explain the presence of hot flashes in thermoneutral environments.

To test these hypotheses, we: 1) determined whether a panicogenic compound would elicit exacerbated tail skin temperature (TST: a correlate of a hot flash) responses in a surgical ovariectomy (OVEX) model of a menopausal state; 2) assessed cellular responses in OX neurons and efferent neurochemically identified targets that regulate arousal, anxiety, and thermoregulation; and 3) assessed orexin gene expression in the PeF, and determined if systemically treating OVEX rats with highly selective and brain-penetrant antagonists that target the two cognate OX receptors at optimal pharmacokinetic timelines would attenuate panicogenic drug induced TST responses.

Results

Experiment 1: OVEX Rats Have Exacerbated TST Responses to a Low Dose of a Panicogenic Drug that is Blocked by Estrogen Replacement.

Compared to sham-OVEX controls, systemically injecting female OVEX rats with a low dose of the panicogenic drug FG-7142 (n=7,5,5) produced a marked 6-7 °C increase in TST, which was blocked with estrogen replacement

(**Fig. 15a-**Representative thermal imaging; **Fig. 15b** - *treatment x time interaction* $F_{(55,770)}$=3.4, p<0.001).

Experiment 2: Alprazolam Pretreatment Prevents Increases in TST in OVEX Rats

Compared to vehicle-treated OVEX controls, alprazolam treatment (3 mg/kg, i.p.) attenuated an increase in TST induced by FG-7142 (3 mg/kg, i.p.) ($F_{(10,70)}$=14.62, p<0.0001; n=3,6), **Fig. 16**.

Experiment 3: FG-7142 induced Increases in TST precedes a decrease in Core Body Temperature (CBT) Decrease in OVEX Rats

Compared to vehicle treated OVEX controls, FG-7142 treated OVEX rats had a dramatic increase in TST within 3 min ($F_{(55,825)}$=18.7, p<0.0001, n=8,9, see thermal imaging of tail in **Fig. 17a**, and TST response on bottom line graphs in **Fig. 17b**) which was followed by a decrease in CBT that occurred 11 min post injection ($F_{(55,825)}$=11.2, p<0.001, n=8,9, see CBT response on top line graphs in **Fig. 17b**).

Experiment 4: Neural Circuits of OVEX Rats that Display Hyperactive Responses Following a Low Dose of a Panicogenic Drug

Among the brain regions implicated in menopause-related symptoms (see **Fig. 18a** for convergent evidence for brain region involvement and **Fig. 18b** for coronal illustration of brain regions assessed), the following brain regions of OVEX rats showed exacerbated cellular c-Fos responses to the low dose of a panicogenic drug FG-7142 (i.e., *OVEX x FG-7142 interaction*, **Fig. 18c**): 1) PeF [n=7,7,7,7, $F_{(1,24)}$=4.5, p=0.044]; 2) central amygdala [CeA, n=7,7,6,7 (area damaged unilaterally on one rat), $F_{(1,23)}$=16.1, 0.001]; 3) dorsolateral region of dorsal raphe nucleus [DRVL, n=6,6,7,7, (area damaged unilaterally on two rats), $F_{(1,22)}$=6.6, p=0.018]; 4) locus ceruleus [LC, n=7,7,7,7, $F_{(1,24)}$=6.1, p=0.021]; 5) raphe pallidus [RPa, n=7,7,7,7, $F_{(1,24)}$=7.4, p=0.012]; 6) rostroventrolateral medulla [RVLM, n=7,7,7,7, $F_{(1,24)}$=4.4, p=0.048]. Cellular responses were not

significantly different in the periventricular hypothalamic nucleus (PVN), medial preoptic hypothalamic area (mPOA), basolateral, lateral, or medial amygdala (BLA, LA, MeA), bed nucleus of the stria terminalis (BNST), dorsomedial or dorsolateral periaqueductal gray (dmPAG, dlPAG), ventral or dorsal part of the dorsal raphe nucleus (DRV or DRD), median raphe nucleus (MRn), or raphe magnus (RMg).

Experiment 5: Neurochemical Circuits of OVEX Rats that Display Hyperactive Responses Following a Low Dose of a Panicogenic Drug

Neurochemical phenotyping of c-Fos responses in OXA-ir neurons in the PeF and adjacent lateral hypothalamus (LH) revealed that OX neurons in the PeF, but not LH had exacerbated responses to FG-7142 in OVEX rats (n=6,6,7,7) [PeF: *OVEX x drug interaction*, $F_{(1,22)}$=4.1, p=0.055; *OVEX effect*, $F_{(1,22)}$=4.1, p=0.055; *FG-7142 effect* $F_{(1,22)}$=4.5, p=0.046; LH data not shown: *OVEX x drug interaction*, $F_{(1,22)}$=1.1, p=0.306; *OVEX effect*, $F_{(1,22)}$=2.0, p=0.169; *FG-7142 effect* $F_{(1,22)}$=0.5, p=0.482] (**Fig. 19a**). This difference in the number of c-Fos-ir OXA positive neurons was unlikely due to differences in numbers of OXA-ir neurons counted since there was no difference in the PeF [see gray bars in **Fig. 19a**: PeF: *OVEX x drug interaction*, $F_{(1,22)}$=0.9, p=0.770; *OVEX effect*, $F_{(1,22)}$=0.3, p=0.601; *FG-7142 effect* $F_{(1,22)}$=0.9, p=0.770; LH: *OVEX x drug interaction*, $F_{(1,22)}$=1.3, p=0.258; *OVEX effect*, $F_{(1,22)}$=3.2, p=0.086; *FG-7142 effect* $F_{(1,22)}$<0.01, p=0.997].

Neurochemical phenotyping of c-Fos responses of tyrosine hydroxylase-ir (TH)- noradrenergic neurons in the LC revealed that these neurons had exacerbated responses to FG-7142 in OVEX rats [**Fig. 19b**, *OVEX x drug interaction*, $F_{(1,22)}$=3.8, p=0.064; *OVEX effect*, $F_{(1,22)}$=7.2, p=0.014; *FG-7142 effect* $F_{(1,22)}$=10.1, p=0.004]. Neurochemical phenotyping of c-Fos responses TPH-ir serotonergic neurons in the DRVL and RPa revealed that these neurons had exacerbated responses to FG-7142 in OVEX rats [**Fig. 19c**, n=6,6,7,7,*OVEX x drug interaction*, $F_{(1,23)}$=9.4, p=0.006 (area damaged unilaterally on two rats); **Fig. 19d**, n=7,6,7,6, *OVEX x drug interaction*, $F_{(1,22)}$=14.7, p=0.001 (area

damaged on two rats)]. No difference was noted in the total number of TH or TPH-ir neurons counted between groups (see gray bars in **Fig. 19c-d**).

Experiment 6: Orexin, Glutamate, GABA Gene Expression in the PeF of OVEX Rats

Gene expression analyses in the PeF region (see **Fig. 20a** for convergent evidence of PeF region involvement) revealed that compared to sham-OVEX controls, OVEX rats had 200% higher expression of prepro-orexin (n=7,6, see **Fig. 20a** for illustration of area micropunched; **Fig. 20b**, $t_{(14)}$=-2.1, p=0.029). A Taqman Low Density Array (TLDA) custom designed for GABA and glutamate-related genes revealed that compared to sham-OVEX rats, OVEX rats had had a 1.81 fold increased gene expression of *GAD1* (aka GAD67, p=0.040) and decreased expression of *Grm4* (-1.83 fold; p=0.003) and *Grin2a* (-1.63 fold; p=0.024) (see volcano plot in **Fig. 20c**).

Experiments 6-7: Systemic Pretreatment with a SORA1 or estrogen is Anxiolytic in OVEX rats

Compared to sham-OVEX controls, OVEX rats spent less time exploring the center region of an open field test (indicating an anxiety-like behavioral response, **Fig. 21a**). Furthermore, pretreating OVEX rats with a SORA1 blocked the OVEX-induced anxiety-associated behavior [*SORA1 effect*, $F_{(1,35)}$=7.1, p=0.012, n=10,10,9,10, an outlier detected and removed from OVEX/vehicle group, Grubb's test z=2.3, p<0.05]. In a second experiment, compared to OVEX rats treated with vehicle, OVEX rats with estrogen replacement spent more time exploring the center of the open field [**Fig. 21b**, n=9,7, failed Shapiro-Wilk normality test p<0.05, Mann-Whitney Rank Sum Test U=5.0, p=0.006].

Experiments 8-12: Systemic Pretreatment with an OX1R, OX2R, or Dual OX Antagonist Attenuates TST Responses to a Panicogenic Drug in OVEX Rats

Here the highest dose of DORA-12 blocked the TST response to the panicogenic drug FG-7142 (**Fig. 22a**; *drug x time interaction*, $F_{(108,1458)}$=1.4,

p=0.006, n=9,10,10]. In the SORA1 experiments, both SB-334867 and Compound 56 attenuated the TST response to the panicogenic drug FG-7142 (**Fig. 22b**; no *drug x time interaction* $F_{(55,715)}$=1.1, p=0.247, but there was a significant *drug effect*, $F_{(1,13)}$=7.6, p=0.016, n=5,10; **Fig. 22c**, *drug x time interaction* $F_{(54,648)}$=2.2, p<0.001, $F_{(1,13)}$=7.6, p=0.016, n=7,7]. Initially, we administered a SORA2 (TCS OX2 29, 5 and 10 mg/kg, i.p.) which, compared to vehicle group, did not alter FG-7142 induced TST responses in OVEX rats (data not shown, *drug x time interaction* $F_{(130,2535)}$=0.5, p=1.00, *drug effect* $F_{(2,39)}$=0.5, p=0.600, n=14,14,14). We later learned that this compound only achieves about ~50% OX2R occupancy and quickly dissociates and is not brain penetrant (P. Bonaventure, personal communication). In a subsequent experiment using a brain penetrant SORA2 called JnJ10397049, which achieves greater (~75%) and sustained (8 h) receptor occupancy an attenuation of the TST response to the panicogenic drug FG-7142 (3 mg/kg, i.p.) was observed at both doses [**Fig. 22d**; *drug x time interaction* $F_{(100,1950)}$=37.4, p<0.001, n=14,14,14].

Discussion

Here we first ovariectomized female rats to model a surgical menopausal state, which increased baseline anxiety-associated behaviors, and when challenged with a low dose of the panicogenic compound FG-7142, OVEX rats had an exacerbated hot flash-associated TST responses >7 °C that preceded a decrease in CBT (suggesting a heat dissipation response), thus providing face validity. Furthermore, ER, which also attenuated anxiety-associated behaviors, almost blocked the TST responses. Thus, predictive validity is provided since ER effectively treats hot flashes and FG-7142 is known to cause hot flashes and/or chills in humans, and also elicits anxiety (Dorow et al., 1983). Alprazolam pretreatment (a benzodiazepine) attenuated an increase in TST following FG-7142, demonstrating that the TST response is specific to drug action at the benzodiazepine site. These data also suggest that there is a loss of GABAergic inhibitory signaling following loss of ovarian function. Consistent with this

hypothesis is that in one clinical study the benzodiazepine oxazepam was effective in reducing hot flashes in a cohort of premenopausal women who underwent oophorectomy (Erkkola et al., 1973). Collectively, these clinical studies and our preclinical studies may explain part of the effectiveness of these treatments for hot flashes. Finally, since this method of inducing hot flash-associated TST responses also produces strong anxiety at higher doses, it suggests that some stress-related stimuli and or existing anxiety states could contribute to hot flash severity and prevalence.

The link between anxiety and stress with hot flashes has been controversial. For instance, although a recent systematic review concluded that anxiety symptoms were low during the menopause transition, they noted that the majority of studies utilized brief and largely non-validated measures of anxiety symptoms (Bryant et al., 2011). Bryant and colleagues further stated that studies that utilized accepted diagnostic assessments did observe an increase in anxiety in postmenopausal women [see (Cagnacci et al., 1997; Pimenta et al., 2012)] that is alleviated with ERT+P (Cagnacci et al., 1997). Furthermore, greater presence of anxiety predicts more frequent and/or severe hot flashes (Freeman et al., 2005), and is also positively correlated with hot flash severity (Gibson et al., 2011). Additionally, women with premenstrual syndrome, which includes high levels of anxiety, are more likely to have hot flashes (Freeman et al., 2004) and hot flashes are common symptom associated with panic attacks that occur in severe anxiety disorders (DSM-V). Perhaps more compelling is that in a recent large (n=3302) multiracial/multiethnic longitudinal investigation [the Study of Women's Health Across the Nation], greater perceived stress, anxiety symptoms, depressive symptoms, and decreased socioeconomic status were associated with more persistent and frequent vasomotor symptoms (Avis et al., 2015). Thus, these data are supportive of the hypothesis that anxiety and stress, which are associated with more severe and persistent hot flashes, may also contribute to hot flash severity and prevalence. This hypothesis is also consistent with a hypothetical review that observed similarities between hot flashes and severe anxiety/panic attacks that included: symptom presentation; neurochemical

circuitry; pharmacotherapy; and psychological treatments (Hanisch et al., 2009) and also suggests that neurochemical systems implicated in generating anxiety-associated emotional, behavioral, and physiological responses may represent novel targets for treating symptoms associated with menopause.

Our subsequent experiments sought to determine if the hypothalamic OX system might represent one such novel therapeutic target. In a series of experiments, we demonstrated that rats with a surgical menopausal state have a two-fold increase in prepro-OX mRNA expression within the PeF region, and exacerbated OX neuronal responses to a subthreshold panicogenic drug that excites this system (FG-7142) (Johnson et al., 2012a). We further demonstrated that efferent targets of OX neurons heavily implicated in hot flash etiology (Freedman et al., 1990; Stearns et al., 2005) also showed exacerbated cellular responses. These included limbic structures such as the amygdala, as well as noradrenergic neurons in the locus ceruleus, and serotonergic neurons in the dorsal raphe nucleus and raphe pallidus. Artificially increasing central OX activity in rodents with intracerebroventricular injections of OXs disrupts sleep (Espana et al., 2002); increases anxiety-associated behaviors (Suzuki et al., 2005); induces thermoregulatory changes (Yoshimichi et al., 2001); elicits cardio-excitation (Samson et al., 1999), and stimulates corticosteroid release (Brunton and Russell, 2003), which is related to greater hot flash symptomatology in menopausal women (Woods et al., 2006a)]. These data are consistent with preclinical data showing that estrogen inhibits OX expression in rodents (Russell et al., 2001), and clinical data showing OX activity is dramatically elevated following menopause (El-Sedeek et al., 2010). Thus, a hyperactive OX system seen here in our surgically-induced menopausal state in rodents and mirrored in postmenopausal women (El-Sedeek et al., 2010) could be contributing to multiple core symptoms that occur during and/or following menopause. An outstanding question for future studies, would be determining if the interactions observed between orexin and estrogen are indirect or direct (e.g., determining if the prepro-orexin gene has an estrogen response element, or whether expression of ERα and/or ERβ are colocalized with orexin neurons).

In a final series of experiments, we systemically treated OVEX rats with highly selective and brain-penetrant OXR antagonists at optimal pharmacokinetic timepoints to determine if antagonizing this system would decrease hot-flash associated increases in TST. There are two forms of OXs, OXA and OXB, and two known receptors. The OX1R has greater affinity for OXA than OXB by an order of magnitude, while the OX2R has similar affinity for both OXA and OXB (Sakurai, 2007). Both receptors are G-protein coupled, and are either co-located or selectively located in specific brain areas (Marcus et al., 2001), suggesting differentiated roles. Here we show that a dual OXR antagonist, DORA-12, blocked the TST response elicited by FG-7142, whereas antagonizing either receptor alone partly attenuated hot flash-associated TST responses. This suggests that these drugs may provide a novel non-hormonal treatment strategy for hot flashes in menopausal women. Interestingly, suvorexant, a DORA, was recently FDA-approved with an indication for insomnia. Sleep problems, broadly defined, are a known problem at midlife and at the menopause (Burleson et al., 2010), and many have posited that hot flashes during sleep contribute to nighttime awakenings/sleep problems. Therefore, it seems reasonable to postulate that targeting either symptom may lead to improvement in the other. The sleep-promoting properties of DORAs are primarily through antagonism of OX2Rs which are exclusive to histaminergic neurons in the tuberomammillary nucleus, which is a brain region that plays a critical role in wake promotion (Huang et al., 2001; Marcus et al., 2001), but also plays a role in regulating thermogenic activity (Yasuda et al., 2004) which could partially explain the ability of the OX2R antagonist to attenuate TST responses. The anxiolytic effects of DORAs may be preferentially through antagonism of OX1Rs (Johnson et al., 2010, 2012a, 2015b; Bonaventure et al., 2015), but also potentially OX2Rs (Li et al., 2010). Anatomically, OX1Rs appear to be more selectively expressed in panic and anxiety-associated neural circuits such as the bed nucleus of the stria terminalis, amygdala, cingulate cortex and exclusively in noradrenergic neurons in the locus ceruleus [(Marcus et al., 2001), see review (Johnson et al., 2012b)], which also play a role in sympathetic mobilization. Yet, anxiolytic effects of

ORX2R antagonism have been demonstrated in the paraventricular thalamus (Li et al., 2010). Thus, DORAs may alleviate sympathetically-mediated hot flashes and or hot flashes resulting from pre-existing anxiety states or in responses to some stress-related stimuli.

Conclusions

Collectively, our previous and current data support the hypothesis that estrogen tonically inhibits the orexin arousal system, and dramatic loss of estrogen tone during menopausal states leads to a hyperactive orexin system that contributes to the cardinal menopausal symptoms such as recurrent hot flashes, insomnia, and anxiety. Additionally, our data suggests that OXR antagonists, possibly in combination with lower doses of ERT, may represent a novel non-hormonal therapy for treating all of these symptoms, with minimal side effects.

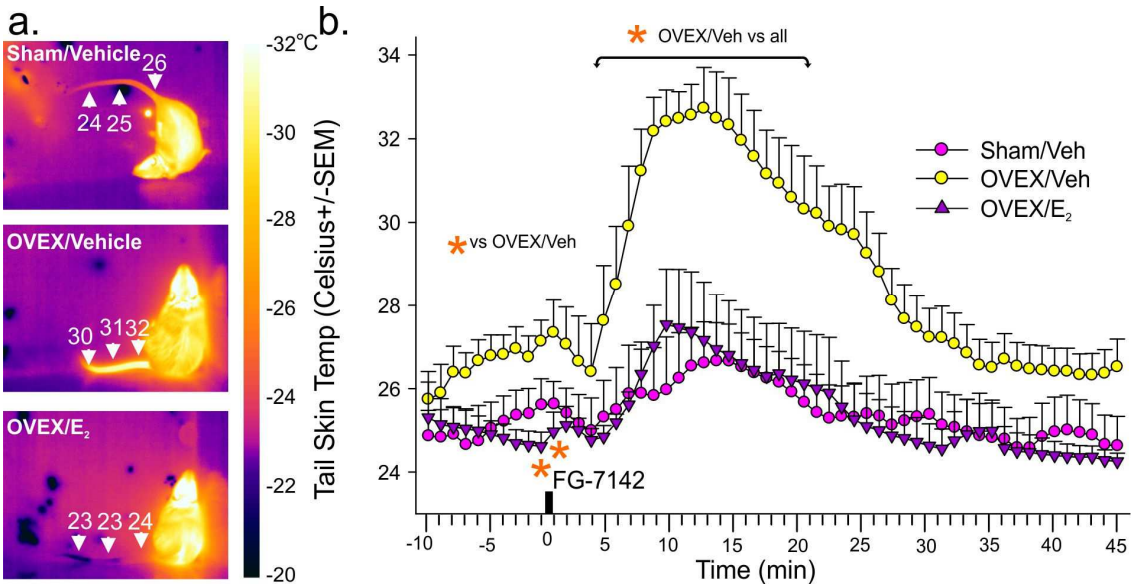


Figure 15. *Rats Modeling Surgical Menopause are Vulnerable to Displaying Hot Flash Associated Cutaneous Vasomotor Responses to a Low Dose of a Panicogenic Drug.* Effects of a low dose of FG-7142 (3 mg/kg) on tail skin temp of female sham-OVEX rats given daily 0.2 ml s.c. injections of sesame oil control vehicle (n=7), or in OVEX rats given daily s.c. injections of control vehicle (n=5) or 0.25 mg/kg 17-β estradiol (n=5). **a)** Thermal images of rats 20 min after FG-7142 injection from each treatment group with scale bar to right. **b)** Line graph with error bars (SEM) represents the mean tail skin temp prior to and following a 3 mg/kg i.p. injection of FG-7142 in each treatment group assessed with a thermistor at base of tail. * denotes significance with a one-way ANOVA with drug as an independent variable and time as a repeated measures $p<0.05$, and significant differences between groups using a Fisher's LSD post hoc test protected by an ANOVA at each time point, $p<0.05$.

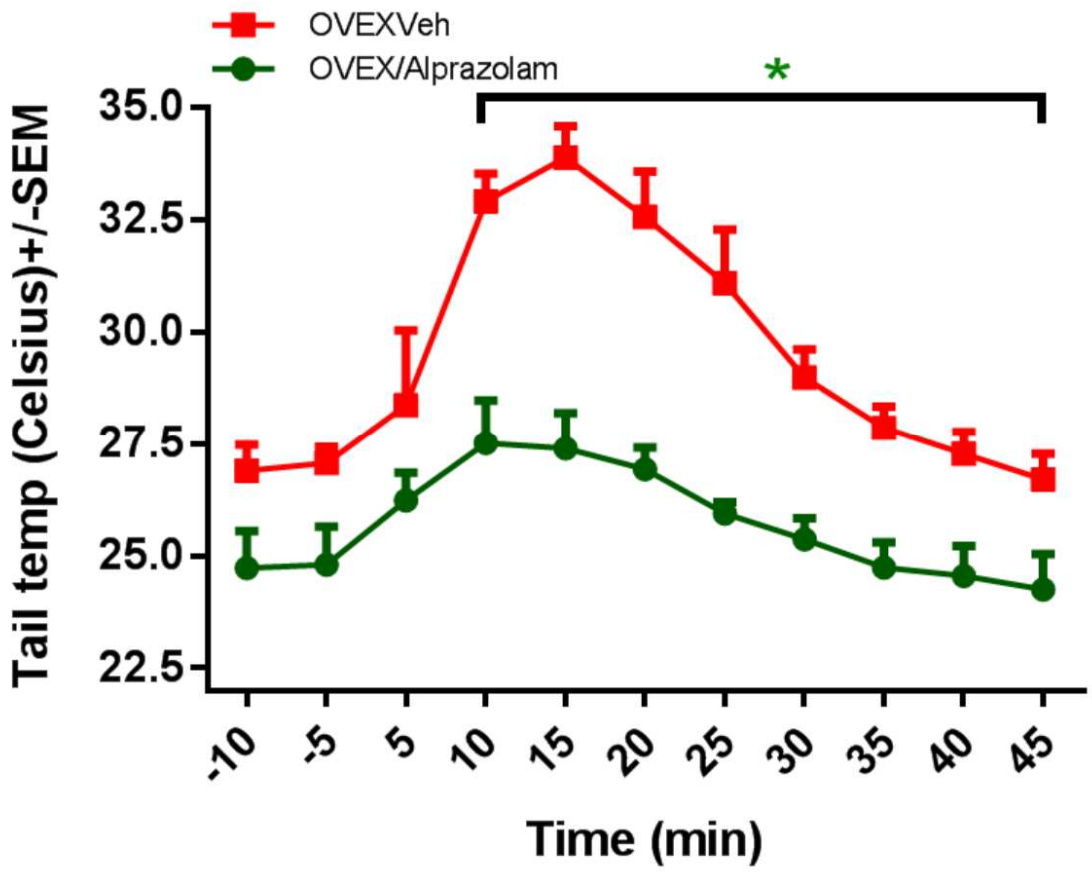


Figure 16. *Alprazolam Pretreatment Attenuates Cutaneous Vasomotor Response to a Low Dose of a Panicogenic Drug.* Effects of a low dose of FG-7142 on tail skin temperature of OVEX rats pretreated with either vehicle (n=3) or alprazolam (n=6; 3 mg/kg, i.p.). Line graph with error bars (SEM) represents the mean tail skin temperature of OVEX rats prior to and following systemic injections of FG-7142 (3 mg/kg, i.p.). *denotes significance with a one-way ANOVA with drug as independent variable, $p<0.05$. Though not shown, there were no baseline differences prior to alprazolam treatment.

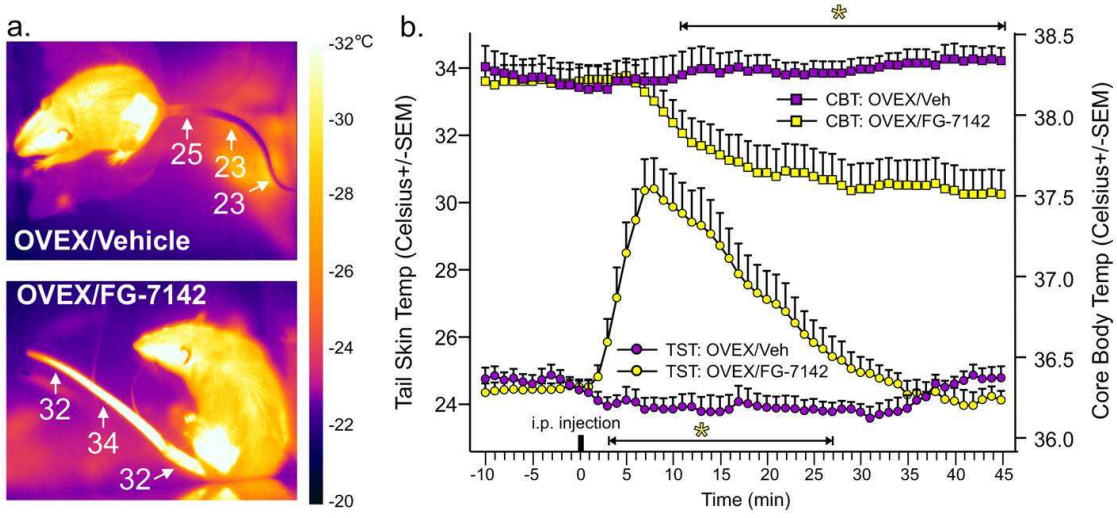


Figure 17. *Panicogenic Drug-induced Cutaneous Vasomotor Responses Precede a Decrease in Core Body Temperature in Rats with Surgical Menopause.* Effects of a low dose of FG-7142 (3 mg/kg) on tail skin temp and core body temperature of female OVEX rats. **a)** Representative thermal images of OVEX rats 10 min after vehicle or FG-7142 injection from each treatment group with scale bar to right. **b)** Line graph with error bars (SEM) represents the mean tail skin temp and core body temperature of OVEX rats prior to and following systemic injections of either vehicle (n=8) or 3 mg/kg FG-7142 (n=9). * denotes significance with a one-way ANOVA with drug as an independent variable and time as a repeated measures $p<0.05$, and significant differences between groups using a Fisher's LSD posthoc test protected by an ANOVA at each time point, $p<0.05$.

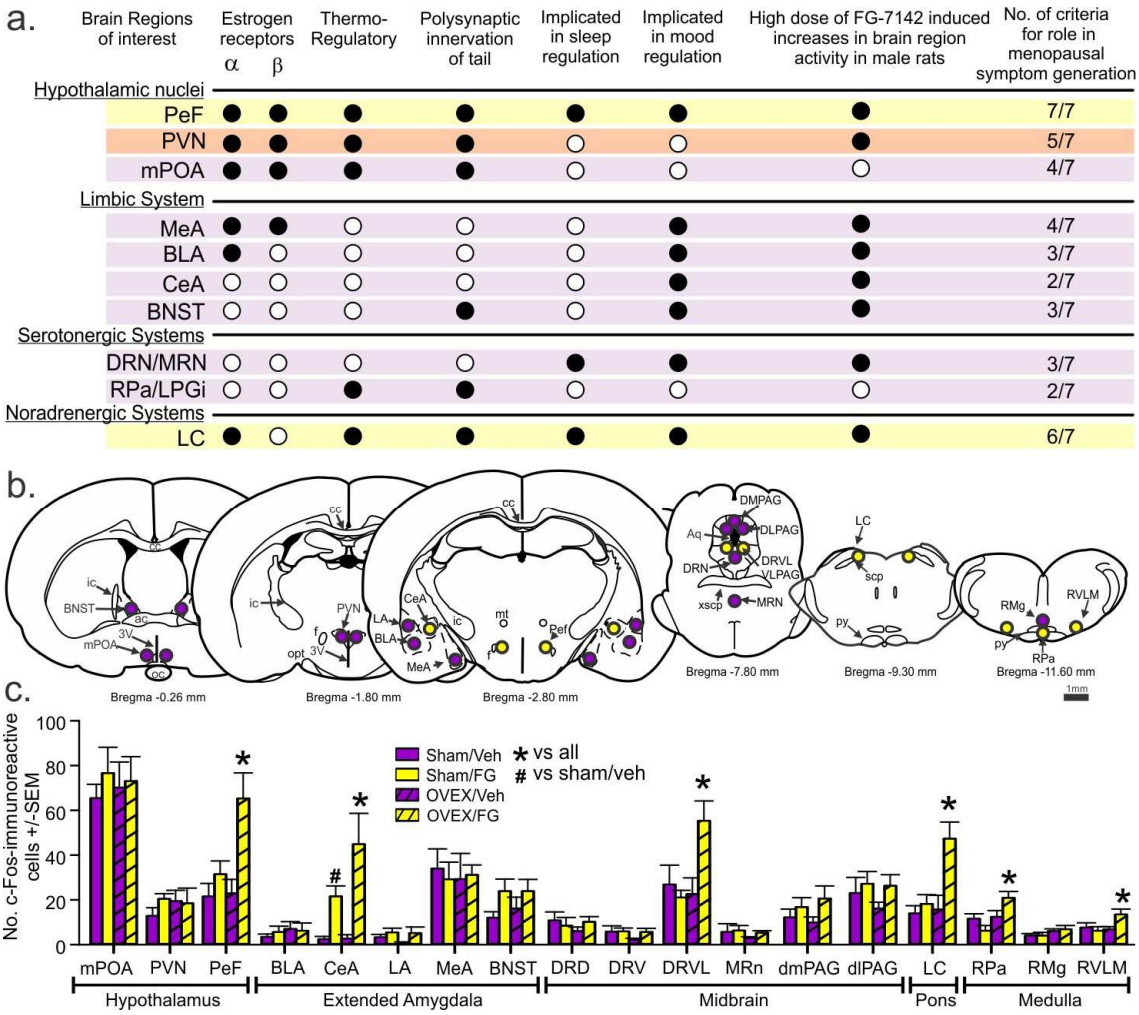


Figure 18. *Rats with Surgical Menopause Display Hyperactive Cellular Responses Following a Low Dose of a Panicogenic Drug in Neural Circuits Heavily Implicated in Menopausal Symptoms.* **a)** Table with convergent evidence of neurochemical circuit involvement in hot flash-associated cutaneous vasomotor responses. Columns respectively represent brain regions and additive criteria to strengthen the likelihood that a particular brain region will be impacted by loss of estrogen, but also involved in menopause-related symptoms. Closed circles means the region meets the criterion, open circles mean the region does not meet the criterion. Yellow shaded rows meeting most criterion, with orange and purple shaded rows meeting less. See results section for anatomical abbreviation definitions. Effects of vehicle or a dose of FG-7142 (that evokes hot flashes in OVEX, but not sham rats) on cellular responses (c-Fos immunoreactive, ir) in brain regions from OVEX and Sham-OVEX rats from Table 1 (n=6,6,7,7). **b)** illustrates coronal brain sections of a rat at different rostrocaudal levels, with yellow circles indicating brain regions where OVEX rats showed hyperactive responses to FG-7142 compared to sham-OVEX rats. Purple circles indicate areas that were counted but had no significant differences between groups. Bars in **c)** represent mean number of single c-Fos-ir cells +/-SEM. * denotes significant differences between groups using a Tukey's HSD post hoc test protected by 2-way ANOVA, $p<0.05$.

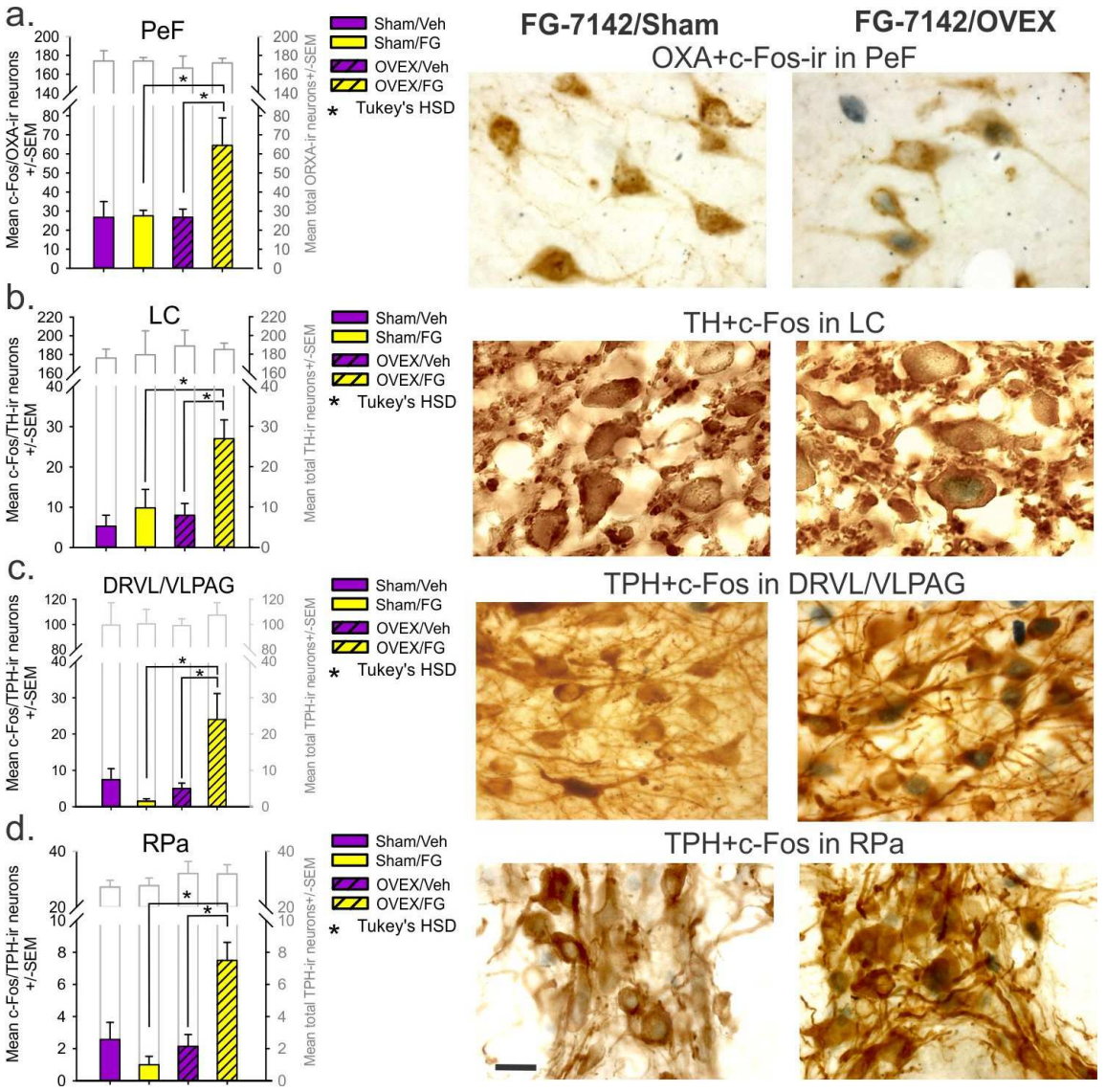


Figure 19. *Neurochemical Circuits of Rats with Surgical Menopause that Display Hyperactive Responses Following a Low Dose of a Panicogenic Drug.* Effects of vehicle or FG-7142 injection (that evokes hot flashes in OVEX, but not sham rats) on cellular responses (c-Fos immunoreactive, ir) in brain regions from OVEX and Sham-OVEX rats from Table 1 (n=6,6,7,7). Bars in **a)** represent c-Fos+OX labeled neurons in the PeF/DMN and LH. Bars in **b)** represent c-Fos+tyrosine hydroxylase (TH) in noradrenergic neurons in the locus ceruleus (LC). Bars in **c-d)** represent c-Fos+tryptophan hydroxylase (TPH) in serotonergic neurons in the ventrolateral region of the dorsal raphe nucleus (DRVL/VLPAG), and in the raphe pallidus (RPa), respectively. * denotes significant differences between groups using Tukey's HSD post hoc test $p<0.05$, protected by a 2-way ANOVA. Photographs in **a-d)** represent high magnification brain sections illustrating dark nuclear immunostaining of c-Fos +/- brown cytoplasmic immunostaining of OX-ir neurons in the PeF, TH-ir neurons in the LC, and TPH-ir neurons in the DRVL/VLPAG and RPa from Sham and OVEX rats treated with FG-7142, respectively. Scalebar in d = 20 μm.

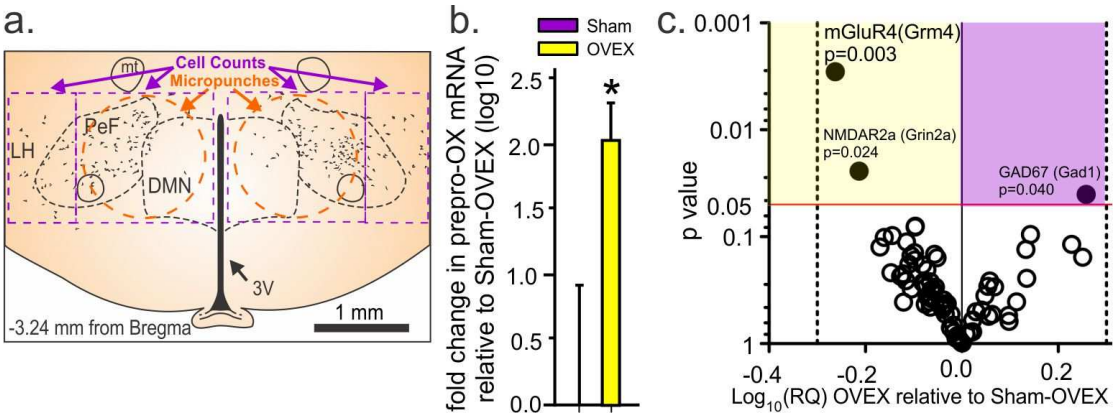


Figure 20. *Surgical Menopause Enhances Baseline Hypothalamic Orexin/Glutamate Gene Expression.* **a)** illustration of a coronal section of rat hypothalamus with the PeF/DMN and LH with representative distribution of OX neurons (black dots) and areas where micropunches were done (orange dashed circles) in a 300 µm fresh frozen tissue section for gene expression data in **b-c)** and where cell counts were done (purple dashed lines) in **Fig. 18-19**). **b)** indicates expression of prepro-OX mRNA in sham and OVEX rats. * indicates $p<0.05$ unpaired t-test. **c)** A volcano plot of hypothalamic mRNA levels of OVEX rats relative to Sham rats, determined by a custom designed Taqman Low Density Array (TLDA). Plotted on the Y-axis are p values from Fisher's LSD test (each gene) and ±fold difference (Log_{10}) on the x-axis. Genes with $p < 0.05$ are indicated by solid circles and all others are open circles. Horizontal red line is p=0.05 and vertical dotted lines represent a ±2 fold change in expression (0.0301 on log_{10} scale).

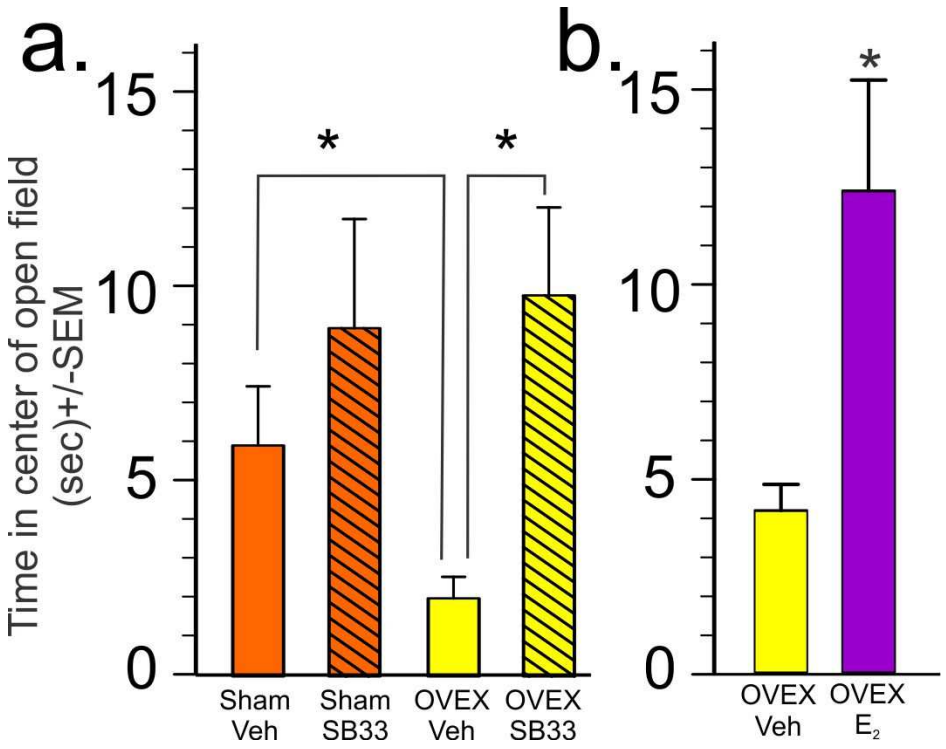


Figure 21. *Systemic Pretreatment with a SORA1 or Estrogen is Anxiolytic in OVEX Rats.* **a)** Bar graphs illustrate the time spent in the center region of an open field test 30 min after injection of vehicle or the SORA1 SB334867 (30 mg/kg, i.p.) in sham-OVEX controls or OVEX rats. **b)** Bar graph illustrates the duration OVEX rats spent in the center region of an open field test following daily subcutaneous injections of estrogen replacement [0.25 mg/kg of 17-β estradiol or 0.2 ml vehicle]. * $p<0.05$. All testing was done 12-13 days after sham-OVEX or OVEX surgeries. Bars represent mean and error bars represent SEM.

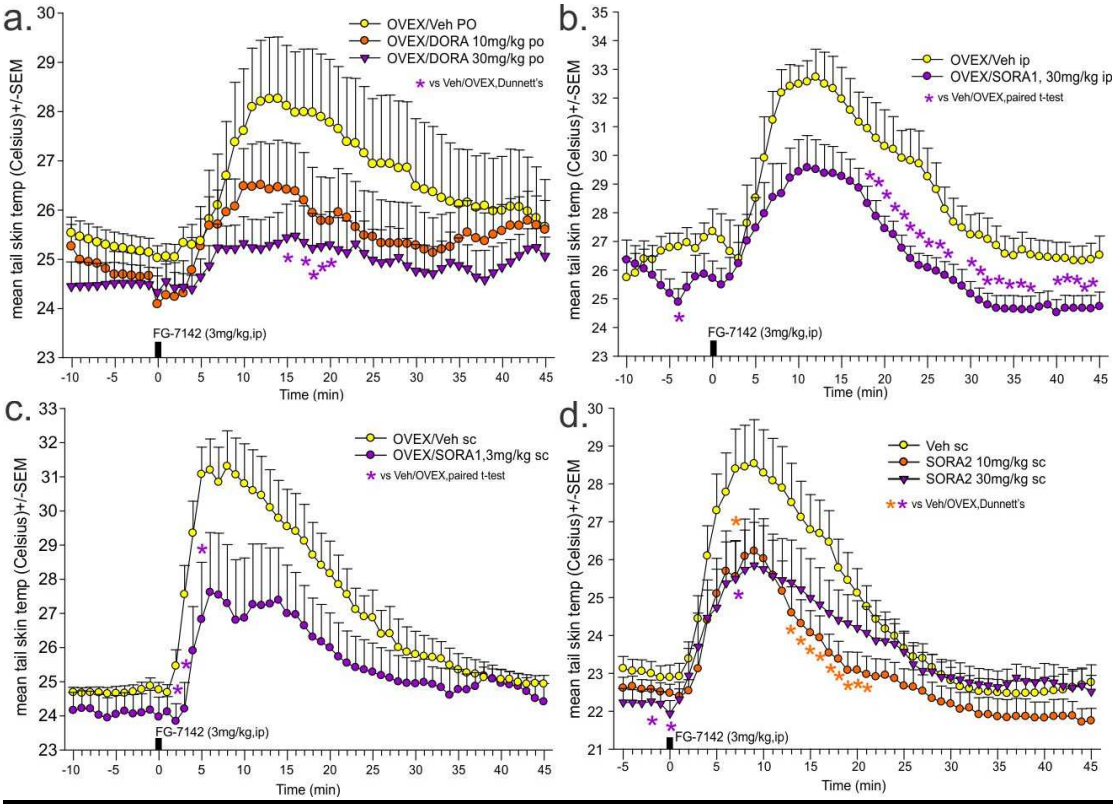


Figure 22. *Systemically Treating Ovariectomized Rats with Centrally Active Orexin 1 and/or 2 Receptor Antagonists Attenuates Hot Flash-Associated Cutaneous Vasomotor Responses to a Low Dose of a Panicogenic Compound.* **a)** Effects of FG-7142 (3 mg/kg) on tail skin temp of female OVEX rats orally pre-treated with vehicle or a dual OXR antagonist (DORA-12. 10 and 30 mg/kg p.o.); **b-c)** Effects of FG-7142 (3 mg/kg) on tail skin temp of female OVEX, or sham-OVEX rats systemically pre-treated with vehicle or an OX1R antagonist (**b**: SB-334867 30 mg/kg i.p.; **c**: Compound 56, 3 mg/kg s.c.);. **d)** Effects of FG-7142 (3 mg/kg) on tail skin temp of female OVEX, or sham-OVEX rats systemically pre-treated with vehicle or an OX2R antagonist (JnJ-10397049, 10 and 30 mg/kg s.c.). * denotes significant differences between groups using a two-tailed Dunnett's post hoc test against vehicle protected by a one-way ANOVA with drug as main factor and time as repeated measures, and a Fisher's LSD post hoc test protected by a one-way ANOVA at each time point, $p<0.05$. All OX receptor antagonist doses, routes, and treatment timelines were done based on published pharmacokinetic information (e.g., brain penetrance, OX1R and OX2R occupancy in the brain, and C_{max} in plasma and brain).

Chapter 3: Anxiogenic CO_2 Stimulus Elicits Exacerbated Hot Flash-like Responses in a Rat Menopause Model

Hot flashes are intense heat sensations that occur primarily in the chest and face and affect the vast majority of women at midlife or following estrogen inhibition therapies or oophorectomy (Kronenberg, 1990; Gallicchio et al., 2006b). In the laboratory setting, the subjective hot flash sensation (as indicated with button press or self-report) correlates well with objectively measured, sympathetically-mediated increases in cutaneous vasodilation which pools 37 °C blood to the skin. This in turn interacts with peripheral thermal sensors in skin to signal heat. Sweating sometimes also occurs during a hot flash, but this induces an evaporative cooling sensation. Under normal circumstances, both cutaneous vasodilation and sweating are effective means of dissipating heat to maintain core body temperature (T_c) in hot environments. While environmental temperature is often thought to affect hot flash parameters (i.e., frequency, severity, duration), many studies have found weak or inconsequential relationships to ambient temperature (Voda, 1981; Stefanopoulou et al., 2014a, 2014b) and in a self-reported survey of hot flash precipitants, temperature was identified less frequently than other stimuli (Stubbs et al., 2008). While exact figures are unavailable, these data suggest that many women may experience hot flashes in thermoneutral environments with no clear identifiable trigger. In these circumstances, the collective heat dissipation mechanisms that occur during a hot flash could lead to decreases in T_c which does occur and could explain chills that also occur in menopausal women [(Low et al., 2008)and see review (Freedman, 2005)].

While there are many variables that influence hot flashes, recent findings from large longitudinal studies have identified the presence of anxiety, early life stress, and lower socioeconomic status as key risk factors for more severe and problematic hot flashes (Thurston et al., 2008a; Avis et al., 2015). Notably, anxiety has been identified as the best predictor of hot flashes, even when controlling for typical confounds, and women with greater anxiety levels

experience more severe hot flashes (Freeman et al., 2005; Avis et al., 2015). Additionally, many women report that stressful stimuli in everyday life precipitate hot flashes (Stubbs et al., 2008), and stressful stimuli in a laboratory setting have been shown to increase objectively measured hot flashes (Swartzman et al., 1990). Taken together, there is evidence that stressful events may provoke hot flashes, and that pre-existing anxiety states or traits may contribute to increased symptoms. However, the mechanisms by which anxiety and stress-related factors contribute to hot flashes remain poorly understood.

One contributing factor to the poor understanding of the mechanisms mediating hot flashes is a scarcity of appropriate, validated animal modeling (Miller and Li, 2004b). Previous models such as the morphine withdrawal model (Simpkins et al., 1983) elicit robust cutaneous vasomotor responses in the tail, but lack an obvious relevance to causes and triggers of hot flashes (Lightman et al., 1981; DeFazio et al., 1984). Consequently, the use of models for mechanistic investigation into the central and peripheral neural circuitry and associated pathways mediating hot flashes has suffered. Recently, we found profound decreases in T_c in rats following an acute 20% carbon dioxide (CO_2) exposure, which signals air hunger and produces anxiety-associated behavior and sympathetic associated cardioexcitation (Johnson et al., 2011, 2012c, 2015). In human studies, 20% CO_2 inhalation induces anxiety symptoms, and in one study the subjects reported having strong heat sensations (Kaye et al., 2004). However, it was unknown if this would have relevance to menopause-associated symptoms.

Therefore, we designed a series of studies to determine if CO_2 exposure represented a novel and valid method of provoking hot flashes. Thus, in a rodent model, we hypothesized that 20% CO_2-induced decreases in T_c were due to cutaneous vasomotor responses in the tail which would increase the tail skin temperature (TST) to dissipate heat in a thermoneutral environment. We further posited that the TST response would be exacerbated in ovariectomized (OVEX) rats, modeling surgical oophorectomy-induced menopause, which is linked to more severe hot flashes (Gallicchio et al., 2006b; Benshushan et al., 2009).

Secondly, recent work has begun to elucidate some of the genetic contributions to increased or problematic hot flashes. The short ('s') version of the serotonin transporter (SERT) gene, whose protein product mediates the termination of serotonin signaling, leads to reduced transcriptional efficiency and less protein, and is linked to anxiety-associated traits in humans (Heils et al., 1996; Lesch et al., 1996; Gonda et al., 2007, 2009) as well as increased climacteric symptoms in menopausal women (Grochans et al., 2013). Therefore, we then hypothesized that OVEX rats with a heterozygous null mutation of the SERT gene ($SERT^{+/-}$) which leads to similar reduced transcriptional efficiency, would have exacerbated and/or prolonged hot flashes (increased TST) in response to CO_2 compared to wildtype (WT) OVEX controls.

Results

Experiments 1-2: Ovariectomized rats exhibit exacerbated hot flash-associated increases in tail skin temperature in response to hypercapnic gas infusion while atmospheric air infusion does not change tail skin temperature

An ANOVA with ovex surgery and hypercapnic gas/CO_2 as main factors and time as a repeated measure revealed that hypercapnic gas infusion produced an exacerbated increase in tail skin temperature in ovex rats while atmospheric air caused no changes in temperature [**Fig 23a**-Representative thermal image; **Fig. 23b**- CO_2 tail skin temperature (n=15/group) overall *CO_2 effect* $F_{(1,38)}$=45.9, p<0.0001, overall *ovex effect* $F_{(1,38)}$=5.3, p=0.027, and overall *CO_2 by time interaction* $F_{(114,532)}$=20.1, p<0.0001 but no *ovex by time interaction* $F_{(14,532)}$=0.23, p=0.999 or *ovex by CO_2 by time interaction* $F_{(14,532)}$=0.34, p=0.989]. Fisher's Least Significant Difference Test was used for post hoc testing to determine specific time point differences between groups, and Dunnett's test was used to determine significance of specific time points against t-1.

Experiment 3: Ovariectomized SERT$^{+/-}$ rats exhibit prolonged hot flash-associated increases in tail skin temperature in response to hypercapnic gas infusion

An ANOVA with genotype as the main factor and time as a repeated measure revealed that hypercapnic gas infusion produced a prolonged increase in tail skin temperature in ovex rats [**Fig 24a**-Representative thermal image; **Fig. 24b**- CO_2 tail skin temperature (n=7,8) overall *time effect* $F_{(13,392)}$=47.8, p<0.0001 and overall *genotype x time interaction* $F_{(15,180)}$=2.1, p=0.013]. Fisher's Least Significant Difference Test was used for post hoc testing to determine specific time point differences, and Dunnett's test was used to determine significance of specific time points against t-1.

Discussion

These studies have demonstrated that an anxiogenic stimulus can precipitate a hot flash-associated increase in TST responses in OVEX rats that is not an artifact of experimental environment. Our studies provide potential insights into triggers of hot flashes in thermoneutral environments and evidence that anxiety or anxiety-generating stimuli may have a causal relationship to hot flashes, not merely an associative one. Earlier clinical provocations used the drug yohimbine to facilitate hot flashes (Freedman et al., 1990), which, at high doses, elicits strong anxiety (Charney et al., 1984; Charney and Heninger, 1986), and also produces robust TST responses in OVEX rats (Morimoto et al., 2011), and thus yohimbine may represent a pharmacological model preclinically and clinically. Other studies have previously documented that stress-related stimuli, including mental arithmetic or emotionally salient films, can increase the rate of objective and subjective hot flashes (Swartzman et al., 1990) and self-reports implicate stressful stimuli (e.g. interpersonal family conflict or social stress) precipitate hot flashes in breast cancer patients (Stubbs et al., 2008). These data complement the emerging literature relating to the role of anxiety and

stress-related factors in hot flashes and may help to start addressing a critical gap in knowledge of hot flash triggers.

In our preclinical rodent experiments, the results exhibit validity in three domains: 1) face validity, as the increase in TST is a rapid heat loss mechanism (cutaneous vasodilation) analogous to and what would be expected during a hot flash; 2) construct validity, as the TST change is exaggerated in ovariectomized rats, which is a model of surgical/oophorectomy-induced menopause in women (a condition with more severe hot flashes (Gallicchio et al., 2006b; Benshushan et al., 2009)); and 3) predictive validity, as the same stimulus causes an exacerbated response in ovariectomized $SERT^{+/-}$ rats, which mimic a common human polymorphism that was recently linked to increased hot flash pathology (Grochans et al., 2013). A small study of 20% CO_2 exposure in symptomatic menopausal women by collaborators in the IU School of Nursing (Dr. Janet Carpenter and colleagues) demonstrated that this stimulus also causes self-reported and objectively-verified (sternal skin conductance) hot flashes (see **Table 2** and **Fig. 25** in Commentary for greater discussion), adding additional validity to our modeling approach.

This modeling approach is unique in that the provocation does not use pharmacological manipulations; rather, it triggers an endogenous challenge to homeostasis by provoking air hunger through CO_2 exposure (which causes acidosis). Currently, the role of blood pH in hot flashes is largely unknown, as only one small study demonstrated a statistically significant, yet very slight, pH reduction following spontaneous hot flashes (Aktan et al., 1998). Paced respiration (which could moderate blood pH) such as controlled breathing of 6-10 breaths per minute, is often recommended as a non-pharmacological treatment for reducing hot flashes, yet clinical trials have not demonstrated significantly greater efficacy than usual treatment or active comparators (Carpenter et al., 2013; Sood et al., 2013; Huang et al., 2015).

Here our challenges utilized systemic administration through environmental exposure, necessitating inhalation leading to systemic exposure. Dissection of the central and peripheral components of the CO_2-induced hot

flashes and TST responses in rats is beyond the scope of this investigation, but the paradigms we used most likely triggered central mechanisms, as previous applications were used to elicit and study anxiety in humans and in rodents that are accompanied by sympathetic associated cardiovascular responses. Evidence for a central role comes from *ex vivo* assessments of cellular responses in rodents where 20% CO_2 exposure increases activity in anxiety and thermoregulatory associated brain circuitry such as the hypothalamus (Johnson et al., 2005, 2011), and noradrenergic and serotonergic neurons in the midbrain pons and medulla (Johnson et al., 2005). However, CO_2 has also been shown to have local cutaneous vasodilatory action in both humans and rodents (Diji, 1959; Ito et al., 1989) (40, 41). Thus, there are likely central and peripheral contributions to hot flashes (Shuto et al., 2011).

Our third study also contributes to an emerging literature concerning the *5-HTTLPR* on climacteric symptoms and anxiety trait vulnerability. Short form '*s'* carriers (*s/s* or *s/l* genotype) of the *5-HTTLPR* experience higher rates of psychiatric disorders later in life, including anxiety and depression (Lesch et al., 1996; Hauser et al., 2003; Gonda et al., 2009), which are two conditions that have been linked to increased hot flashes (Avis et al., 2015; Gallicchio et al., 2015). Analyses from the multiethnic Study of Women's Heath Across the Nation Mental Health Study found that early life stress (childhood abuse or neglect) also predicted later vasomotor symptoms (Thurston et al., 2008a). Menopausal women with the short form ('*s'* carrier) of the *5-HTTLPR* also experience increased climacteric symptoms (Grochans et al., 2013), which could be related to increased anxiety traits associated with this polymorphism. This animal model could contribute mechanistic insight into the interactions between early life events, genetic contributions, and later anxiety and physiological phenotypes, including hot flash-associated responses, which may ultimately lead to a better understanding of the role of serotonin and its complement of proteins to menopausal symptoms.

Indeed, this is an important research area in women's health at midlife, as drugs that target the serotonin transporter are among the most frequently used

pharmacotherapies for vasomotor symptom relief and currently represent the only FDA-approved non-hormonal treatment for hot flashes (Simon et al., 2013; Orleans et al., 2014). Despite this indication, SSRIs do not fully alleviate hot flashes and treatment switching is common due to tolerability issues (Handley and Williams, 2015). Some of this variability in response and side effects may be due to differences in SERT genotype, as persons with the s/s genotype have been shown to have a poor response to SSRIs (for treating anxiety disorders) (Stein et al., 2006). Specifically, *s/s* carriers are at greater risk for treatment-emergent side effects from these medications (Perlis et al., 2003). Investigations into *5-HTTLPR* genotype by hot flash treatment efficacy could prove useful in advancing personalized medicine by informing treatment strategies, though to our knowledge, no such studies have been performed. This is likely to be a substantial challenge, as there is evidence that the effect of the polymorphism can be complex and modulated by ethnic background; for example the *'l'* allele, as opposed to the *'s'* allele, confers increased risk for anxiety in a Chinese population (Long et al., 2013).

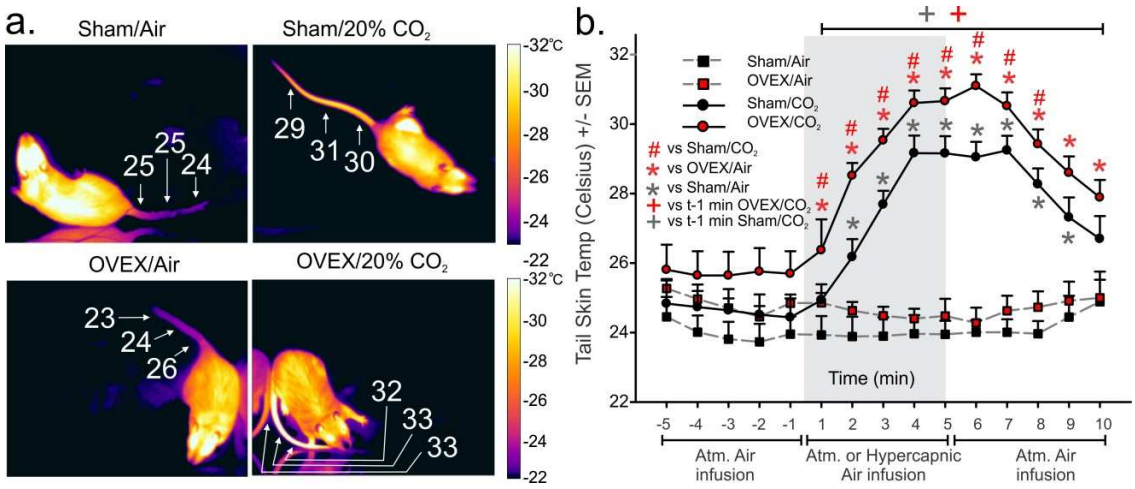


Figure 23. *Rats Modeling Surgical Menopause Have Exacerbated Hot Flash-Associated Tail Skin Temperature Responses*. Effects of ovariectomy or sham surgery on baseline tail skin temperature in response to hypercapnic or atmospheric air infusion. **a)** Representative thermal images with scale (to the right) of a sham-OVEX (top) or OVEX rat (bottom) after 5 min exposure to atmospheric air (left) or 20% CO_2 (right). **b)** Line graph with error bars (SEM) represents mean tail skin temperature prior to and following atmospheric air or hypercapnic gas challenge in OVEX or sham-OVEX groups assessed with a tail thermistor at the base of the tail (n=15/group for CO_2 challenge and n=6/group for atmospheric air challenge). *denotes significance of surgical treatment at specific time points in (b) with Fisher's Least Significant Difference test protected by an ANOVA, and + denotes significant differences over time from t-1 as measured by Dunnett's test.

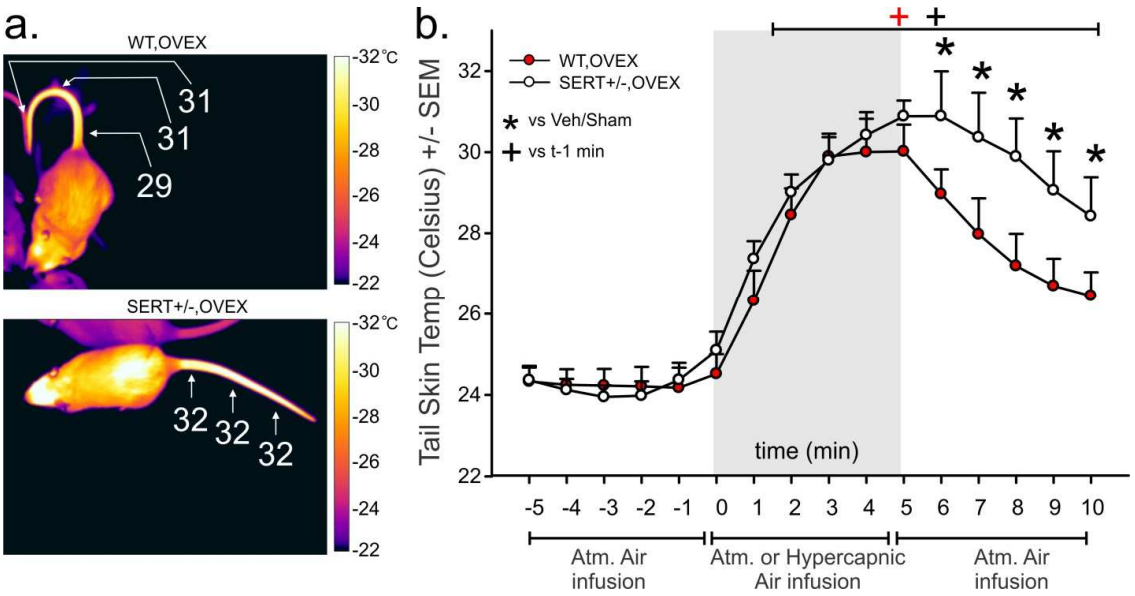


Figure 24. *Rats with a Heterozygous Null Mutation of the Serotonin Transporter (SERT$^{+/-}$) have Prolonged Hot Flash-Associated Tail Skin Temperature Responses to Hypercapnic Gas Infusion.* Effects of ovariectomy in SERT$^{+/-}$ and WT rats tail skin temperature in response to hypercapnic gas infusion. **a)** Representative thermal image with scale (to the right) of a WT-ovex (top) or SERT$^{+/-}$ rat (bottom) after 5 min exposure to 20% CO_2. **b)** Line graph with error bars (SEM) represents mean tail skin temperature prior to and following hypercapnic gas challenge in ovex SERT$^{+/-}$ or WT rats assessed with a tail thermistor at the base of the tail (n=8,7). *denotes significant effect of estrogen treatment with a two-way ANOVA p=0.001 in (a). *denotes significance of genotype at specific time points in (c) with Fisher's Least Significant Difference test protected by an ANOVA, and + denotes significant differences over time from t-1 as measured by Dunnett's test.

Commentary

Overall statement of significance

Hot flashes are a beguiling phenomenon, and research to date has yet to determine how estrogen loss leads to hot flashes and how estrogen replacement relieves them (Kronenberg, 2010). Undoubtedly, the mechanisms that mediate these effects are likely to be complex, and elucidating such complexity through clinical investigation exclusively is likely to be unsuccessful, if not impossible, and certainly untimely. Therefore, an alternative tool is needed, and that is where animal modeling can contribute to understanding not only hot flashes, but other menopausal symptoms. My studies demonstrate key roles for anxiety-associated pharmacological stimuli/triggers and anxiety states in animal models that meet key criteria for validity. The elucidation of potentially critical brain structures and novel neurochemical systems provides many avenues for future investigation to better understand how dramatic loss of sex steroid hormones leads to hot flash vulnerability, and these findings could lead to the development of novel non-hormonal treatment strategies for women that desperately need them. Furthermore, while striking similarities between panic, anxiety, and hot flashes have been identified in the literature, direct evidence was lacking. The studies in this work provide evidence that anxiety or panic-provoking stimuli (depending on the dose) can elicit rapid and profound hot flashes in a model of simulated/surgical menopause. These findings further strengthen the link between anxiety and hot flashes that has been reported in many well-controlled longitudinal studies, and it is my hope that future clinical studies will stringently examine this link in order to elucidate the details mediating this relationship.

Brief recapitulation of studies

Based in part on early observations of hypothermia occurring during panic provocations, and later verification of surface/tail temperature changes, I explored the relevance of anxiety-associated factors in hot flash induction in ovariectomized rats. The studies in the major part of this work have focused on

understanding the neural circuits and associated neurochemicals through a novel hot flash provocation, a low dose of an anxiogenic drug, the beta carboline FG-7142 (a $GABA_A$ receptor partial inverse agonist at the benzodiazepine allosteric site). My studies demonstrated this provocation leads to a rapid and profound increase in tail skin temperature and concomitant drops in core body temperature in ovariectomized rats, but not sham controls or estrogen-replaced rats, providing evidence of face, construct, and post-dictive validity. Importantly, FG-7142 provokes anxiety and hot flashes in humans [for review, see (Evans and Lowry, 2007) and (Dorow et al., 1983)], though menopausal women's sensitivity to it is unknown. Furthermore, this approach implicated GABA in hot flashes. As discussed in the introduction, GABA has not been a major target of basic research in hot flashes, and clinical investigation is extremely limited. A handful of small studies have examined the efficacy of the GABA analogue gabapentin (largely in cancer patients), which may increase GABA content in the brain, for the amelioration of hot flashes and found efficacy. However, only one study examined the effectiveness of a benzodiazepine on hot flashes in a cohort of women with oophorectomy and used an early benzodiazepine (oxazepam); there have been no subsequent studies of any benzodiazepine in a larger sample of naturally menopausal women or cancer patients.

To elucidate the neurochemistry and circuitry involved in mediating the hot flash-associated response, I performed a series of immunohistochemical studies. The results of the initial study (single labeled c-fos) revealed hyperactivity in drug-treated ovariectomized rats in key sites, including the dorsomedial/perifornical hypothalamus, locus ceruleus, raphe pallidus, rostroventrolateral medulla, and dorsoventral-ventrolateral periaqueductal gray, key thermoregulatory structures in animals (Morrison and Nakamura, 2011). Brainstem structures have been shown to activate before the perception of a hot flash, but resolving the precise nuclei is currently beyond the limits of detection, as the voxel size (spatial resolution) currently exceeds some small nuclei (Diwadkar et al., 2014). Therefore, it is possible that the raphe pallidus and rostroventrolateral medulla are activated, but this is another unknown. Drug

effects in both ovariectomized and sham-ovariectomized rats revealed heightened activity in the central nuclei of the amygdala, which has also been implicated in animal models of thermoregulation, and may be particularly relevant in the context of emotion-related flushing (Yu and Blessing, 2001). Secondary neurochemical phenotyping studies confirmed the noradrenergic identity of A6 locus ceruleus neurons and serotonergic neurons in the RPa and RVLM, further adding to the construct validity of the model. The c-fos hyperactivity in the dorsomedial/perifornical area of the hypothalamus, a thermoregulatory and panic-generating site, co-expressed orexin-A, and thus revealed the orexin peptide system as a potential novel target in hot flash-associated tail skin temperature responses.

Subsequent experiments targeted the orexin system by blocking receptors with systemically administered antagonists to determine if this could decrease both flushing and anxiety behaviors. Pre-treatment with a dual orexin receptor antagonist (DORA-12), completely blocked the response to FG-7142. Blockade of either the orexin-1 receptor (OX1R) or orexin-2 receptor (OX2R) with selective, well-characterized, brain-penetrant antagonists prior to hot flash provocation partially attenuated the increased tail skin temperature. An initial assessment of anxiety-related behaviors in ovariectomized rats found that pretreatment with the selective orexin-1 receptor antagonist (SORA1) SB-334867 attenuated ovariectomy-induced anxiety. Further experiments using an improved SORA1 (Compound 56, donated by Janssen Research and Development) or DORA-12 that were successful in attenuating hot flash-associated responses also proved anxiolytic, yet in these cohorts there were no differences in baseline anxiety behaviors between ovariectomized and intact rats. Difficulties in assessing sex-specific behaviors are discussed below.

A final series of studies utilized a different type of anxiety induction, hypercarbic gas, which elicits hypercapnia (decreased blood pH/acidosis), in conjunction with animals predisposed to greater levels of anxiety behaviors. This approach models an interoceptive stimulus, or one that does not reach conscious appreciation under baseline conditions. A 5 min challenge of 20% CO_2-enriched,

normal oxygen content air elicited a profound increase in tail skin temperature that was greater in ovariectomized rats compared to intact controls. Importantly, atmospheric air did not change tail skin temperature, so the effects observed were not due to the novelty of the experimental environment. To validate this modeling approach and determine if the sensations evoked were consistent with a naturally-occurring hot flash, our collaborators (Dr. Janet Carpenter, Sarah Dorsey, and Connie Krier) tested two similar CO_2 inhalation paradigms in a small sample of highly symptomatic menopausal women. Overall, 5/6 women experienced a mild hot flash in response to breathing 20% CO_2 (40 sec) or taking two vital capacity inhalations of 35% CO_2, as shown in **Fig. 25**. Participants reported that these sensations were consistent with a hot flash, and all subjectively reported hot flashes were verified with an objective hot flash monitor (sternal skin conductance). Interestingly, the subjective intensity of the hot flash response and anxiety rating following CO_2 inhalation positively correlated with diary-reported daily hot flash frequency. Lastly, and as another validation of CO_2 hot flash induction, I tested for increased hot flashes in ovariectomized (serotonin transporter) $SERT^{+/-}$ and $SERT^{+/+}$ rats, and found a prolonged response to 20% CO_2. This deletion mimics a human polymorphism of the SERT that is linked to increased anxiety and increased climacteric symptoms in menopausal women (Grochans et al., 2013). This series of studies represents some of the first evidence that direct manipulation of anxiety leads to hot flashes, and may be a useful method of provoking hot flashes in the clinical setting.

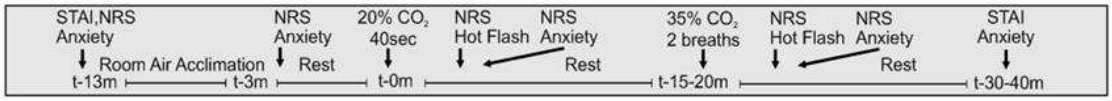
Critique of modeling approach

Methodological considerations

Ovariectomy

Animal models, like all models, have limitations, and this one is no exception. The induction of menopause by ovariectomy is highly clinically

a.



b.

Subjects	Age	7 Day Daytime Hot Flashes	7 Day Nighttime Hot Flashes	Average Daily Hot Flashes	STAI Anxiety baseline	NRS Anxiety baseline	NRS Anxiety Control Air	NRS Hot Flash post 20%/35%CO2	NRS Anxiety post 20%/35%CO_2	NRS Anxiety Rest	STAI Anxiety Rest
1	46	25	16	5.86	40	1	1	3/0 (1.5)	2/0 (1)	0	40
4	49	24	23	6.71	40	0	0	0/1 (0.5)	3/1 (2)	1	39
5	53	44	34	11.14	40	5	6	-/4 (4)	-/7 (7)	7	40
6	52	55	24	11.29	40	0	3	4/- (4)	4/- (4)	0	40
3	49	95	20	16.43	40	0	0	5/- (5)	9/- (9)	0	39

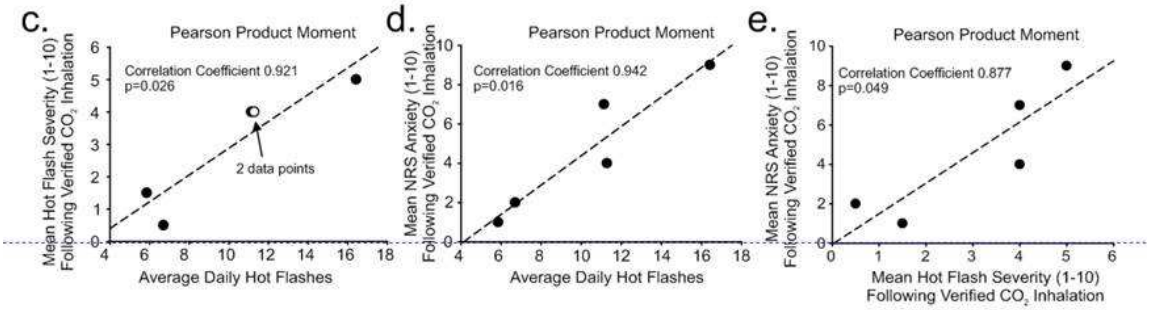


Table 3 and Figure 25. *Schematic of Clinical Study of Hot Flash Provocation with CO_2 Challenges in Highly Symptomatic Women and Participant Characteristics*. **a)** Depicts the timeline for the study procedures. **b)** Table illustrates subjects assigned number, age, total number of hot flashes during the day and night for a seven day period prior to the study, average number of hot flashes per day, baseline STAI and NRS anxiety, NRS anxiety post control air, NRS hot flash and anxiety (respectively) severity post 20% and 35% CO_2 inhalation, followed by the NRS Anxiety and STAI anxiety at rest. Participants are ranked in order of increasing daily hot flashes. Three of the four participants with confirmed inhalations of 20% CO_2 reported hot flashes within 5 min of the challenge, and two of the three participants with confirmed inhalations of 35% CO_2 reported a hot flash within 5 min of the challenge; hot flashes were rated from mild to moderate. Numbers below NRS hot flashes or anxiety (columns 9 and 10, respectively) post CO_2 respectively represent 20% CO_2 and 35% CO_2 with the parenthetical number representing the mean of the confirmed inhalations ("-" indicates no confirmed inhalation for respective challenge). Correlation analyses revealed that the frequency of daily hot flashes (total per 24h day) was positively correlated with the **c)** mean severity of the hot flash post confirmed CO_2 inhalations and **d)** mean severity of the anxiety response post confirmed CO_2 inhalations. Correlation analysis also demonstrated that the **e)** mean hot flash severity following verified CO_2 inhalation was positive correlated with the mean anxiety rating following verified CO_2 inhalation.

relevant, as women that undergo this procedure for a variety of medical reasons, including cancer prevention and/or treatment, have rapid and severe hot flashes. From this perspective, it has high utility. In the United States, an estimated 2,000,000 women reach menopause every year; about 300,000 of these women have ovarian removal (half for ovarian cancer prevention, half for "benign disease"), so approximately 15% of menopausal women are acutely menopausal (Parker et al., 2005; Rocca et al., 2006). Furthermore, for translational reasons, I elected to use freely cycling, sham-ovariectomized rats as controls in the majority of my experiments. This choice was more relevant clinically, as oophorectomized women are not frequently given estrogen replacement. However, freely cycling controls were a source of greater and often unequal variability because I did not assess stage of estrous to perform testing at particular stages. Future studies could control for stage of the estrous cycle, determine differential responses relating to phase of estrus. Recent work in this laboratory has shown this is especially relevant for behavioral studies, which sometimes include an estrogen replacement group.

Timing and Treatment Rationale

All of my testing was done within a set time frame following recovery from ovariectomy, starting 12 days after surgery. Estrogen loss is accomplished within 48 hours following ovariectomy, but this longer time frame was chosen based on previous work in these laboratories that demonstrated an induction of anxiety at this time frame. The initial focus of this project was anxiety, not hot flashes, and decisions were made in this context at first. Then, initial attempts at modeling hot flashes with CO_2 were successful at this time point, and so were continued in subsequent experiments for consistency. Yet, based on the clinical literature, hot flashes eventually subside for *most* women. It would be of great interest and further validate the model if vulnerability to these provocations were assessed over time and found to subside as time from ovariectomy increased.

Similarly, the doses of estrogen used in the initial study that established vulnerability to FG-7142 were supraphysiological (0.25 mg/kg/day), and were,

chosen in the context of efficacy for relief of ovariectomy-induced anxiety behaviors from previous work in the laboratory. Therefore, I used this dose in the model, and it blocked the increase in tail skin temperature in this provocation. While it is high, it parallels studies of estrogen replacement in symptomatic women. The current guidelines for hormonal therapy recommend the lowest possible dose for the shortest period of time (North American Menopause Society position statement), and clinical studies have examined the effectiveness of different doses of estrogen therapies. While lower doses (e.g., 0.30 and 0.45 mg/day) of estrogen replacement reduce hot flashes greater than placebo, the standard dose (0.625 mg/day) is still more effective at reducing hot flashes (compared to both doses), and is the only treatment that provided complete relief (Utian et al., 2001).

Provocation Techniques

Pharmacological Provocations

My methods of provocation are mostly pharmacological (yohimbine, FG-7142, d-fenfluramine) with the exception of CO_2. While these provocations may seem artificial, all have demonstrated to induce anxiety responses and flushing ability in humans, and symptomatic menopausal women exhibit vulnerability to yohimbine (Dorow et al., 1983; Charney et al., 1984; Freedman et al., 1990). Furthermore, the pilot study of CO_2 challenges described in **Ch. 3** in symptomatic menopausal women in Dr. Carpenter's laboratory provide evidence that an interoceptive stimulus may be a real trigger of hot flashes; the women reported that CO_2 inhalations "felt just like a hot flash".

Interestingly, I also challenge the $SERT^{+/-}$ rats with FG-7142 (3 mg/kg, i.p.) and d-fenfluramine (5 mg/kg, i.p.) and found no genotype differences to either challenge. It may be that the $SERT^{+/-}$ rats are sensitive to some, but not all, anxiogenic stimuli, and the defect in their serotonergic system could underlie that difference. Serotonin neurons are known to be chemosensitive and responsive to CO_2 (Hodges and Richerson, 2010), and so this difference may represent a

proclivity toward stimulus-dependence. Furthermore, and unlike the first series of studies using Sprague-Dawley rats, these rats were pair-housed with littermates (due to the breeding procedures), and so perhaps the lack of differences is due to reduction of isolation stress, to which these rats may be particularly vulnerable.

Measurement Technique

During the provocations, the animals were non-restrictively tethered with a thermistor for many of the physiological experiments, which permitted a quick, inexpensive, and easy to use assessment of tail skin temperature. Because these experiments were not assessing behavioral responses, this type of tethering is a minor concern. Experiments where I used implanted telemetric probes with internal thermistors (allowing the animals to move completely freely and monitor core and tail temperature simultaneously) provided comparable flushing results. These probes would be ideal for both long-term periodic assessment of provocation vulnerability and continuous monitoring over several days or even weeks. The current design of the probe, even with customized modifications, can slip out of the tail and provide a measurement of core temperature; methodological refinement or more sophisticated implants may eliminate the need for tethering with thermistors.

Difficulties in assessing anxiety behaviors

There are several critical problems to assessing anxiety behaviors in animals, and additional complications in working with female rats. First, the tests most commonly used to assess anxiety are dependent upon locomotion, which can easily be confounded. For example, high doses of caffeine are anxiogenic, and an experiment in our lab administered a large dose of caffeine to rats. The rats were placed in the center of the open field arena, and did not move for the duration of the test. Conventions of the test dictate that the greater time spent in the center and away from the walls of the box indicate less anxiety-related behavior, yet this is a known anxiogenic drug. These types of testing only

provide fairly crude measures and cannot measure internal state or emotions, only overt evidence of them. Second, there are critical sex differences that make testing anxiety behaviors more or less feasible in female vs. male rats. For example, one of the more sensitive anxiety tests (in males) is the Social Interaction tests, wherein two rats are placed in an arena, and the duration and nuances of time spent interacting (scoring the test rat) is quantified. However, this test is difficult to perform in females because of interference from the estrous cycle (Frye et al., 2000), requiring that stage of estrous is controlled for, which eliminates natural sources of variability that were not controlled for in different experiments. There is also conflicting evidence that estrogen is actually "anxiogenic" in the SI test, or at least when measured by spatial parameters (Koss et al., 2004). Another example is the assessment of conditioned fear, measured by the time spent freezing after a tone (conditioned by a subsequent mild shock); females exhibit less freezing behavior when re-exposed to the conditioning chamber (Maren et al., 1994). Additional groups have demonstrated that both contextual freezing and cue-induced freezing is estrus-stage dependent (Markus, 1997; Milad et al., 2009). Finally, in my hands, the anxiety state induced by ovariectomy proved to be inconsistent (or at least was not always adequately reproduced in the behavioral tests that I used). Initial studies demonstrated a decrease in exploratory behaviors in the open field test (and was prevented by SB-334867), but further studies failed to find anxiety-like behavior consistently, with some tests showing differences in anxiety between surgical groups but other tests were the same. While other orexin receptor antagonists tested in these studies were anxiolytic, there were no interactions with surgical treatment. All tests were performed within the same time frame after surgery, and so observed differences in anxiety could have been due to experimenters and/or any handling procedures between experiments.

Recent work in the laboratory (by Aline Abreu Rezende) has focused on developing more sophisticated behavioral analyses; specifically, the elevated T maze (ETM) and defensive burying. The first test can give measures of escape and avoidance behaviors, and is a multi-day, multi-trial protocol commonly used

to assess panic-associated behavior. Early evidence for ETM's use in assessing behavior in females looks quite promising (see **Figure 26** on next page), and has shown estrus stage-dependent effects. Another more ethologically relevant test is also being developed for use in females, defensive burying. This test examines active (burying) versus passive (avoidance) coping by placing a shock probe in a cage of bedding, and quantifying time spent avoiding the probe and/or burying the probe.

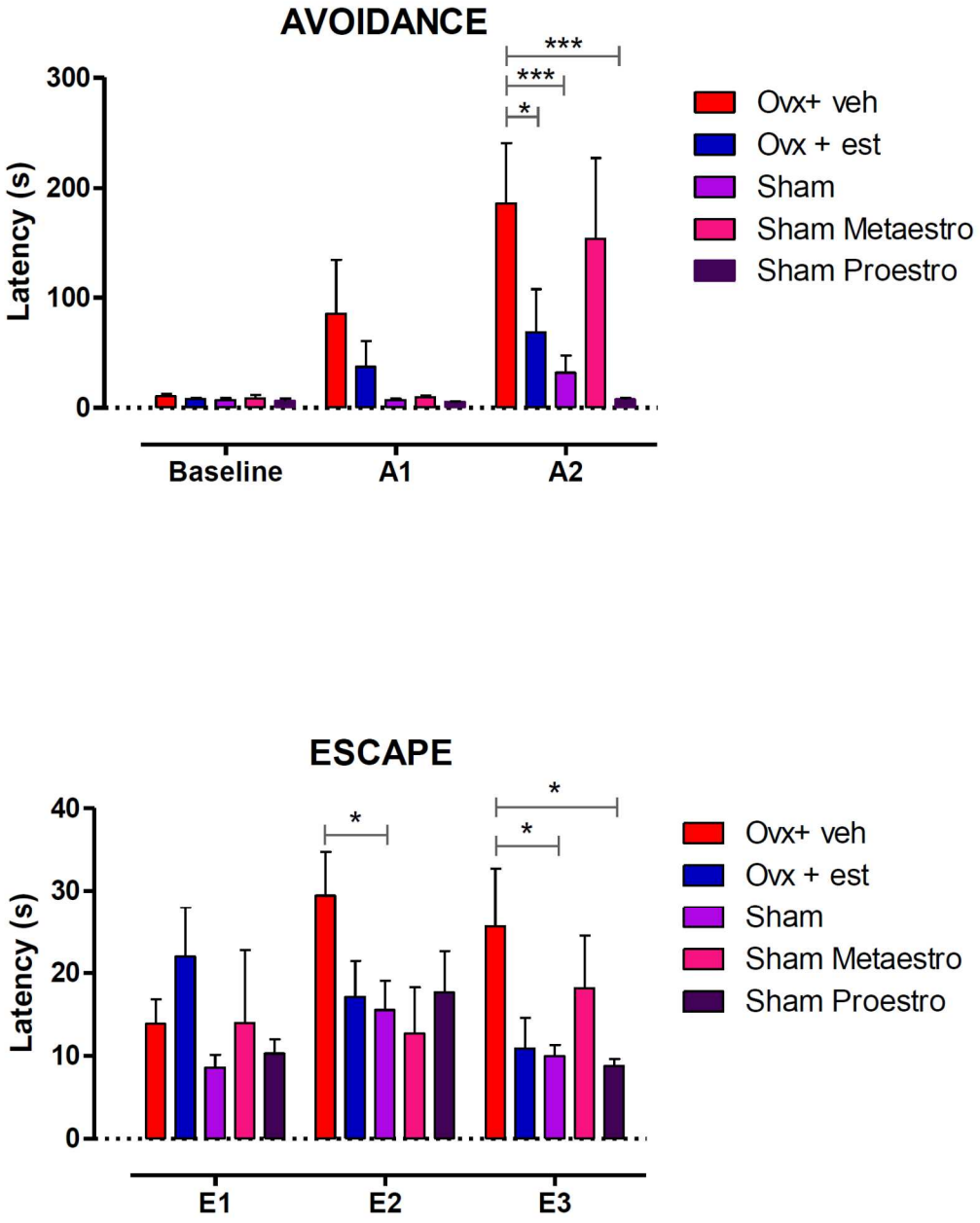


Figure 26. *The Elevated T-maze Test of Anxiety-Associated Behaviors in Female Rats Across the Estrus Cycle.*

Future Directions

Manipulation of neurochemical systems

While these models exhibit critical face, construct, predictive, and postdictive validity (estrogen replacement), additional validation should be performed. As mentioned above, assessing the long-term vulnerability to the FG-7142 challenge and other neurochemical challenges would add to the validity of the model, particularly if there is a time-dependent vulnerability. The immunohistochemical studies that I performed using c-fos and neurochemical phenotyping demonstrated construct validity by implicating the serontonergic and noradrenergic systems, but these systems have not yet been systematically manipulated and tested for effects on hot flash-associated responses.

Future studies should determine what foci of these systems are necessary for hot flash provocation or if stimulation of any particular sites are sufficient to cause a hot flash on their own. This could be achieved with neurochemical lesioning techniques (i.e. saporins targeting the serotonergic system) in a site-specific manner (i.e. the raphe pallidus or dorsal raphe nucleus) followed by baseline assessment and pharmacological provocation. Specifically, the targets that were indicated by the immunohistochemical studies could be removed, including the perifornical hypothalamus, rostral raphe pallidus, rostroventral lateral medulla, and dorsal raphe. Following this, baseline temperature assessment should be performed, and the ability of a particular provocation (i.e. FG-7142, CO_2, or a thermal provocation, for example) could be determined. Presently, these techniques are being validated in this laboratory for different research questions involving panic and anxiety, and implications for hot flash vulnerability are as yet unanswered.

Refinement of Orexin's Role in Hot Flash Associated Responses

Based on prior evidence in this laboratory, it seems that orexin's role in thermoregulation may be complicated. Results from panic studies indicate that antagonizing the orexin system prior to panic provocation with sodium lactate or

CO_2, respectively, can attenuate both an increase *and* decrease in core body temperature. These studies both had dramatic increases in orexin levels, either by the provocation itself (CO_2) or as a mechanism (by decreased GABA in the PeF through an inhibitor of GAD (Johnson et al., 2010). Extant literature reports similarly divergent effects on core temperature and tail skin temperature when directly injecting orexin into the brain. For example, a 2 µg i.c.v. injection of orexin-A in cold-adapted, conscious *female* rats elicited significant hypothermia and an increase in tail skin temperature to approximately 30 °C in a 15 °C room with no change in metabolic rate (Balasko et al., 1999). Unfortunately, the data analysis was limited, with only a representative trace shown and no reporting of group data or the statistical significant of tail skin temperature changes. Another group, after injection of 280 pmol of OX-A i.c.v., induced a 1.3 °C hypothermia in conscious, male rats 60 min following injection, but did not assess tail skin temperature (Jaszberenyi et al., 2002). Perhaps the strongest evidence that supports the role of OX-A in eliciting hypothermia and consequent flushing comes from a microinjection study wherein a 30 pmol injection of OX-A into the raphe pallidus (in conscious rats) elicited a strong tail flush within 60 minutes (Luong and Carrive, 2012) and no overt change in body temperature (measured primarily with thermography).

However, a number of other studies have demonstrated hyperthermia following i.c.v. OX-A injection (Monda et al., 2001, 2003a, 2003b, 2004, 2005). In contrast to Luong and Carrive's study, a study of OX-A microinjections into the raphe pallidus (under cool body and skin conditions) elicited increased BAT thermogenesis and sympathetic nerve activity, and increased core body temperature (Tupone et al., 2011). In this particular case, TST did increase significantly, but baseline values were in excess of 35 °C, so it is impossible to discern the magnitude of the contribution of OX-A to TST. Importantly, these studies were performed under anesthesia and on a heating pad, so it is possible that these conflicting reports could be due to the experimental procedures. Another important point is the handling (picking the rats up) and data fragmentation involved in many of these studies, due to relatively crude

measures of assessing core temperature using rectal probes instead of implanted, telemetric devices for continuous measurement. These factors are likely causing stress-induced hyperthermia that could be confounding the meaning of the results. Most importantly, some of the conflict could be due to dose; Monda's group consistently used 1.5 nmol/5 µL injections (and male rats) whereas Balasko's group used 2 µg/5 µL injections; perhaps at very high doses, hypothermia occurs; according to Monda, his dosing regimen produces a sub-maximal dose for hyperthermia. An important series of experiments to address these discrepancies include 1) replicating an OX-A flush in both normal male and female rats (across the estrus cycle, ideally) in a dose-response design; and 2) determining whether ovariectomized rats have an exacerbated TST response to an OX-A infusion compared to sham or estrogen-replaced controls. Collectively, it seems clear that orexin regulates tail skin vasomotion, and future studies will be necessary to determine precisely how this is occurs under normal physiological conditions and following precipitous hormone loss.

Identification of key nodal sites for orexinergic activity in hot flash responses

Results from neurochemical studies consistently implicated the orexin system and its output sites as hyperactive in ovariectomized rats treated with FG-7142. Thoughout this work, every attempt has been made to use selective orexin receptor antagonists with high brain penetrance/receptor occupancy that are relatively sustained. As I have always delivered these drugs systemically in testing their potential therapeutic efficacy, I have not delineated the sites within the orexin system (e.g. perifornical hypothalamus versus raphe pallidus or dorsal raphe) that are critical for the tail skin temperature response. One approach to determining the necessity of a given site could be an orexin-conjugated saporin, though previously available molecules have been removed from the market.

Identification of hypothalamic mechanisms

This project has not yet definitively identified critical mechanistic targets in the hypothalamus. An initial microarray analysis of GABA and glutamate-related

genes were performed (with tissue punched from the perifornical hypothalamus in ovariectomized or sham-ovariectomized rats), and significantly altered targets revealed the 2A subunit of the NMDA-type glutamate receptor (up-regulated in ovex rats) and mGluR4 (a Group III mGluR; down-regulated in OVEX rats). I performed a pre-treatment study with the NMDA receptor antagonist memantine, and a moderate dose (5 mg/kg, i.p.) appeared to attenuate flushing induced by FG-7142. Interestingly, in a few rats, this treatment combination elicited seizure-like events, with hyperlocomotor activity and what can best be described as backflips, consistent with prior work [see (Loscher and Honack, 1990)]. Importantly, memantine is a nonselective drug and has effects at multiple targets. It is an antagonist at 5-HT_3 receptors and nicotinic acetylcholine receptors (α7) and an agonist at the dopamine D_2 receptor *in vitro* (Rammes et al., 2001; Aracava et al., 2005; Seeman et al., 2008). Stimulation of these targets probably mediates some of these effects.

Secondly, to examine the contribution and/or potential therapeutic efficacy of augmenting metabotropic glutamate receptors in hot flash provocation, I performed a study using a systemically-administered mGluR2 (a G_i-coupled, pre- and postsynaptic receptor that can also couple with ERα) positive allosteric modulator, CBiPES. This was a treatment that our laboratory had previously shown to be effective in reducing sodium lactate-induced panic-associated physiological and behavioral responses in an animal model (Johnson et al., 2013). Intriguingly, systemic CBiPES pre-treatment caused an immediate, high-amplitude increase in tail skin temperature in OVEX rats; yet, after returning to baseline, the hot flash-associated response provoked with FG-7142 was *attenuated* in the CBiPES-treated group. The distribution pattern of mGluR2 may provide some clues. mGluR2 is heavily expressed in several nuclei of the amygdala, including the basolateral, basomedial, and terminals of the central part of the lateral amygdala also express mGluR2 (Ohishi et al., 1993, 1998; Gu et al., 2008). As demonstrated in **Ch. 2, Fig. 18c,** the central amygdala was responsive to hot flash provocation, which suggests that this area may contribute to emotionally-relevant flushing. This latter point suggests that future studies

should use site-specific treatments to modulate mGluRs within the amygdala and4 finely explore the relevance of these receptor systems with respect to thermoregulation, which would answer more basic, rather than translational, questions.

Indeed, the role of the amygdala in thermoregulatory responses overall may be especially meaningful in light of emotionally-relevant stimuli. A neuroimaging study of 12 volunteers exposed to fearful faces (and non-face scenes of the same emotional valence, in additional to geometric shapes controls) found that skin conductance (the most commonly measured clinical indicator of a hot) and blood-oxygen level dependent (BOLD) fMRI responses in the amgydala increased in response to the faces only (Hariri et al., 2002). The amygdala response to fearful faces can be potentiated with a subthreshold dose of the anxiogenic drug, dextroamphetamine (Hariri et al., 2002b). It appears that thermoregulatory sweating (sweating induced by temperature increase) is dissociable from emotional sweating, which occurs predominantly on the palms and soles. In patients with Idiopathic Pure Sudomotor Failure, a condition in which thermoregulatory sweating is absent, emotional sweating is preserved (Nakazato et al., 2004). Conversely, a case report of a 21-year old woman with limbic encephalitis/mesial temporal lobe lesions found reduced emotional sweating in response to tasks including mental arithmetic, exercise, or tactile sensation that was recovered as she improved clinically (Asahina et al., 2011). Similarly, patients with Urbach-Wiethe Disease, a rare condition in which the amgydala is calcified, exhibit blunted skin conductance responses in anticipation of a CO_2 inhalation (Feinstein et al., 2013). The distinction between emotional and thermoregulatory sweat responses in hot flashes is an outstanding and unanswered research question. The localization and distinction between different inductions of sweating could be highly relevant to clinical studies of hot flashes, as skin conductance is often assayed in discrete areas, often the chest. Measuring skin conductance on the palms or soles of the feet could reveal significant information about the nature of hot flashes induced by different paradigms (i.e., CO2 inhalation compared to warming techniques). Further, this

could be related to the affective dimensions of hot flashes, such as through standardized assessments of anxiety given after each of a series of challenges.

Tamoxifen

Lastly, because hot flashes are a serious side effect of some cancer treatments, this project could branch into exploring hot flash pathology in menopause induced by tamoxifen treatment. Prior work in this lab demonstrated anxiety-like behavior following a short course of tamoxifen treatment in female rats (unpublished data), but this work was discontinued as the animals lost weight and appeared to be unwell. However, improved methods of administration with subcutaneous pumps and refinement of dosing may improve outcomes. This investigation could explore differences in the neural circuitry and neural mechanisms between the two models, and any sensitivity to the current pharmacological flushing agents would add validity to the vulnerability to challenges in rats with ovariectomy. It may be that there are particular differences that would lead to more specialized targets despite precipitous estrogen loss of function in both cases; for example, in tamoxifen-treated women, the circadian periodicity to hot flashes has not been found (Carpenter et al., 2001). Additionally, though, there are groups working on developing endoxifen directly, so non-hormonal treatment options may be greater for cancer patients if these efforts are successful.

Gaps in knowledge: Clinical literature

Hot flashes have only been subject to serious investigation for about the last 25-30 years, and much of the early literature was highly descriptive and involved small numbers of participants. It was not until the early 1990s that large, prospective cohort studies (such as the WHI and SWAN) started to be assembled and studied over many years. Clearly, such studies are expensive and require coordination of multiple investigators over many sites to ensure that representative samples are recruited and adherence to standardized methodology is met. Many of the early studies focused predominantly on

Caucasian women, and there does appear to be a few consistent associations with race or ethnicity as discussed in the introduction. The factors mediating these differences have yet to be elucidated. In fact, the mechanisms by which most of the known risk factors increase risk of hot flashes is unclear, and the precise mechanisms underlying estrogen's role in ameliorating hot flashes is similarly murky. Many studies have failed to find any correlation or have found only weak correlations between estrogen levels and hot flashes, which suggests that other hormones, neurochemicals, and/or proteins are involved. Because of this gap, treatment strategies have similarly failed to advance much beyond estrogen derivatives. Importantly, knowledge of estrogen's mechanisms would inform treatment in other aspects of women's health, especially post-menopausal bone loss and increased risk of cardiovascular events and stroke and cognitive complaints.

There is also bias in some studies for participant selection, like those that include women who experience hot flashes in situations of increased ambient temperature or direct heating paradigms. Certain studies that have examined the relationship between ambient or outdoor temperature have failed to find any significant associations (see the introduction for details), and the inclusion of these women to the exclusion of others creates the opportunity to only study one type of hot flash, if there are multiple 'types' of hot flashes. It could be that there are 'subtypes' of hot flashes, each with distinct physiology and etiological factors. This is an area that needs systematic investigation, and studies with larger numbers of women are needed. Imaging studies of hot flashes at present are extremely limited, which has hindered understanding of the neural mechanisms underlying these events. At present, the voxel size in magnetic resonance imaging is larger than small subnuclei in the hypothalamus or brainstem regions (Diwadkar et al., 2014), and advancements in spatial resolution could greatly improve our understanding of the brain regions mediating hot flashes in women.

Recent large cohort studies have used standardized assessments, and this is a great boon to the literature. Many studies that examined anxiety in menopause used non-standardized or non-validated measures (see (Bryant et al., 2012)),

which had led to the contention that anxiety is not a symptom of menopause. More current literature has demonstrated that anxiety and stress-related stimuli are important factors in the hot flash experience, and further studies to finely elucidate how these factors contribute to hot flashes are essential. It is unclear whether it is the experience of the hot flash(es) that contributes to anxiety, or if anxiety states heighten the probability of having hot flashes. The study by (Swartzman et al., 1990) suggests that it may be the latter, as stressful stimuli increased the likelihood of having hot flashes in a laboratory setting. However, this may be artificial, and long-term ambulatory monitoring would be helpful in trying to fine-tune this relationship.

The strong placebo effect in hot flash trials is intriguing. The placebo response rate for hot flashes is among the highest of all conditions studied, with rates ranging up to 58% in trials of estrogen replacement (Maclennan et al., 2004). Comparisons between studies demonstrate that, in some cases, a placebo is more effective in reducing hot flashes than the test compound. A pooled analysis of treatment studies demonstrated the time course for placebo for hot flashes, and, like estrogen, the effects accumulated each week, which is curious since the compound is, by definition, inactive (Freeman et al., 2015). Strikingly, the factors that predicted response to placebo had substantial overlap with those factors that have demonstrated risk for hot flashes, including African-American race, cigarette smoking, and greater hot flash severity. Presently, it is unknown whether the mechanisms by which estrogen and placebo ameliorate hot flashes are the same or differ, and furthermore, it is not well understood if placebo mechanisms are the same between conditions (i.e. does the placebo work differently when used for chronic pain than hot flashes?). Understanding the mechanism(s) underlying the placebo response could provide new treatment strategies that potentially have less risk of harm, or may provide strategies to enhance the placebo response and avoid using drug treatments altogether.

Reliance on self-report and question of measurement

The vast majority of studies investigating hot flashes have used self-report diary-type measures, either paper or electronic versions wherein a woman notes the time and characteristics of a hot flash (i.e. intensity, duration). The use of self-report measures has several drawbacks, including failure to report hot flashes due to time constraints or interference with daily activities or forgetfulness. Objective physiological monitoring has used a few different methodologies, including monitoring of skin temperature, core temperature, and skin conductance or sweat rate, and the best correlations with subjective sensation were found with skin conductance (Tataryn et al., 1979). Using both subjective and objective assessments of hot flashes allows for multiple dimensions of the hot flash experience to be studied. Initially, studies of objective monitoring in the laboratory provided evidence that the association between an increase in sweating and the subjective label of a "hot flash" was quite high, approaching 100% (Freedman, 1989). However, the use of these monitors in *ambulatory* settings has not replicated this concordance (Carpenter et al., 1999, 2004), and there may be important cultural differences in these measures (Sievert et al., 2002, 2008; Brown et al., 2009). These monitors, while effective, can be bulky and rather conspicuous, and the thermal effects of clothing can interfere with monitoring; advancements in monitoring could greatly expand our knowledge of typical thermoregulatory patterns and hot flashes overall. As Sievert discovered, there are important cultural or ethnic variations in sweating patterns during hot flashes among symptomatic women, and measuring sternal skin conductance to the exclusion of other sites, such as the back of the neck, may preclude the detection of a hot flash. Furthermore, while these measures are useful in determining frequency, the magnitude of the sweat response does not provide an objective indicator of intensity or distress (Carpenter et al., 2005).

So the question then becomes, what exactly is a hot flash? Is it the sensation alone, or must it be accompanied by an increase in temperature and sweat rate? Sievert reports that not all hot flashes are accompanied by sweating, and

sweating in the absence of cutaneous vasodilation would lead to a cooling sensation (under conditions of appropriate relative humidity). If not sweating, than an increase in skin temperature or blood flow? Again, the magnitude of the increase or total duration of the increase does not correlate very well the subjective sensation. So what psychological factors are involved in labeled a feeling of heat of varying amplitude, with or without sweating, as a hot flash? These are outstanding key questions in this field, (as summarized by (Miller and Li, 2004)). The answers as to what neurochemicals and hormones contribute to certain components of hot flashes is a complex problem, and careful dissection of the systems implicated insofar using animal modeling may provide new avenues for treatment.

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Curriculum Vitae

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Education

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Honors and Awards

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2010-2011	IUPUI University Fellowship
2007-2010	IUPUI Salutatorian Scholarship
2007, 2009	Rabe Vermillion Scholarship
2007-2009	Avery Scholarship
2007	Kiwanis Scholarship
2007	Hoosier Scholarship
2007	Set A Good Example Foundation Scholarship

Manuscripts

2016 Yoder KK, Albrecht DS, Dzemidzic M, Normandin MD, **Federici LM**, Graves T, Herring CM, Hile KL, Walters JW, Liang T, Plawecki MH, O'Connor S, Kareken DA. Differences in IV alcohol-induced dopamine release in the ventral striatum of social drinkers and nontreatment-seeking alcoholics. Drug and Alcohol Dependence 2016 Jan. 13

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2011 Yoder KK, Albrecht DS, Kareken DA, **Federici LM**, Perry KM, Patton EA, Zheng QH, Mock BH, O'Connor S, Herring CM. Test-retest variability of [^{11}C]raclopride-binding potential in nontreatment-seeking alcoholics. Synapse. 2011 Jul;65(7):553-61.