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Integration of Metabolic and Reproductive Cues in the Neural Control of Feeding

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Integration of Metabolic and Reproductive Cues in the Neural Control of Feeding

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy in

Neuroscience

by

Megan Gina Massa

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ABSTRACT OF THE DISSERTATION

Integration of Metabolic and Reproductive Cues in the Neural Control of Feeding

by

Megan Gina Massa

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2022

Professor Stephanie Correa van Veen, Chair

Metabolism and reproduction are linked homeostatic processes. This linkage becomes particularly important for animals who gestate their young, as gestation and postpartum care are energetically taxing life stages. Indeed, long- and short-term measures of metabolic reserve and availability, adiposity and feeding, gate reproductive processes in mammals with ovaries. Many neuronal nodes and circuits that help mediate this tradeoff are located in the hypothalamus. As an important component of energy intake, feeding nodes have been investigated as responsive to reproductive cues such as gonadal hormones for many decades. Feeding is a complex behavior, tapping into both homeostatic and hedonic mechanisms. As such, there are many locations where metabolic and reproductive status may integrate and affect feeding behavior. This thesis explores how the tuberal nucleus (TN), a relatively new feeding node, may integrate metabolic and reproductive cues to affect food intake. Using the Cre-lox system and viral stereotaxic injections, somatostatin neurons in the TN (TN^{SST}) were selectively manipulated to interrogate neuronal function. Chemogenetic activation of TN^{SST} neurons

increased food intake across sexes, but cell autonomous caspase ablation only decreased food intake in female mice (mice with ovaries) during the night of proestrus, when circulating hormones like estradiol are high. This apparent effect of estradiol was only evident in animals with a low body weight, and the inverse correlation of food intake and body weight during proestrus was completely eliminated with TN^{SST} neuron ablation. Further analysis revealed that this body weight effect may be primarily determined by adiposity, as high levels of hypothalamic estradiol seem to increase communication between TN^{SST} neurons and various adipocyte depots. This may be a direct effect of estradiol on TN^{SST} neurons, as these neurons are both estrogen sensitive and responsive. Ongoing fat transplantation studies confirm the adipose dependency of this effect. Together, this dissertation proposes a model whereby TN^{SST} neurons activate during periods of fertility to induce food intake when body reserves are low. Thus, sex differential recruitment and/or activation of TN^{SST} neurons may work to mitigate the effects of sex steroids on behavioral feeding output. This dissertation research illustrates how gonadal steroid modulation of neuronal circuits can be context-dependent and gated by other physiological signals. Furthermore, this project illustrates how sex as a singular, coherent biological variable is insufficient for current explorations into the contributions of sexed physiologies to feeding behavior. Thus, this dissertation also proposes a framework shift to a “sex variables” paradigm that recognizes the limitations of binary, internally consistent sex in favor of a more contextual and expansive definition of sex and sexed physiologies.

The dissertation of Megan Gina Massa is approved.

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Chapters 3, 4, and 5 are currently in preparation for submission. I will be the primary author and performed data collection, most major statistical analysis, figure generation, results interpretation, and am writing the first draft of the manuscript. Rachel Scott, Sahara Ali, Betty Tesfaye contributed to data collection and animal care, and Sahara Ali performed all histological analyses on white adipose tissue (Figure 4-2). J. Ed van Veen and Amanda Misquez injected and imaged reportER mice (Figure 4-3A&D), respectively. J. Ed van Veen also performed FlowSeq dissections, dissociations, FAC sorting, and DEG analysis (Figure 4-3B) which formed the basis for Figure 4-4. Collaborator Marcus Seldin performed all GTEx co-correlation analyses (Figure 4-4). Ally Cara assisted in preliminary and ongoing fat transplantation studies through collection of donor fat (Figure 4-5). Stephanie Correa van Veen is the corresponding author and project director.

Chapter 6 is currently under revision at *Nature* as a commentary with the title "'Sex' is not a coherent biological variable – a sex variables framework." I am the primary and co-corresponding author on this publication. MJ Hill was formative in sex variables framework generation and manuscript preparation. Krisha Aghi will be contributing to data analysis methods in line with the developed framework.

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Ali S & **Massa MG**[#]. Effects of Sex Hormones on Food Intake, Body Weight, and Fat Composition: A Cross-Species Analysis. *J Student Research* 10 (3), 1-10 (2021). https://doi.org/10.47611/jsr.v10i3.1337

Other Scholarly Works

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Chapter 1

Metabolism and Reproduction: Homeostatic Tradeoffs

1.1 Introduction

Metabolism and reproduction are two homeostatic processes that are intrinsically linked. These bi-directional interactions are apparent in everyday life. Athletes and patients with disordered eating, namely anorexia, exhibit reproductive dysfunction (Chavarro et al., 2015; Cunningham et al., 1999; Fontana and Della Torre, 2016; Frisch, 1990; De Souza and Metzger, 1999). Conversely, reproductive quiescence often promotes large alterations in metabolic profile, including changes to energy expenditure and fat distribution (Hansen et al., 2013; Palmer and Clegg, 2015; Tchernof et al., 2004).

Teleologically, such homeostatic trade-offs between metabolism and reproduction may have evolved to promote successful sexual reproduction through increased offspring viability during times of metabolic security and inhibit it during times of food scarcity (Fontana and Della Torre, 2016). Indeed, early studies found that body weight, but not height or age, determined onset of menstruation (Frisch, 1972, 1990; Frisch and Revelle, 1970), and fecundity (as measured by time-to-pregnancy) correlates inversely with weight[1] (Hassan and Killick, 2004). Recent studies have confirmed the inverse correlation between fat mass percentage and age of pubertal onset in humans (O'Keeffe et al., 2020). Though these integrations are apparent across all individuals of a species (such trade-offs have even been seen in nematodes; reviewed in Fontana and Della Torre, 2016), most research investigating these tradeoffs focus on female mammals, defined in this case as animals born with ovaries and internal developed Müllerian system, due to the evident metabolic demands of offspring gestation and nutrition postpartum. Despite this focus, average body weight has also been found to inversely correlate to fertility in sperm producers as well (Sermondade et al., 2015). This may similarly be related to metabolic environment and prospective nutritional availability for future offspring.

[1] Weight in this study was measured via body mass index (BMI), a flawed metric of racist origins (Springs, 2019) that inappropriately assesses risks associated with adiposity and body composition (Jackson et al., 2009).

1.2 Adiposity Gates Reproductive Processes

As a component of bodyweight, it is specifically adiposity which communicates metabolic capability for reproductive success. In particular, sufficient fat reserves have been found to be crucial for pubertal onset and continued maintenance of reproductive function (Cunningham et al., 1999), with leptin being the crucial permissive adipokine signal (reviewed in Tena-Sempere, 2007). Early studies of leptin-deficient *ob/ob* mice showed hypothalamic-pituitary-gonadal (HPG) axis dysfunction across all sexes (Swerdloff et al., 1976) that could be rescued through leptin treatment (Chehab et al., 1996). Leptin was soon demonstrated to be required for pubertal onset. In a particularly elegant study, pubertal onset was accelerated in leptin-treated animals as compared to controls despite decreased food intake, and delayed pubertal onset due to decreased food intake through a pair-feeding was recovered through leptin treatment (Cheung et al., 1997). Later studies demonstrated that leptin and leptin receptor was necessary for pubertal onset, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion from the anterior pituitary in all sexes, and the LH surge particularly in ovulating animals (Quennell et al., 2009; Tena-Sempere, 2007). These effects were found to be elicited through indirect regulation of gonadotropin-releasing hormone (GnRH) neurons, possibly through the modulation of kisspeptin production and release acutely (reviewed in Tsatsanis et al., 2015) or through modulation of kisspeptin circuitry over a lifetime (reviewed in Navarro, 2020). Interestingly, leptin can also act directly on the gonads to influence steroidogenesis, inhibiting estrogen-specific steroidogenesis in granulosa cells (Ghizzoni et al., 2001; Spicer and Francisco, 1997), inhibiting ovulation (Duggal et al., 2000), and decreasing testosterone steroidogenesis in Leydig cells (Tena-Sempere et al., 1999).

The adipokine adiponectin has also been found to alter the HPG axis and thereby affect reproduction. As an adipokine that negatively correlates with visceral fat mass in mammals (Fontana and Della Torre, 2016), adiponectin acts in a manner opposite to that of leptin. Higher levels of adiponectin signal lower levels of visceral body fat, and thus the adipokine acts to

inhibit LH secretion through various mechanisms, including through downregulation of the GnRH receptor (Rodriguez-Pacheco et al., 2007). It can also directly inhibit testosterone production through actions on the testis (Caminos et al., 2008)

1.3 Feeding Requirements for Reproduction

While internal fat stores might indicate long-term metabolic availability and bodily capacity for reproduction, food intake provides a more immediate metric of available nutritional environment across species. Early studies demonstrate that food deprivation in female rats temporarily suppressed reproduction, only to rebound later in life when food scarcity was relieved (Ball et al., 1947). Interestingly, this rebound superseded the time-based decrease in pregnancy success, revealing that food availability may shift the reproductive window within the lifespan of an individual. A similar effect was seen in female mice, where food restriction resulted in acyclicity and refeed restored cyclicity during a time when similarly aged mice were now acyclic due to the aging process (Nelson et al., 1985). This remains in line with studies demonstrating that acute food deprivation delays but does not prevent pubertal onset in female rats, as refeeding allows for the normal progression of puberty (reviewed in Cunningham et al., 1999). This interruption of reproductive function is due to the impact of food scarcity on LH cyclicity in animals with active ovarian cycles. A short, 48-hour food deprivation decreases LH cyclicity, frequency, and amplitude in intact, cycling animals, but this effect is eliminated following ovariectomy (OVX; Cagampang et al., 1990). The dependency of this effect on active ovarian presence indicates that the effect of food deprivation on hypothalamic or pituitary function requires reproductively relevant activity from the downstream ovary, further solidifying the salience of immediate nutritional availability for successful reproductive function.

The stomach “hunger” hormone ghrelin provides a mechanism by which hunger cues are communicated to the HPG axis to temporarily inhibit reproduction. Puberty in rodents is delayed following chronic elevation of ghrelin levels, and systemic ghrelin administration can

diminish GnRH frequency (reviewed in Roa and Tena-Sempere, 2014). This effect may be mediated through kisspeptin neurons in the arcuate nucleus of the hypothalamus. Known for mediating estrogen-mediated negative feedback in the hypothalamic-pituitary-ovarian axis, arcuate kisspeptin neurons decrease kisspeptin production (normally a potent stimulator of GnRH neurons) in response to ghrelin administration, subsequently decreasing LH pulse frequency (Forbes et al., 2009). Another potential mechanism of interaction may occur at the level of gonadotropin inhibiting hormone (GnIH). GnIH has been shown to increase during periods of fast and may act on both the reproductive and metabolic axes to decrease GnRH release (inhibiting reproduction) and increase feeding behavior through interactions with arcuate hypothalamic feeding circuits, specifically the melanocortin system (reviewed in Tsutsui and Ubuka, 2016).

Long-term and short-term nutritional cues can also interact to produce relevant reproductive phenotypes. Sufficient adiposity levels have been shown to counteract decreased food availability cues induced by short-term food deprivation. A study in female hamsters demonstrated a weight-dependency of the effects of food deprivation, namely that animals fed high-fat diet (HFD, shown to increase adiposity) showed normal reproductive markers – estrous cyclicity, lordosis, ovulation, vaginal discharge, and uterine weight – following 48-hour fast whereas lean animals were impaired on these metrics (Schneider and Wade, 1989). Pharmacologically blocking fatty acid utilization in high-adipose animals during food deprivation prevented the protective effects of high adiposity (Schneider and Wade, 1989), indicating that it is not just adipose store levels which protect against reproductive disruption from food deprivation, but the ability to metabolize and utilize those metabolic stores.

Fewer studies have investigated the effects of food deprivation on the hypothalamic-pituitary-testicular axis. However, evidence suggests that immediate food availability can similarly affect this reproductive axis in some ways. Dietary restrictions and alterations in both male mice and rats inhibit fertility through decreasing both ependymal fluid production and

overall sperm count (Brinkworth et al., 1992). These effects are probably also manifested at the level of the hypothalamus and pituitary as in ovarian animals. A one-day fast in male rhesus monkeys significantly reduced LH pulse frequency but not amplitude, subsequently also decreasing testosterone secretion (Cameron and Nosbischf, 1991). Refeed increased LH pulsatile frequency above that of baseline, indicating a post-inhibitory rebound and perhaps overactivation of reproductive capacity in the face of nutritional abundance (Cameron and Nosbischf, 1991).

Some evidence indicates that there are divergences in how animals with different reproductive strategies respond to food deprivation. For instance, meadow voles do not display changes in male proceptivity and receptivity in response to food deprivation (Ferkin, 2017). Similarly, starvation conditions actually result in an increase in food intake in mice with testes in an effort to increase fat stores, whereas the same conditions produce a decrease in energy expenditure to preserve existing fat stores in mice with ovaries (Mauvais-Jarvis, 2015). Thus, while food deprivation may similarly repress the reproductive axis in animals with testes, metabolic strategies to combat nutritional deficit may differ based on gonadal status and subsequent hormonal milieu.

1.4 Conclusion: Locating Metabolic and Reproductive Interactions in the Hypothalamus

These interactions between reproduction and metabolism occur across varying levels of physiology, but their importance in regulating behaviors associated with these homeostatic processes suggest central neuronal control in this integrative process. The hypothalamus is a prime location for such integration, as nodes within this highly evolutionarily conserved region contribute to various aspects of reproduction and metabolism. Specific cell types within the lateral hypothalamus, and arcuate, paraventricular, and dorsal medial nuclei modulate feeding behavior (see Chapter 2, also reviewed in Andermann and Lowell, 2017), and the dorsal medial hypothalamus, arcuate nucleus, preoptic area, and the ventrolateral region of the ventromedial

nucleus of the hypothalamus modulate various aspects of energy expenditure and thermogenesis (Correa et al., 2015; van Veen et al., 2020; Zhang et al., 2021). Some of these regions, including the ventromedial, arcuate, and medial preoptic nuclei also contribute to reproductive behaviors (reviewed in Kammel and Correa, 2019 and Micevych and Meisel, 2017). Indeed, well-established metabolic cell types have also been shown to contribute to reproductive success. Chronic activation of Agouti-related peptide (AgRP) neurons in the arcuate nucleus, a canonical population of feeding neurons, leads to reproductive dysfunction independent from changes in food intake (Padilla et al., 2017).

In a similar fashion, this thesis explores how a relatively new node of the feeding circuit, the tuberal nucleus of the hypothalamus (Luo et al., 2018), integrates across reproduction and metabolism. Somatostatin (SST)-producing neurons in this region (TN^{SST}) differentially regulate feeding across female (ovarian) and male (testicular) mice. In female mice, these neurons seem to promote food intake during fertile periods when energy reserves are low. When endogenous adiposity levels are high, on the other hand, TN^{SST} neurons no longer promote metabolic compensation over that of reproduction. The experiments herein demonstrate how the neuronal detection of metabolic and reproductive cues alter feeding intake within the context of appropriate metabolic reserves.

This context-dependency of a seemingly sex-specific effects prompts discussion of the continued usefulness of the binary heuristic of “sex” and the use of “sex as a biological variable” across biological studies. Without attention to the physiologies within the large categories of “female” and “male,” important interactions such as those described herein would be obscured. Thus, the language within this thesis will challenge the assumptions of internal concordancy within sex categories, and this thesis ends with a proposal of a new “sex variables” framework within which to situate future studies interested in sexed physiologies.

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Chapter 2

Sexes on the Brain: Sex as multiple biological variables in the neuronal control of feeding

2.1 Abstract

Neuronal interactions at the level of vagal, homeostatic, and hedonic circuitry work to regulate the neuronal control of feeding. This integrative system appears to vary across sex and gender in the animal and human worlds. Most feeding research investigating these variations across sex and gender focus on how the organizational and activational mechanisms of hormones contribute to these differences. However, in limited studies spanning both the central and peripheral nervous systems, sex differences in feeding have been shown to manifest not just at the level of the hormonal, but also at the chromosomal, epigenetic, cellular, and even circuitry levels to alter food intake. In this review, we provide a brief orientation to the current understanding of how these neuronal systems interact before dissecting selected studies from the recent literature to exemplify how feeding physiology at all levels can be affected by the various components of sex.

2.2 Introduction

Food and eating play dual roles in global society, providing both biological and cultural capital. Humans eat for more than mere sustenance; food can provide a social back drop and an emotional reprieve, while also producing anxiety over body image. These socioemotional factors can lead to extreme modulations in food intake, including over- and under-eating, resulting in detrimental health outcomes. Increased food intake of calorie-dense foods can increase the likelihood to develop obesity and its comorbidities, whereas binge and under-eating can result in dysmenorrhea, cardiovascular problems, starvation, and even death. Though both obesity and eating disorders may have multifactorial metabolic, psychological, and societal etiologies, the modulation of food intake remains both a symptom of and exacerbating factor in these conditions. Both obesity and eating disorders are also significantly gendered – women are the fastest growing constituent of obese and extremely obese individuals (despite body mass tending to be higher in men than women, NIH National Institute of Diabetes and Digestive and

Kidney Diseases (NIDDK), 2017), and women and girls engage in disordered eating up to three times more than men and boys (Hudson et al., 2007).[2] Thus, sex factors (a set of biological variables typically associated with reproduction) may predispose people of a certain gender (a combination of our social reaction to the phenotypes resulting from those variables and internal identification) to modulate food intake in response to various environmental pressures. Much research investigating sex differences in feeding has focused on hormonal contributions to food intake. However, these studies often result in an incomplete understanding of sex difference etiology. This is most likely because sex is not a simple dichotomy of estrogen vs. testosterone, but a complex and interacting network of related but distinct factors, including not only hormones but also sex chromosomes, parental imprinting, and sex-specific environment, which may interact, counteract, or synergize with one another. An understanding of how, then, specific components of sex shape and act upon feeding circuitry is vital to our understanding of the neuronal control of food intake. In this review, we will first provide a brief overview of the vast overlap in the central nervous system's control of homeostatic and hedonic feeding. We will then explore how sex, sex differences, and the various facets of sex affect all levels of feeding physiology.

2.3 The Neuronal Circuitry of Feeding – Homeostatic and Hedonic Cooperation

The physiology of feeding is typically construed as a universal homeostatic process, which, due to its vital nature, remains relatively physiologically conserved across the animal kingdom. Across vertebrates, both the hypothalamus and hindbrain prove crucial to the control and regulation of food intake (reviewed in Schneeberger et al., 2014; Sternson and Eiselt,

[2] These epidemiological surveys do not report how they determine sex or gender. As there are typically no reported physical examinations, karyotypes, or hormone levels, we assume that this is gathered by self-report and therefore it is probable that these studies only consider cis individuals. This is a vast oversight in the field, as the trans population is at an increased risk for eating disorders due to a multiplicity of factors, including a higher incidence of gender dysphoria, body dysmorphia, and varied gender expression (Cella et al., 2013; Jones et al., 2018; Vocks et al., 2009).

2017). Despite this apparent conservation of structure and function, patterns and uses of food intake vary both across and within species. In a variety of animals, including rodents and humans, homeostatic circuitry is integrated with hedonic, motivational, reward, and higher-order top-down circuitry. This integration lends flexibility to the system yet increases chances for circuitry "malfunction." Most current literature separates this homeostatic and hedonic circuitry. These systems, however, are inextricably intertwined and interdependent (Andermann and Lowell, 2017; Castro et al., 2015; Ferrario et al., 2016; Figure 2-1). For instance, when canonical homeostatic regions are developmentally ablated, hedonic mechanisms can provide compensatory support (Denis et al., 2015).

The interaction of homeostatic and hedonic circuitry spans across all phases of feeding behavior. The melanocortin system canonically consists of the appetitive agouti-related peptide (AGRP)/neuropeptide Y (NPY) neurons and the satiety-related proopiomelanocortin (POMC) neurons within the arcuate nucleus of the hypothalamus (ARC) along with second order α-melanocyte stimulating hormone receptor cells in the paraventricular nucleus of the hypothalamus (reviewed in Oldfield et al., 2016). While this system has been characterized as promoting homeostatic foraging and food seeking (Burnett et al., 2016; Chen et al., 2015b), there is also evidence for AGRP/ NPY neuronal recruitment of learning/reward circuitry, as these neurons both induce a negative valence state (Betley et al., 2015) and promote fearlessness in the face of starvation through projections to the amygdala (Padilla et al., 2016). In the lateral hypothalamus, GABAergic, orexin, and/or melanin-concentrating hormone neuronal subpopulations involved in homeostatic feeding (Bonnavion et al., 2016; Jennings et al., 2015; Navarro et al., 2016) exhibit extensive communication with motivational circuitry (reviewed in Rossi and Stuber, 2018). Connections, in some cases bidirectional, with regions including the ventral tegmental area (Barbano et al., 2016), bed nucleus of the stria terminalis (Jennings et al., 2013), the core and shell of the nucleus accumbens (reviewed in Bonnavion et al., 2016; Ferrario et al., 2016), and hedonic hotspot the parabracheal nucleus (Smith et al.,

2010; Tokita et al., 2014) allow the lateral hypothalamus to integrate homeostatic signals of metabolic need as well as motivational and hedonic cues to promote eating behavior for the duration of a meal. Even regions functioning as homeostatic nodes of satiety, satiation, and meal cessation have significant overlap with stereotypical hedonic circuitry. The nucleus of the solitary tract (NTS) receives a significant amount of vagal afferent synapses to communicate hormonal and mechanosensory markers of satiety to the brain (reviewed in Andermann and Lowell, 2017). These signals then integrate with hedonic circuitry on multiple levels. Glucagon-like peptide 1 neurons in the NTS send direct processes to dopaminergic neurons in the ventral tegmental area, which then in turn project to the nucleus accumbens shell (Wang et al., 2015). NTS populations expressing norepinephrine and cholecytoskinin recruit the hedonic/homeostatic parabracheal nucleus. From here, calcitonin gene-related peptide neurons promote meal termination by way of projections to the central amygdala and other connections (Andermann and Lowell, 2017; Carter et al., 2013).

As is evident, these canonically homeostatic nodes regularly interact with hedonic, motivational, and learning pathways (Figure 2-1). Interestingly, it has been hypothesized that dysregulation of homeostatic/hedonic balance promotes disordered eating (Avena and Bocarsly, 2012). And as the propensity for disordered eating in humans is gendered, the effects of sex on both homeostatic and hedonic nodes, and how they interact, must be considered when investigating the neuronal control of feeding.

2.4 Current Understanding of Neuronal Sex Differences in Feeding

In mice and rats, males consume more food daily than do female conspecifics. Hence, sex differences in feeding have been long appreciated (for review see Asarian and Geary, 2013). Phenotypic sex is comprised of various factors, including the organizational and activational effects of hormones, chromosome complement and expression, and sex-based experiences (Arnold, 2017). Despite this, the bulk of neuroscience research regarding sex

differences in feeding has focused on the effects of sex steroid hormones, namely 17β-estradiol, and its interactions with estrogen receptor subtype α (ERα). A recent review highlighted multiple nodes across homeostatic and hedonic circuitry wherein estradiol acts, typically through ERα (Geary et al., 2001; Santollo et al., 2010), to decrease food intake across species (Rivera and Stincic, 2018), though the particular effects and mechanisms of estradiol varies from species to species. Rats and guinea pigs provide models of feeding that are tightly correlated to estrogen levels. In these organisms, increased levels of endogenous ovarian estrogens decrease food intake: feeding decreases following the high estradiol phase of the estrous cycle (Asarian and Geary, 2002, 2013; Eckel, 2011) and ovariectomy significantly increases feeding and subsequently body weight (Asarian and Geary, 2002; Clegg et al., 2007; Eckel, 2011). This anorexic effect of estradiol appears to be mediated by affecting both the appetitive and meal-cessation stages of feeding. In the melanocortin system, estrogens have been shown to decrease food intake in the ARC (Santollo et al., 2011) through POMC neuron activation via both ERα and the g protein-coupled membrane estrogen receptor known as GqMER (Roepke et al., 2007, 2010; Stincic et al., 2018a, 2018b), as well as by altering both the effects and expression of NPY and α-melanocyte-stimulating hormone (Ainslie et al., 2001; Santollo and Eckel, 2008). In the brainstem, estradiol has been found to generally interact with signaling processes of the orexigenic stomach hormone ghrelin (Clegg et al., 2007) and the adipokine leptin (Ainslie et al., 2001; Clegg et al., 2003), while also proving necessary for cholecytoskinin (Asarian and Geary, 2006; Clegg et al., 2007), and glucagon-like peptide 1 (Maske et al., 2017) signaling in the NTS to promote meal cessation. This modulation of homeostatic feeding by estradiol can act in addition to, in opposition to, or synergistically with estrogenic alteration of hedonic, motivational, and reward circuitry. Endogenous and replacement estradiol has been found to reduce food-motivated reward in the ventral tegmental area (Richard et al., 2017). Furthermore, both ERα and estrogen receptor subtype β (ERβ) have been found to be expressed and affect cellular activity in regions such as the bed nucleus of the stria terminalis,

amygdala, ventral tegmental area, and nucleus accumbens (reviewed in Becker and Chartoff, 2019).

In mice, however, the picture becomes complicated. Whereas some studies have found a typically mild effect of endogenous estrous cycle (Olofsson et al., 2009; Petersen, 1976), ovarian hormones (Chen et al., 2012, 2015a; Geary et al., 2001), and/or estrogen receptor presence (Geary et al., 2001; Musatov et al., 2007; Xu et al., 2011) on food intake, others have reported no clear estrogenic effects (Eckel, 2011; Naaz et al., 2002; Witte et al., 2010). This lack of consensus indicates that caution must be employed when interpreting how the discovered effects of estrogens on feeding and metabolism in one species apply to another. Interestingly, the more robust and consistent phenotype in mice following depletion of estrogen signaling is a sex-specific decrease of energy expenditure (Correa et al., 2015; Musatov et al., 2007; Xu et al., 2011). As weight gain in post-menopausal women also does not result from an increase in food intake and is instead primarily due to a decrease energy expenditure (Hodson et al., 2014), mouse models may be more applicable to humans than other rodents when considering the effects of estrogens on feeding and metabolism.

Comparatively less research has focused on testosterone and androgenic effects on feeding. Castration in adulthood has been shown to decrease food intake, while replacement and/or long-term testosterone treatment increases feeding (Chen et al., 2015a; Gentry and Wade, 1976; Rowland et al., 1980). Though the precise mechanisms of these effects remain unknown, the effect of testosterone on male feeding is most probably not due to aromatization and is therefore androgenic in nature (Rowland et al., 1980). During development, on the other hand, neonatal androgen exposure decreases POMC mRNA content and projections from the ARC (Nohara et al., 2011), indicating that lifetime increase of food intake in males may be partially due to developmental inhibition of the anorexic arm of the melanocortin pathway. Interestingly, the effects of neonatal androgen on feeding patterns may also require further

development during puberty, as perinatal testosterone decreased female rats' risk for binge eating only after mid-puberty (Culbert et al., 2018).

This approach to sex, viewing it as a single variable alterable by presence or absence of various steroid hormones and their respective receptors, is useful but incomplete. When applied to feeding, adulthood hormone manipulations alone are insufficient to elucidate the role of sex differences on neuronal physiology. For example, in a recent tracing study, vagal afferents were found to have sex differences both within and beyond simple hormone manipulations (Figure 2-2). Female and male Wistar rats exhibit variations in both density and localization of heavily myelinated (A) and unmyelinated (C) peripheral fiber innervation into the brainstem, including the NTS (Ciriello and Caverson, 2016). While peri-pubertal ovariectomy decreased and subsequent estradiol replacement increased innervation density in females, the NTS of females in both conditions was still more densely innervated with non-myelinated fibers as compared to males. Furthermore, regardless of hormonal manipulation in females, females and males exhibited a differential pattern of peripheral fiber innervation into the brainstem (Ciriello and Caverson, 2016). Together, these results indicate that vagal-NTS connective anatomy is modulated by sex variables above and beyond that of pubertal and post-pubertal hormone levels. While the organizational effects of the neonatal testosterone surge on this innervation pattern have yet to be explored, some sex differences exist beyond these early-life hormonal influences as well.

In recent work, sex differences in food anticipatory behavior in mice were found to exist independent of various hormonal manipulations (Aguayo et al., 2018). Due to the circadian nature of feeding, scheduled food-restriction can result in increased animal movement in anticipation of food delivery, particularly in males (Li et al., 2015; Michalik et al., 2015). One study searching for the etiology of this sex difference investigated the effects of hormones in adolescence and in early life. Neither peripubertal gonadectomy nor neonatal injections of estradiol, accepted to be the primary neurologically-bioactive compound in rodents during the

organizing testosterone surge, increased female food anticipatory activity (Aguayo et al., 2018). However, this study does lie in contrast to earlier work in which early adulthood gonadectomy in males decreased and ovariectomy in females increased food anticipatory activity, abolishing the sex difference (Li et al., 2015). These conflicting results may be due to methodological differences in the timing of female ovariectomy, indicating a possible organizational role for pubertal hormones in addition to other sex factors.

Together, these and other studies exemplify how we must cease viewing sex as an all-encompassing heuristic or as a function purely of gonadal hormones and their receptors. In order to fully understand the complexities underlying feeding physiology, we must instead adopt a model of sex as a set of diverse, covarying, but independently acting components (Reue, 2017).

2.5 Room for Investigation: The Components of Sex as Contributors to Sex Differences in the Neuronal Control of Feeding

The prominence of neuroendocrinological examinations of sex differences in feeding is due to not only the relative ease of hormonal manipulations, but also the historical dominance of the organization/activational paradigm with regards to sexual differentiation. Initially pioneered in the 1950s, this hypothesis hinges on the idea that the neonatal testosterone surge, primarily through its metabolite estradiol in rodents, “organizes” the male brain by permanently defeminizing and/or masculinizing brain circuitry. Gonadal hormones, elevated during puberty and into adulthood, are then able to reversibly act on, or “activate,” the circuit as hormone levels fluctuate (Phoenix et al., 1959). In the years following its initial introduction, the hypothesis has been amended to include puberty as an organizational event as well (Sisk and Zehr, 2005). The stand-alone primacy of the organizational/activational hypothesis in forming and creating sex differences in the mammalian brain has been repeatedly challenged (Arnold, 2009a; McCarthy and Arnold, 2011) despite this and other amendments. That is not to say that the effects of sex

steroid hormones, particularly the neonatal testosterone surge, on structural differences in neuronal populations (de Vries and Södersten, 2009), cellular expression (Nugent et al., 2015), and epigenetic modulation (McCarthy and Nugent, 2015) are not real, varied, and persistent. However, by viewing these effects as absolute, uniform, and all-encompassing, we neglect how other components of sex may interact with these to compensate for or contribute to physiological heterogeneity in the brain (McCarthy, 2015). Hence, by viewing sex as a binary of "testosterone or estradiol" instead of a spectrum created by the composition of interrelated parts, we miss how the components of sex work together or separately to affect feeding physiology and dysfunction. In the sections that follow, we will interrogate how other sex components – sex chromosomes, parental imprinting, and environmentally-induced epigenetic modifications – interact with feeding circuitry.

Sex chromosome complement

As has been repeatedly seen across species, sex chromosome complement (e.g., XX, XY, etc. in most mammals, and ZW, ZZ, etc. in birds) can affect both physiology and behavior. This can occur not only through gene expression, but also through epigenetic autosomal regulation by the sex chromosomes (Wijchers and Festenstein, 2011). Indeed, sex differences can occur at a cellular level prior to gonadal hormone exposure (Burgoyne et al., 1995; Dewing et al., 2003; Werner et al., 2017). The difficulty in assessing sex chromosome effects independent of gonadal hormones is that the two are typically linked. The Y chromosome canonically contains *Sry*, the testis-determining gene, which typically results in the development of testes from the bipotential gonad in mammals. The four core genotypes mouse model allows for the dissociation of sex chromosome complement and gonadal type due to the translocation of *Sry* to an autosome, resulting in four offspring of varying sex components: XX + ovaries, XY + ovaries, XX + testes, and XY + testes. The four core genotypes model can thus be used to discover hormonal/gonadal (ovaries vs. testes) or sex chromosome complement (XX vs. XY)

contributions to sex differences, as well as the interactions between these factors (De Vries et al., 2002).

The effects of sex chromosome complement can manifest in several ways. In mammals, both gene differences on the X and Y chromosomes (namely the presence of a Y chromosome) and X gene dosage may contribute. Typically, to compensate for a doubling of the X chromosome in XX individuals, one mammalian X chromosome will undergo inactivation by the gene *Xist*. This occurs randomly on an individual cell-by-cell basis, leading to a mosaic organism where some cells have the paternal, and other cells the maternal, X chromosome silenced (Arnold, 2017; Arnold et al., 2016). However, X-inactivation is an incomplete process. Some genes, known as "X escapees," evade *Xist*-mediated epigenetic inactivation and remain expressed at higher levels in individuals more than one X chromosome (Arnold et al., 2016; Xu and Disteche, 2006). Thus, a difference in phenotype between XX and XY mice independent of gonadal hormones could be explained by either X or Y gene effects. To separate these, the XY* mouse model can be used. This mouse model contains a Y chromosome which has an altered pseudoautosomal region (Y*), resulting in abnormal recombination producing genotypes similar to XO, XX, XY, and XXY (Arnold, 2009b; Chen et al., 2012). Therefore, this model can be used to determine whether effects of sex chromosome complement are due to X effects (due to dosage or imprinting) and/or the presence or absence of the Y chromosome.

Both of these approaches have been applied to feeding and metabolism in a fairly broad capacity. In four core genotypes mice, average body weight was found to result from an interaction between gonadal type and sex chromosome complement (Chen et al., 2012). Prior to gonadectomy, mice with testes weighed more than those with ovaries, regardless of sex chromosome complement. XX mice also tended to be heavier than their XY counterparts within testicular and ovarian groups. Following gonadectomy, a strong sex chromosome complement effect emerged, with XX mice gaining weight more rapidly than those with an XY sex chromosome complement (Figure 2-3A). Interestingly, there also appeared to be an interaction

between sex chromosome complement and gonadal status, as XX mice formerly having ovaries gained weight much quicker than XX mice previously having testes. Such a seemingly counteracting nature of typically-paired sex components (XX counteracting ovarian effect, XY counteracting testicular) is not uncommon. Physiological sex differences have been found to both reinforce and counteract others in order to mitigate differences in behavior in various organisms and contexts (Arnold, 2009a; McCarthy and Arnold, 2011; De Vries, 2004).

This body mass phenotype was propagated, at least partially, by a preceding alteration in food intake. Four weeks following gonadectomy, prior to the divergence of body weights, mice formerly having ovaries consumed more grams of chow during the dark period as compared to mice which had had testes, regardless of sex chromosome complement (Figure 2-3B). During the inactive daytime period, however, XX mice ingested more grams of chow than XY mice, and XX + ovaries mice tended to have higher food intake than XX + testes mice (though this was not statistically significant, Figure 2-3B). These joint effects of gonadal hormones and sex chromosome complement on food intake were no longer apparent by 10 months following gonadectomy. And while the XY* mouse model revealed that this effect of sex chromosome complement on body weight was due to X dosage and not Y presence (Figure 2-3C, Chen et al., 2012), it is interesting to note that this is not true of all strains. On another background, both X dosage and Y presence contributed to an increase in high fat diet food consumption, with XXY mice consuming almost 4 g in one 12-hour nighttime period (Chen et al., 2013). Thus, sex chromosome complement may have varying effects on alternate genetic backgrounds and when consuming different diets. Importantly, none of these effects were localized to any particular neuronal circuitry, though subsequent investigations revealed that sex chromosome complement and/or gonadal type contribute to various components of meal architecture (Chen et al., 2015a). It is possible that these effects are due to combined peripheral and central effects, as both gonadal type and sex chromosome complement were found to affect adiposity (and resulting leptin circulation), energy expenditure, muscle activity, and liver function (Chen et

al., 2012, 2013, 2015a). Regardless, if we wish to fully understand how the nervous system controls feeding behavior, more research into where the effects of sex, including those of sex chromosome complement, manifest within the homeostatic and hedonic feeding circuit must be conducted.

Parental imprinting

Closely linked to sex differences due to sex chromosome effects is the possible influence of parent-of-origin genomic imprinting. In this phenomenon, certain genes are subject to epigenetic modulation based on parental inheritance. Such cis epigenetic modulation typically takes the form of silencing through DNA methylation or histone tail modifications to alter the chromatin conformation and thereby decrease transcription. Few genes have been found to be under the control of parent-of-origin effects, though many imprinted genes have been found to be important for hypothalamic development and function (Keverne, 2007). In the human population, parent-of-origin genomic imprinting has been found to be associated with obesity (Dong et al., 2005). Quite possibly the most famous imprinting occurs on the 15q11-q13 chromosomal region to produce Prader-Willi Syndrome, which is characterized by both severe hyperphagia and hypothalamic hypogonadism (Gurrieri and Accadia, 2009). Interestingly, this syndrome occurs specifically when paternal imprinting is evident in this region; maternal imprinting, on the other hand, results in the phenotypically distinct, non-hyperphagic Angelman Syndrome. This phenotypic variance from differential imprinting may be due to the combinatorial effects of differences in methylation site during the imprinting process along with specific parental allelic expression in certain brain regions (Gurrieri and Accadia, 2009). Parental imprinting to varying degrees has also been found in specific nodes of the feeding circuit and affiliated nuclei, including the ARC and dorsal raphe nucleus (Bonthuis et al., 2015). Given that parental imprinting appears to have such a large effect on hypothalamic development and metabolism (and these effects may be more common than initially believed, Cheverud et al.,

2008), this mechanism may also play a role in the development of neuronal feeding circuitry and sex differences within it.

When discussing sex differences due to parental imprinting, it is typically assumed that this phenomenon occurs only on the X chromosome, as mammalian XX individuals alone could be subjected to paternal imprinting, thereby leading to even further modulation of transcript expression aside from random X-inactivation by *Xist* (Davies et al., 2006). However, some studies have found that parental imprinting effects can be specifically "targeted" towards one sex or another. In a study of mouse embryonic germ cell lines derived from the genital ridge of four core genotypes mice, sex-specific methylation patterns of a paternally-imprinted gene showed that a large degree of XX-specific demethylation was not dependent on gonadal type (Durcova-Hills et al., 2004). Due to lack of quantification, no interactions were evident between sex chromosome complement and gonad type, though the data presented indicate this may be possible. One study demonstrating similar sex-differential parental imprinting in the brain found various loci specifically affiliated with growth and weight exhibiting parent-of-origin imprinting in one sex but not the other (e.g., females showing paternal imprinting of a specific gene whereas males show no epigenetic regulation, Hager et al., 2008). Together, this evidence suggests that sex-specific parental imprinting may interact with sex chromosome complement or other sex variables to produce, exacerbate, or diminish sex differences in the known nodes of the feeding circuit.

Environmental effects

Too often, environmental effects on the brain are not taken into consideration when investigating sex differences. This becomes particularly relevant when determining how different treatment of the sexes may result in altered gene expression (through epigenetic mechanisms) or even strengthening/weakening of neuronal connections (through learning) in a relatively sex-segregated fashion. In human societies, said differential treatment, learning, and expectations

constitutes gender socialization, a culturally defined phenomenon based on assumed sex. Such cultures create sex-biased environments that, like other environmental variables, can act on the brain to induce or exacerbate neuronal “sex” differences during both development and adulthood (Ritz et al., 2014). These gender socialization differences can even register in brain imaging studies. In one small neuroimaging study, women displayed higher activation (as compared to men) in the dorsolateral prefrontal cortex when presented with images of hedonic food following a eucaloric diet and fast (Cornier et al., 2010). As this region is late to develop in humans and is primarily concerned with executive function and top-down inhibitory control, it is not radical to consider that this difference in brain activation might be largely due to gender socialization instead of purely endogenous differences in neuronal activation. Though the biological underpinnings responsible for societally induced changes on the brain are difficult to study in the human population, differential sex treatment in early life by rodent dams has provided an interesting model by which to study an environmentally exacerbated (or created) sex difference.

Olfactory cues, such as higher levels of testosterone and/or its metabolites secreted by pup urine, result in rodent dams preferentially licking the anogenital region of male offspring over that of female pups (reviewed in Moore, 1992). This differential treatment has been shown to be vital to the development of sex-specific sexual behaviors in both male (Moore, 1984) and female rats (Cameron et al., 2008). More recent research has revealed that maternal care and grooming results in epigenetic modifications (Weaver et al., 2004) to alter steroid hormone receptor expression (Szyf et al., 2005), including ERα, in various hypothalamic subnuclei (Cameron et al., 2011; Champagne et al., 2003) and other deep-brain regions (Edelmann and Auger, 2011). Beyond gene expression, such early-life care and exposure has the potential to alter neuronal and dendritic outgrowth (Lenz and Sengelaub, 2006), activity (Cameron et al., 2011; Nguyen et al., 2015), and plasticity (Nguyen et al., 2015). In humans, one might consider such differential treatment by perceived biological sex a construction of gender socialization,

whereby infants and children receive differential treatment and have varying expectations placed upon them due to this perception and its interaction with social norms. Thus, it is quite plausible that gendered constructions may alter gene expression through epigenetic means to produce, reinforce, or counteract endogenous biological sex differences (Cortes et al., 2019). This potentiation of sex differences via differential treatment of offspring by sex retains the possibility of intersection with the feeding pathways. Much of the research concerning differential epigenetic modification following maternal licking or treatment has investigated the modulation of steroid hormone receptors. However, low maternal grooming has also been found to significantly decrease offspring body weight (Lenz and Sengelaub, 2006), indicating possible long-term modulation of feeding and/or other metabolic circuitry. Furthermore, there have been ample studies investigating how epigenetic modification, such as DNA methylation, histone modification, and micro-RNAs, alter feeding through the expression of key genes regulating feeding, including POMC and NPY (for review, see McCarthy and Nugent, 2015). It is therefore not unlikely that sex-specific environmental impacts, exemplified by maternal anogenital licking in rodents and gendered expectations in humans, may also result in epigenetic modifications on the feeding circuit. Thus, the role of differential environment due to perceived sex should not be ignored as a potential source of sex and gender differences in future study of rodents and humans, respectively.

2.6 Bringing It All Together: How Sex Variables Can Alter Feeding Circuits

Cellular effects

Effects of the components of sex can manifest individually and together to result in divergent and/or convergent neuronal activity. Altering cellular activity may result in both divergent and/or convergent behavioral outputs. In one vein, sex differences may result in differential activation of the same neuronal population. In a study demonstrating that female rats consume more palatable food than males, post-mortem analysis demonstrated increased

presence of the immediate early gene *Fos*, a marker of neuronal activation, in the nucleus accumbens of females as compared to males (Sinclair et al., 2017). This indicates that either basal cellular excitability level differences (as no control provided excludes this possibility) or differential activation of this region by the same stimulus might result in altering behavioral output as it relates to food intake. Interestingly, this *Fos* difference was only evident when specifically quantifying the nucleus accumbens shell, designated as a “hedonic hotspot” in the hedonic feeding literature (Sinclair et al., 2017; Smith et al., 2010). While this difference in hedonic activation has not been attributed to any of the aforementioned components of sex in isolation or combination, this region expresses ERα, ERβ, and the androgen receptor (Becker and Chartoff, 2019b). How sex components like gonadal hormones result in differential activation in this case is not known. One mechanism of action could be similar to that examined in serotonin neurons in the dorsal raphe nucleus. Here, estradiol was found to activate neurons through the inhibition of inward-rectifying, small conductance calcium-activated potassium (SK) current in an ERα-dependent fashion, and this activation was associated with a suppression of ovariectomy-induced binge eating (Cao et al., 2014). In this study, the existence of a similar mechanism in males was not explored, nor were the possible genetic, etc. etiologies investigated for differences in dorsal raphe nucleus organization that may lead to hormonally dependent binge eating pathology in females (Klump et al., 2013). Indeed, evidence from outside the feeding circuitry indicates that equivalent cell populations in females and males contribute to similar but distinct outputs. Aromatase neurons in the medial amygdala have been shown to mediate aggression in both females and males. However, the behavioral manifestations of maternal and intermale aggression, respectively, are distinct in form and function (Unger et al., 2015). Which, if any, component(s) of sex contribute to and set up this difference in aromatase neuron function (whether by differential gene expression, axonal projection, or something of the like) has yet to be determined.

In the periphery, sex differences in vagal nerve activity, particularly as it relates to heart rate and overall vagal tone, have become apparent. Not only have baseline sex/gender[3] differences in human vagal tone during sleep been reported (Valladares et al., 2008), but these differences persist in the context of disease, where depressed women exhibit greater cardiac vagal control than depressed men (Chambers and Allen, 2007). Interestingly, the reports of this simple human sex/gender difference in vagal reactivity are muddled. The same physiology may have different behavioral consequences within the context of assigned sex. Vagal reactivity as measured by respiratory sinus arrhythmia was negatively associated with maternal assessments of irritability & oppositional nature in boys but was positively associated with such in girls (Vidal-Ribas et al., 2017). Given the evidence of vagal innervation into regions of central feeding circuitry (Ciriello and Caverson, 2016), it is plausible that this vagal reactivity and context−/sex-dependency may also affect gut-brain communication as it relates to ingestion feedback (Dal et al., 2014; Green et al., 2009). A few human studies have suggested that this may be the case, namely those indicating that infant diet and early life exposures differentially affect children of differing assigned sex (Kühne et al., 2016; Pivik et al., 2015). However, little basic research investigating the mechanisms and factors by which these apparent effects of sex/gender manifest has been conducted.

Anatomical effects

The components of sex can also affect anatomical morphology and connectivity within neurological feeding pathways. As previously stated, neonatal testosterone was found to decrease POMC projections from the ARC and result in a masculinization of food intake (Nohara et al., 2011). Interestingly, this projection pattern, while mimicking that of control males,

[3] Here, we are using the combined term “sex/gender” as in (Jordan-Young and Rumiati, 2012) because, in the context of human studies, the endogenous effects of sex variables and the exogenous effects of gender socialization are inextricably linked and arguably unable to be dissociated.

does not fully explain sex differences in adulthood feeding, as this masculinization of food intake did not quite reach the level of male controls. These results may be confounded by the effects of treatment on peripheral energy processing, as neonatal testosterone failed to defeminize/masculinize leptin signaling and white adipose tissue storage in any discernable pattern (Nohara et al., 2011). Nonetheless, this study makes apparent that neonatal testosterone's “masculinization” of POMC projections from the arcuate is merely one factor in how the components of sex alter the neurological control of feeding.

In the human brain, a similar pattern emerges. The use of brain imaging technologies has allowed for investigations into the living human brain. And while sex/gender differences unveiled in these studies may be somewhat inconclusive depending on their interpretations, careful analysis and conservative extrapolations of the data can allow for a deeper understanding of human physiology. In a diffusion tensor imaging study, women (pre-menopausal, apparently naturally-cycling, and scanned during the approximated menstrual follicular phase) exhibited higher connectivity between nodes associated with reward circuitry as compared to men (age-matched and presumably cis; Gupta et al., 2017). While the specific pattern of connectivity strength did interact with body mass index, the overall sex/gender difference in connectivity strength remained within reward circuitry nodes. Even understanding the limited extrapolations brain structure can convey to function (de Vries and Södersten, 2009), this evidence is nonetheless intriguing given the hypothesis that disordered eating, a woman/girl-skewed trait, potentially results from an imbalance of homeostatic and motivational/reward circuitry (Avena and Bocarsly, 2012; Ferrario et al., 2016). And while this imaging study excluded individuals with diagnosed eating disorders, the underlying circuitry differences may result in a biological predisposition to extreme feeding modulation in certain contexts (though whether this apparent connectivity difference is endogenous, the result of gender socialization, or both remains to be explored).

As noted above, it is currently difficult to dissociate the relative contributions of the endogenous components of sex from the exogenous effects of gender on the brain. Endogenously, the organizational and activational effects of sex hormones may indeed contribute, but not fully explain, sex and gender differences in feeding circuitry to differentially promote disordered eating. In a study using 2D:4D digit ratio as a proxy for neonatal testosterone levels (Lutchmaya et al., 2004) along with salivary estradiol levels, researchers found that inferred lower neonatal testosterone and higher circulating adult estradiol levels independently correlated with disordered eating as defined by the Minnesota Eating Behaviors Survey (MEBS; Klump et al., 2006). However, inferred neonatal testosterone and adult estradiol accounted for only ~5% and 6–11% of the population variability in survey score, respectively, suggesting that other factors are needed to explain the sex difference. Indeed, some portion of these sex differences may be due to sex chromosome complement. Moving back to mice, intact XX animals of either gonadal type show increased food-reinforced habit formation than XY counterparts (Quinn et al., 2007), indicating that sex differences in hedonic/motivational feeding circuitry is probably due to a combination of at least gonadal hormone and sex chromosome complement effect.

2.7 Conclusions

The contributions of sex differences are multifaceted and varied in any context, including the neuronal control of food intake. Instead of providing a complete account of all studies that report a sex difference, we have chosen a few examples to illustrate how the components of sex can act individually or in combination to affect behavioral output. Sex can modulate many facets of neuronal feeding circuits, from individual cells to circuitry connections, in the homoeostatic and hedonic nodes, and at all stages of feeding. It is vitally important to remember that effects of one component of sex (e.g., gonadal hormones in adulthood) do not exist in isolation. Both synergism (De Vries, 2004) and compensation from other sex components can further

differentiate or mitigate resulting behavioral output, respectively (McCarthy and Arnold, 2011; Ritz et al., 2014). Not only this, but the degree of “masculinization” or “feminization” may differ from tissue to tissue and brain region to brain region. Such heterogeneity may result from the effects of differing sex components, context, and environment (Joel, 2012; McCarthy, 2015). Thus, it is more useful to view both central and peripheral neuronal controls of feeding as being possibly differentially influenced by orthogonal individual components of sex (Joel and McCarthy, 2017).

When we take this view, it is not surprising that sex differences remain apparent in vagal innervation following both pre-pubertal ovariectomy and after subsequent estradiol replacement (Ciriello and Caverson, 2016) as previously referenced (Figure 2-2). While the effects of neonatal testosterone surge should undoubtedly be explored, so too should the effects of sex chromosome complement, parental imprinting, early life experience, and other environmental impacts. This would allow for a more complete understanding of how these factors manifest in an alteration of peripheral-to-central circuitry and potentially change responsiveness to gustatory and/or ingestion cues. Hence, future research investigating sex differences in feeding must collectively pay heed to how all the varying components of sex intersect to modulate and shape cells and circuitry in the hypothalamus, brainstem, periphery, and beyond. This approach will improve our understanding of how sex (and even gender) factors modulate food intake to predispose people of certain genders to modulate food intake to the degree of under- or over-nutrition. We believe that this multifaceted approach is crucial for a fundamental understanding of feeding physiology.

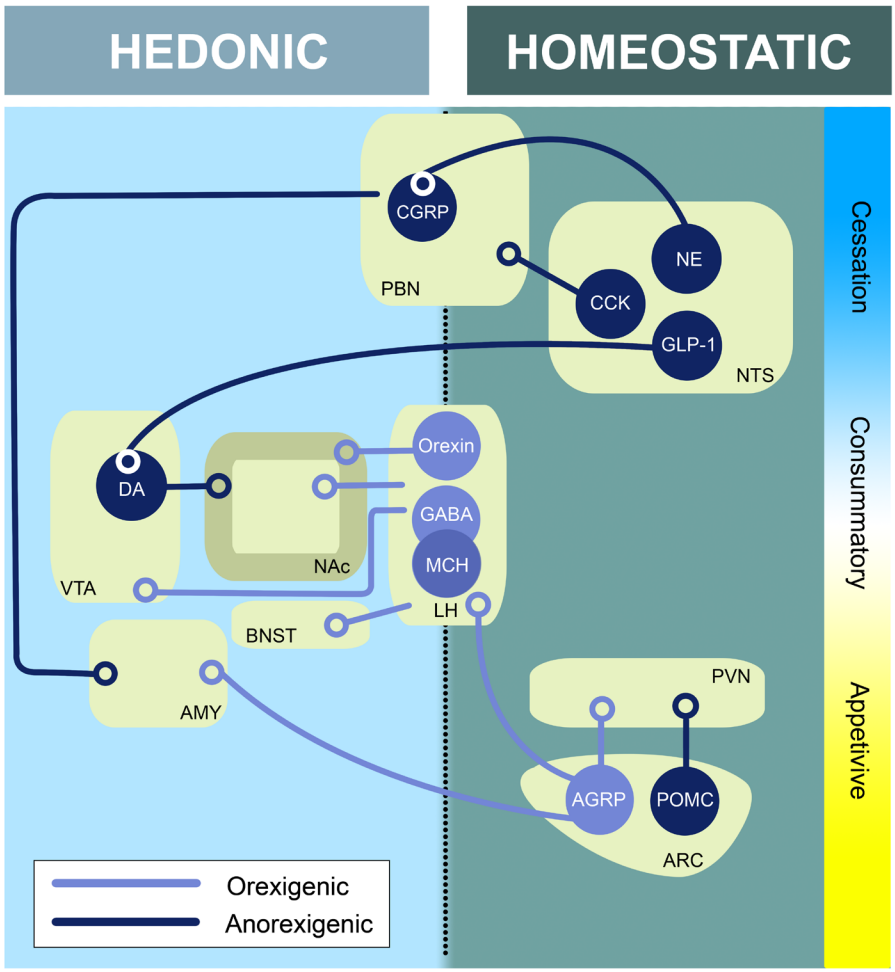

Figure 2-1. Circuit diagram of homeostatic/hedonic interaction between feeding nodes in the brain. This non-exhaustive figure provides a rough overview of the large degree of overlap and communication between canonically hedonic and homeostatic feeding nodes across the appetitive, consummatory, and cessation stages of feeding. Processes do not denote excitation/inhibition, merely recruitment. Light blue lines denote typically orexigenic pathways, dark blue typically anorexigenic. Terminal peripheral outputs not depicted. AGRP: agouti-related peptide; AMY: amygdala; BNST: bed nucleus of the stria terminalis; ARC: arcuate nucleus of the hypothalamus; CCK: cholecytoskinin; CGRP: calcitonin gene-related peptide; DA: dopamine; GLP-1: Glucagon- like peptide 1; LH: lateral hypothalamus; MCH: melanin-concentrating hormone; NAc: nucleus accumbens; NE: norepinephrine; NTS: nucleus of the

solitary tract; PBN: parabrachial nucleus; POMC: proopiomelanocortin; PVN: para- ventricular nucleus of the hypothalamus; VTA: ventral tegmental area.

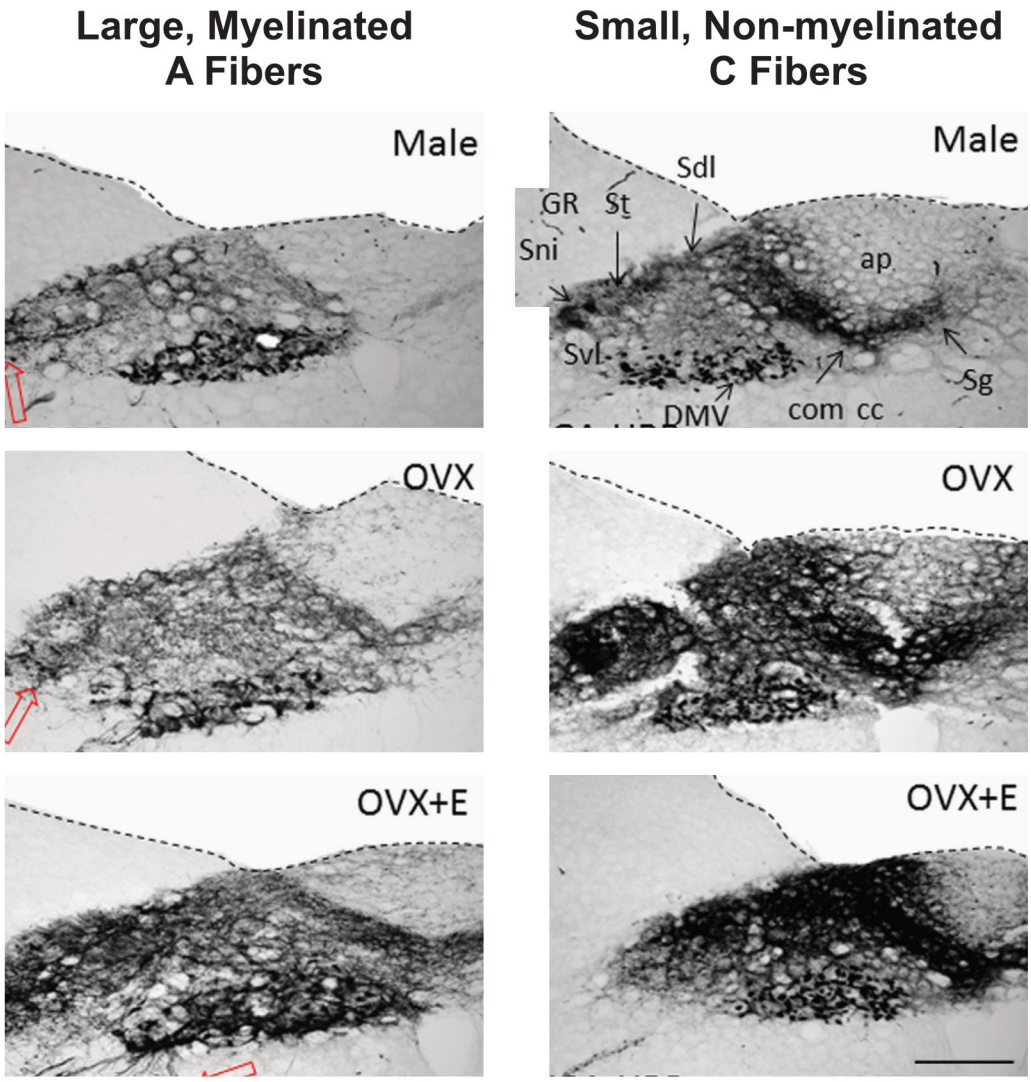

Figure 2-2. Sex difference in vagal fiber innervation of NTS is not fully explained by adult circulating ovarian hormone presence. Adapted figure from (Ciriello and Caverson, 2016) under fair use copyright showing innervation densities and patterns in the NTS. Innervation density is affected by estradiol in females, but innervation pattern of both myelinated and non-myelinated fibers into the NTS remains distinct in female and male animals despite hormone manipulation in females. E: estradiol; OVX: ovariectomy

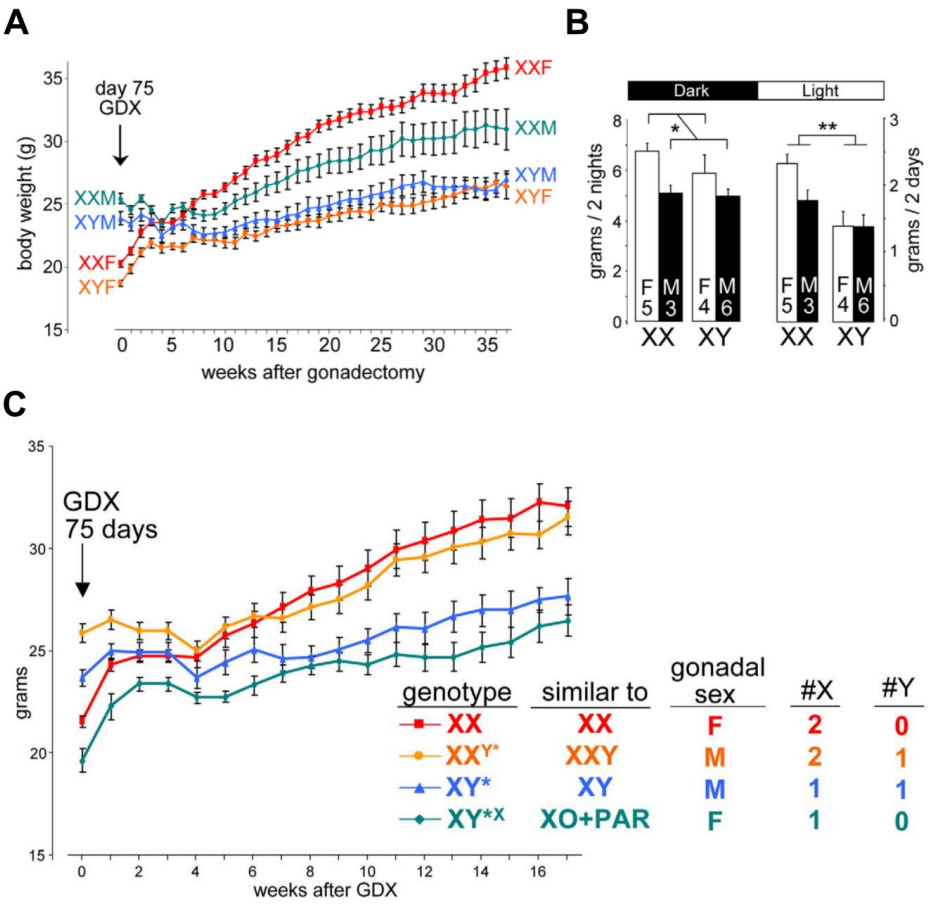

Figure 2-3. Effect of sex chromosomes on body weight and food intake. Adapted from (Chen et al., 2012) under fair use copyright. (**A**) Experiments in four core genotypes model demonstrate that body weight is primarily driven by gonadal type prior to gonadectomy (GDX), after which the effect of sex chromosome complement (and interactions with previous hormonal state) are revealed. (**B**) This effect on body weight is contributed to by an effect of sex chromosomes (particularly X dosage as revealed by the XY* model, **C**) on daytime food intake four weeks following GDX. PAR: pseudoautosomal region; XXF: XX + ovaries; XXM: XX + testes; XYF: XY + ovaries; XYM: XY + testes.

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Chapter 3

Sex Differences in the Tuberal Nucleus of the Hypothalamus

3.1 Introduction

The results of this thesis support my assertion in Chapter 2 that a multifaceted approach to the contributions of sex variables is vital to investigate the neuronal control of feeding. What began as a simple study in sex differences (Chapter 3) expanded and was complicated by evident interactions with metabolic cues (Chapter 4). Without attention to negative data and proper positive controls, this complex and perhaps vital role of a newly discovered hypothalamic feeding node may have been overlooked.

In 2018, the tuberal nucleus of the hypothalamus (TN) was identified as a novel node of the hypothalamic feeding circuit (Luo et al., 2018; Mickelsen et al., 2019). Cells expressing the neuropeptide somatostatin (SST) were found to integrate into the canonical melanocortin system, releasing γ-aminobutyric acid (GABA) in the paraventricular nucleus (PVN) to elicit increases in food intake (Luo et al., 2018). The role of these neurons, while novel, was not quite surprising. While SST was originally named growth hormone inhibiting hormone (GHIH) in the central nervous system (Painson and Tannenbaum, 1991), central SST has been long known to affect food intake through somatostatin receptor 2 (SSTR2; Beranek et al., 1999; Campbell et al., 2017; Danguir, 1988; Karasawa et al., 2014; Lin et al., 1989; Stengel et al., 2015, 2010a, 2010b, 2010c, 2011, 2013; Tachibana et al., 2009). Indeed, many revealed projections of SST neurons in the TN (TN^{SST}) express SSTR2, including the amygdala, PVN, and periaqueductal gray (PAG; Fehlmann et al., 2000; Luo et al., 2018). Subsequent papers from the same group revealed integration of TN^{SST} neurons into food context learning and perhaps hedonic circuitry in males[4] (Mohammad et al., 2021).

The TN straddles the mediobasal and lateral hypothalamic area, with some viewing this region as closely related to the ventrolateral region of the ventromedial hypothalamic nucleus

[4] In all external papers discussed, no definitions for sex category were ever provided. In mice, I assume that females were defined as short anogenital distance and post-mortem confirmation of ovaries; males long anogenital distance and testes.

(VMH_{VL}; Canteras et al., 1994) and others categorizing this region as a constituent of the lateral hypothalamus (Mickelsen et al., 2019). Regardless, given this region's unique situation within the brain near regions known to be involved in sex-specific modulation of energy homeostasis (Correa et al., 2015; Musatov et al., 2007; van Veen et al., 2019; Xu et al., 2011), its possible sensitivity to circulating reproductive hormones (Canteras et al., 1994), and its detection of metabolic hormones such as ghrelin (Luo et al., 2018), it is likely that the TN also displays yet-to-be defined sex differences in its modulation of food intake.

The overarching question of this chapter is: do TN^{SST} neurons display sex differences in the modulation of food intake? To answer this question, I used female and male mice (defined as having small anogenital distance at weaning and a subsequent vaginal opening, estrous cycle, and postmortem ovary presence; long anogenital distance at weaning, no vaginal opening, and postmortem testis presence, respectively) in conjunction with the Cre-lox system and stereotaxic viral injection to selectively manipulate TN^{SST} neurons and examine their effects on food intake. With enough mice to detect statistical differences, TN^{SST} neurons exhibit differential control of feeding in female and male mice, with TN^{SST} neuronal ablation decreasing food intake only in females. Further analysis revealed this effect was primarily due to a decrease in food intake during proestrus, when circulating hormones are at high endogenous concentrations. Perplexingly, this effect was unable to be recapitulated using reversible TN^{SST} neuron inactivation or in an ovariectomy paradigm.

3.2 Materials and Methods

Mice

Female and male mice expressing the *Sst-Cre* driver transgene (stock no. 013044, $Sst^{tm2.1(cre)Zjh}$/J) were maintained on a C57BL/6 genetic background. Heterozygotes and/or wildtype littermates were used for all studies. Genotypes were determined as per JAX protocol 28317. Experiments were performed on cycling females and intact males unless otherwise

stated. Mice were maintained on a 12:12 light cycle, with ad libitum access to food and water (unless otherwise specified), under controlled humidity conditions, and in single-housed cages with non-caloric paper bedding to ensure accurate food intake assessment. All studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. UCLA is AALAS accredited, and the UCLA Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

Estrous cycle staging

Vaginal lavages were performed on females daily, between ZT 0 and ZT 4, using 30 mL of standard PBS. Samples were deposited onto slides for further staining. Males were subjected to similar handling during this time to ensure roughly equivalent handling stress. Giemsa staining was carried out to visualize cellular composition of the vaginal cavity. Stock Giemsa stain was prepared at least one week in advance of use. An 18.5% solution of Giemsa powder (Fisher G146-10) in glycerin was heated to 60^{o}C and cooled before diluting 9:14 with 100% methanol. Stock was diluted 1:30 in PBS before use, shaking vigorously before a one-hour incubation at room temperature. Prior to staining, slides were fixed by dunking ten times in 100% methanol. Staging was assessed via light microscopy as in (Cora et al., 2015), and stages were assigned via a modified behavioral method (Becker et al., 2005), with morning swabs indicating the prior night's stage (Figure 3-1). This staging method was confirmed by core body temperature waveform alignment (Sanchez-Alavez et al., 2011).

Surgical procedures

Mice received analgesics (0.074 mg/kg buprenorphine two times daily, 7.11 mg/kg carprofen one time daily) on the day of and one day post-surgery. Mice were anaesthetized with 3% isoflurane and maintained within a range of 1.25-2.5%. Cre-dependent viruses were bilaterally injected into the TN of adult mice (coordinates relative to Bregma: A-P -1.65 mm, M-L

±0.75, D-V -5.45; scaled when Bregma-Lamda distance was not equivalent to 4.2 mm) at a rate of 5 nL/s using a glass-pulled needle. See Table 1 for titers and injection volumes. Controls consisted of both wildtype animals injected with the experimental virus (virus controls) and cre positive animals injected with cell-filling green fluorescent protein (GFP; genotype controls). Ovariectomy surgeries included complete removal of gonads from adult mice. Gonadectomies occurred immediately prior to stereotaxic viral injections within the same surgical period. In telemetry experiments, G2 eMitters (Starr Life Sciences) were implanted intraperitoneally on the same day as viral injection. Experiments were conducted following at least two weeks recovery from surgical proceedings.

Transient activation food intake assay

Clozapine-n-oxide (CNO; MilliporeSigma #0832) was used to activate TN^{SST} neurons in animals expressing designer receptors exclusively activated by designer drugs (DREADDs). Stock solution of 20 mg/mL in DMSO was stored at -20°C and diluted to a working solution of 0.03 mg/mL in sterile saline also stored at -20°C. Saline control (0.15% DMSO) or CNO (10 μL/g body weight, dose of 0.3 mg/kg) working solution were administered IP to both DREADDed animals and controls in a counterbalanced design. Experiments were completed in duplicate. Mice were transferred to the experimental room at least 15 minutes prior to experimentation onset. Experiments were begun between ZT 2-3 and terminated between ZT 6-7. Following injection, food intake was measured at 0.5, 1, 2, and 4 hr. Female mice were lavaged after experiment conclusion to prevent stress interference with food intake. All mice were injected with CNO 90 minutes prior to sacrifice to enable neuronal activation validation via cFOS immunohistochemistry.

Caspase ablation experiments

Gross movement and core body temperature were passively measured every other week for eight weeks using VitalView software (Starr Life Sciences). Body weight was measured every week. Food assay was performed when mice were not on telemetry pads. At ZT 0.5 on the start day of the experiment, 2/3 of the non-caloric paper bedding was removed and regular cotton bedding was provided. A pre-measured amount of food was delivered, and mouse body weight measured. Food in hopper was weighed at ZT 0.5 and ZT 11.5 every day until experiment conclusion. After 96 hours, food and all bedding were collected to account for food spillage. For some experiments, 4-5 hour fasted glucose tolerance tests were performed prior to sacrifice. In ovariectomy experiments, two food assays were performed back-to-back, non-fasted resting glucose levels were collected, body composition was measured via NMR, and indirect calorimetry was performed in Oxymax metabolic chambers (Columnbus Instruments) at room temperature. Upon experiment completion, all brains were collected in an RNase-free manner. Intrascapular brown adipose tissue (BAT), inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), liver, soleus muscle, and gastrocnemius muscle were collected for both RNA and histology analyses. Oviduct and gonad (if present) were collected for histology only. In ovariectomy experiments, bilateral oviducts and vaginal canal were weighed as a proxy for estrous stage (Bingel et al., 1975).

Transient inactivation fast-refeed food assay

Salvinorin B (SalB; Cayman Chemical #23582) was used to inhibit TN^{SST} neurons in animals expressing the modified κ-opioid receptor inhibitory DREADD (KORD). Solution of 5 mg/mL in DMSO was stored at -20°C and warmed prior to use. DMSO control or SalB (2 μL/g body weight, dose of 10 mg/kg as in Luo et al., 2018) were injected subcutaneously in the intrascapular region. Mice were fasted within one hour of lights-off (ZT 11-12), with cages changed to eliminate all food access. The next day, mice were transferred to a new room

(between ZT 1.5 and 2.5) and allowed to acclimate for thirty minutes. Mice were then injected with DMSO or SalB and allowed to rest for one hour before refeeding. Food was weighed every hour for 3 hours. Mice were weighed at food introduction and at the conclusion of the 3 hour refeed to perform feed efficiency calculations. Female mice were lavaged after experiment conclusion to prevent stress interference with food intake. Experiments were carried out in a counterbalanced design and in duplicate. All mice were fasted for 16 hours and then injected with SalB 90 minutes prior to sacrifice in an attempt to visualize neuronal inactivation via cFOS immunohistochemistry.

In situ hybridization (ISH) and immunostaining (IHC)

RNA probe generation was accomplished as in (van Veen et al., 2019). Briefly, *Sst* PCR products were amplified using Allen Brain Institute-derived reference primer sequences and cloned into pCR 2.1 TOPO vectors (Invitrogen). Plasmid DNA was then isolated from bacterial cultures (ZymoPURE II Plasmid Midiprep kit), linearized, and purified (Zymo DNA Clean & Concentrator). *Sst* sense and antisense probes were transcribed using a DIG or FITC RNA labeling kit (Roche) and purified with RNA Clean & Concentrator (Zymo Research). ISH protocol was carried out on 35μm-thick slices as per (van Veen et al., 2019) and co-stained with ERα or cFOS primary antibodies and animal-specific secondaries. For IHC only cFOS stainings, polyclonal rabbit anti-c-Fos (Synaptic Systems # 226003) was used at a dilution factor of 1:200, and secondary goat anti-rabbit antibody at a dilution of 1:500.

Statistical Analyses

All statistics were carried out in R. Sex difference presence was determined by interaction terms between genotype and sex (caspase experiments) or genotype, treatment, and sex (DREADD and KORD experiments). In caspase experimentation, animals meeting both the criteria of outlier by Cook's distance as well as "miss" (no hit or unilateral hit) were excluded.

In KORD experiments, animals meeting the criteria of either outlier by Cook's distance or "miss" (no hit or unilateral hit as defined by less than 5% of targeted cells infected) were excluded.

3.3 Results

Chemogenetic activation of TN^{SST} neurons increases food intake in female and male mice

To test whether activation of TN^{SST} neurons resulted in differing behavioral outputs across sexes, we delivered a Cre-dependent GqDREADD virus to the TN of *Sst-Cre* mice (Figure 3-2A). Hits were validated by immunofluorescent microscopy and validated for increased activation in the TN via cFOS immunohistochemistry (CNO delivered 90 minutes prior to sacrifice, Figure 3-2B). Unilateral hits were removed from analysis. Mice were then subjected to treatment with activating CNO or saline in a counterbalanced, within-subjects design.

Overall, activation of TN^{SST} neurons increased daytime food intake over the four-hour testing period in both females and males (Figure 3-2C). Control animals without expression of GqDREADD confirmed a lack of endogenous CNO effect, while within-subjects comparisons of animals expressing GqDREADD in TN^{SST} neurons indicated an increase in feeding upon CNO-induced cellular activation. There was a significant interaction between neuronal activation (genotype) and treatment (saline v. CNO) over time ($F(2,105)=3.2964$, $p=0.0409$) and independent of time ($F(1,105)=35.2054$, $p<0.0001$). Within sex, only males exhibited an effect of activation and treatment over time ($F(2,45)=3.2793$, $p=0.0468$), though both females and males exhibited an effect independent of time ($F(1,60)=12.7928$, $p=0.0007$ and $F(1,45)=25.1794$, $p<0.0001$, respectively). This effect was most prominent in both sexes at 4 hours post-CNO injection (females: $F(1,12)=10.0208$, $p=0.0081$; males: $F(1,9)=12.1521$, $p=0.0069$), though males also exhibited a significant activation-by-treatment interaction at 2 hours post-CNO ($F(1,9)=8.6957$, $p=0.0163$). *Post-hoc* within-subjects analyses of animals bilaterally infected with GqDREADD virus indicated a significant increase during activation by CNO as compared to

treatment with saline control (females overall: t(31)=2.8486, p=0.007732; males at 2 hours: t(5)=2.9701, p=0.0311; males at 4 hours: t(5)=3.3263, p=0.0286).

Caspase ablation of TN^{SST} neurons decreases food intake only in females

To investigate whether permanent inactivation of TN^{SST} neurons resulted in differing feeding behavior across sexes, a Cre-dependent modified caspase virus was stereotaxically delivered to the TN of *Sst-Cre* mice (Figure 3-3A). Hits were validated by *in situ* hybridization (Figure 3-3B) and unilateral hits were removed from the analysis. Mice were then subjected to two 96-hour food assays along with a battery of other metabolic tests.

The effect of caspase ablation differed by sex (sex-by-ablation interaction: F(1,38)=4.6852, p=0.0368), an effect which *post-hoc* t-tests revealed to be cared by a decrease in food intake specifically in females (t(15.671)=-3.0561, p=0.007686, Figure 3-2C). ANOVA analysis also revealed an overall effect of sex where males consume more food than females, as to be expected (F(1,38)=14.1896, p=0.0006). This sex difference was not previously reported (Luo et al., 2018), though collapsing these data across sex results in females carrying the effect to show a decrease in food intake with caspase ablation (t(39.86)=-2.2713, p=0.0286), so previous studies may have been underpowered in this regard.

This sex difference appears to be determined by estrous cycle stage. Animals were assigned nightly stage by morning vaginal lavage and average nightly food intake per stage was examined. There was a significant interaction between caspase ablation and estrous stage (F(3,41)=3.2946, p=0.0298) which was predominantly due to a decrease in food intake due to caspase ablation specifically in proestrus (t(5.902)=-2.6044, p=0.04104, Figure 3-2D), when ovarian hormone levels are high. Within subjects analysis revealed that this amount of nightly food intake during proestrus was also slightly decreased when compared with consumption during metestrus (t(7)=-1.976, p=0.0887) and diestrus (t(7)=-2.3276, p=0.0528).

Despite this effect on food intake, no other metabolic measures were affected by caspase ablation. TN^{SST} neuron ablation did not affect telemetry measures of activity/movement (Figure 3-3E) or core body temperature (Figure 3-3F), nor response to fasting glucose tolerance test (Figure 3-2G). Surprisingly, despite a decrease in food intake, ablation also did not affect body weight (Figure 3-3H).

Chemogenetic inactivation of TN^{SST} neurons nominally recapitulates sex-specific decreases in food intake

To ensure that neuronal ablation did not result in permanent compensatory effects to result in this sex difference, TN^{SST} neurons were transiently inactivated using KORDs. A Cre-dependent KORD virus was stereotaxically delivered to the TN of *Sst-Cre* mice (Figure 3-4A). Hits were validated through visualization of the mCitrine conjugated to the KORD protein (Figure 3-4B) and unilateral hits were removed from analysis. Mice were fasted overnight before being subjected to a refeed paradigm following treatment with inhibiting SalB or DMSO control in a counterbalanced, within-subject design.

Overall, inactivation of TN^{SST} neurons nominally decreased food intake during refeeding in females but not males (Figure 3-4C). Control animals without expression of KORD confirmed a lack of endogenous SalB effect, while within-subject comparisons of animals expressing KORD in TN^{SST} neurons revealed an interaction between neuronal inactivation (genotype), treatment (DMSO v. SalB), and sex ($F(1,110)=11.70327$, $p=0.0009$) alongside an overall interaction between sex and time ($F(2,110)=4.3169$, 0.0157). Three hours post treatment demonstrated the most relevant effect, showing an interaction across neuronal inactivation, treatment, and sex ($F(1,22)=4.4371$, $p=0.0468$). Within sex, only females exhibited a decrease in food intake with neuronal inactivation ($F(1,60)=11.7172$, $p=0.0011$), but *post-hoc* tests were underpowered given inter-mouse variability and thus revealed no timepoint-specific within-

subject decreases, though inactivation decreased food intake at three hours after treatment across subjects ($F(1,12)=6.5706$, $p=0.0248$).

Ovariectomy fails to recapitulate TN^{SST} neuronal ablation-mediated decrease in food intake

To test whether ovarian hormones were required to reveal an effect of TN^{SST} neuronal ablation, we performed a 2x2 ovariectomy experiment, where both ablated and not ablated animals were either ovariectomized or given a sham procedure. Interestingly, not only did ovariectomy decrease food intake compared to sham controls ($F(1,70)=3.5479$, $p=0.0638$), but the positive control sham group did not reveal a decrease in food intake upon caspase ablation (Figure 3-5).

3.4 Conclusions

Together, these data suggest that a sex-specific function for TN^{SST} neuronal contributions to feeding. Whereas activation of TN^{SST} neurons increases food intake across sex, ablation (and, to some degree, transient inactivation) of these neurons decreases food intake only in females. While this effect appears to predominantly occur during periods of high circulating ovarian hormones, ovariectomy experiments were inconclusive due to the failure of the sham positive control.

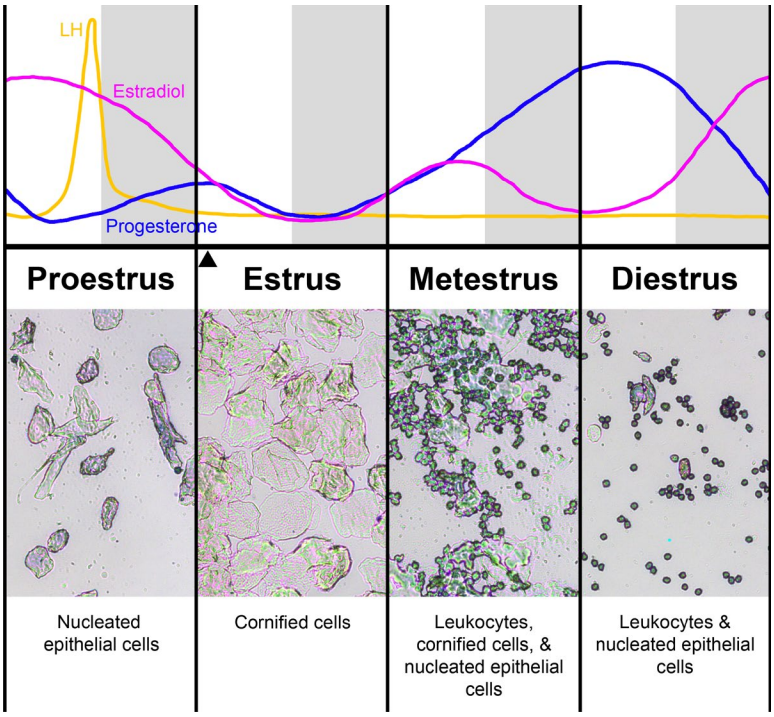

Figure 3-1. Method of estrous cycle determination. Relative hormonal fluctuations (adapted from McLean et al., 2012 under fair use copyright) corresponding to vaginal lavage cell collection. Swabs collected the morning after each estrous stage was used to assess the previous day's stage (e.g., proestrus was assigned via vaginal cytology displaying nucleated epithelial cells at the black arrow).

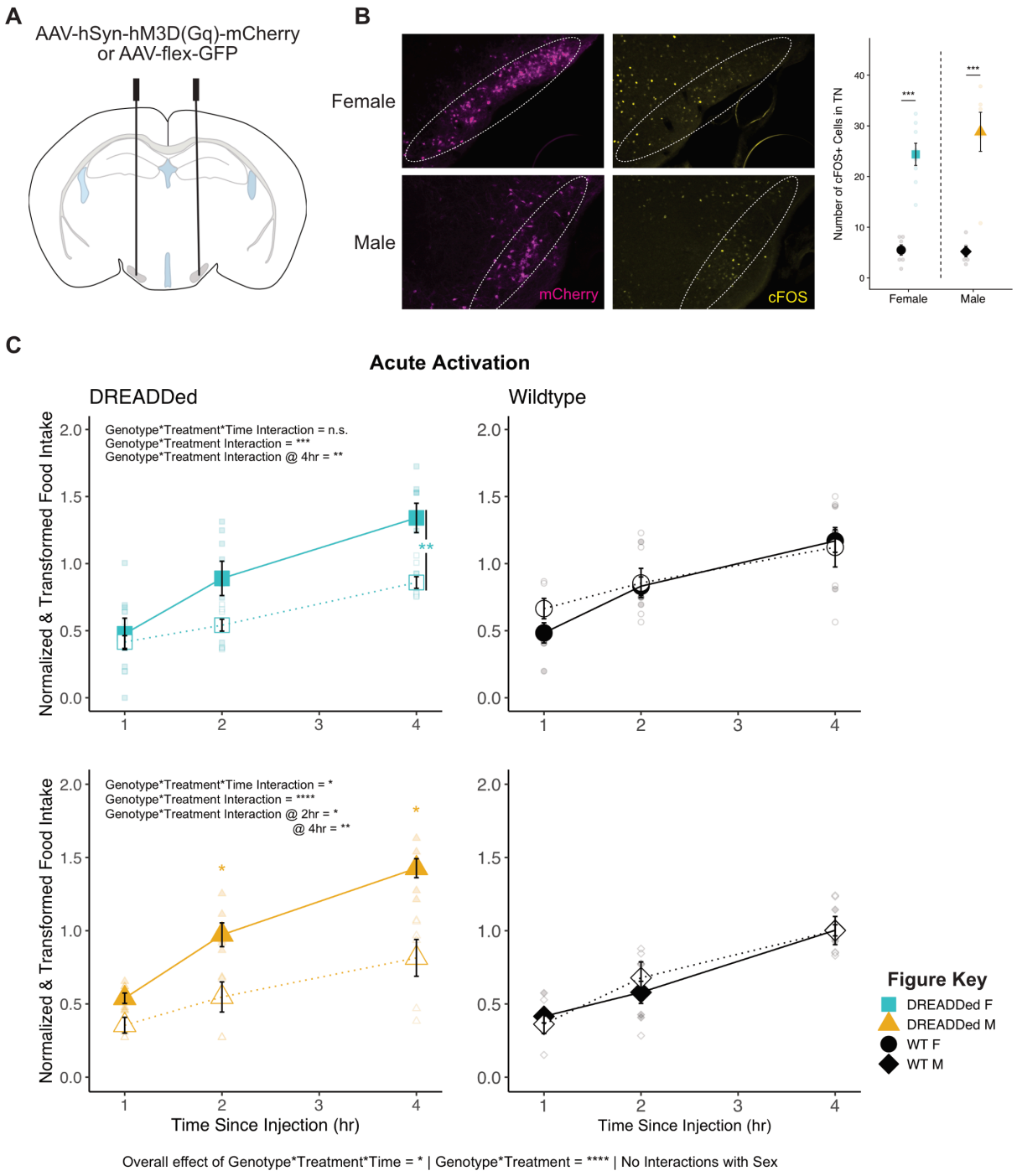

Figure 3-2. Transient activation of TN^{SST} neurons increases food intake across sexes. (A) Schematic of experimental paradigm. AAV viruses of GqDREADD or GFP contained within Flip-Excision (FLEX) cassettes were injected bilaterally into the TN of *SST-Cre* mice. (B) DREADDed animals show mCherry expression and increased neuronal activation in the TN as

seen through an increase of cFOS positive cells 90 minutes after CNO injection. (C) Activation of TN^{SST} neurons in both female and male mice show an increase in food intake within the four-hour daytime testing period (left column). CNO alone did not affect food intake (right column). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

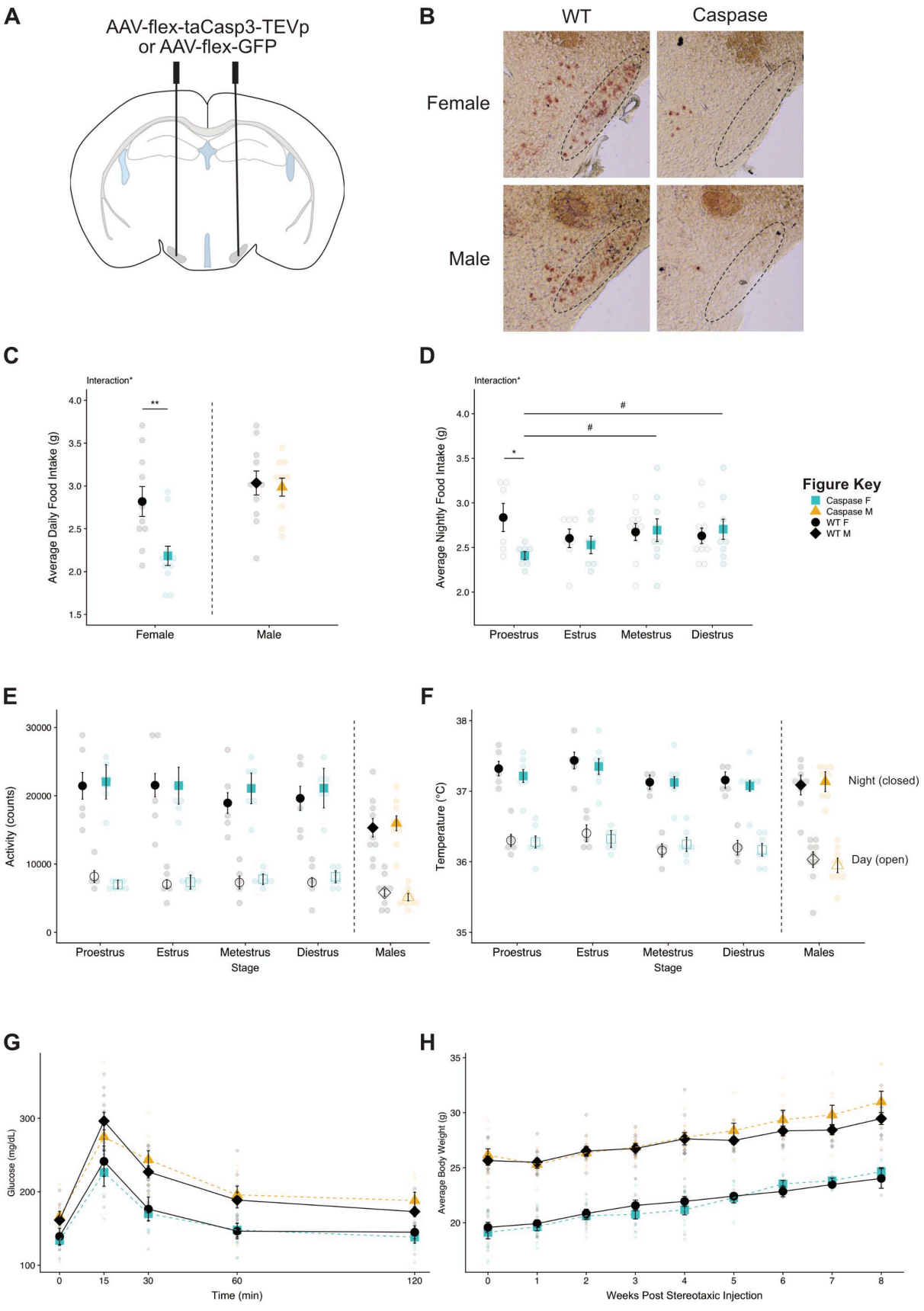
A
AAV-flex-taCasp3-TEVp
or AAV-flex-GFP
B
WT
Caspase
Female
Male
C
Interaction*
**
Average Daily Food Intake (g)
Female
Male
D
Interaction*
#
#
*
Average Nightly Food Intake (g)
Proestrus
Estrus
Metestrus
Diestrus
Figure Key
Caspase F
Caspase M
WT F
WT M
E
Activity (counts)
Proestrus
Estrus
Metestrus
Diestrus
Males
Stage
F
Temperature (°C)
Night (closed)
Day (open)
G
Glucose (mg/dL)
Time (min)
H
Average Body Weight (g)
Weeks Post Stereotaxic Injection

Figure 3-3. Caspase ablation of TN^{SST} neurons decreases food intake only in females. (A) Schematic of experimental paradigm. AAV viruses of modified caspase 3 or GFP contained within FLEX cassettes were injected bilaterally into the TN of *SST-Cre* mice. (B) Representative images of wildtype (WT) and ablated animals. (C) Permanent TN^{SST} neuronal ablation decreases average daily food intake in females but not males. (D) This decrease in food intake is predominantly carried by a decrease in nighttime food intake during proestrus. TN^{SST} neuronal ablation did not affect other metabolic measures, including activity (E), temperature (F), glucose tolerance (G), and average body weight (H). Mean ± SEM; between subjects: *$p<0.05$, **$p<0.01$; within subjects: $^{\#}p<0.05$

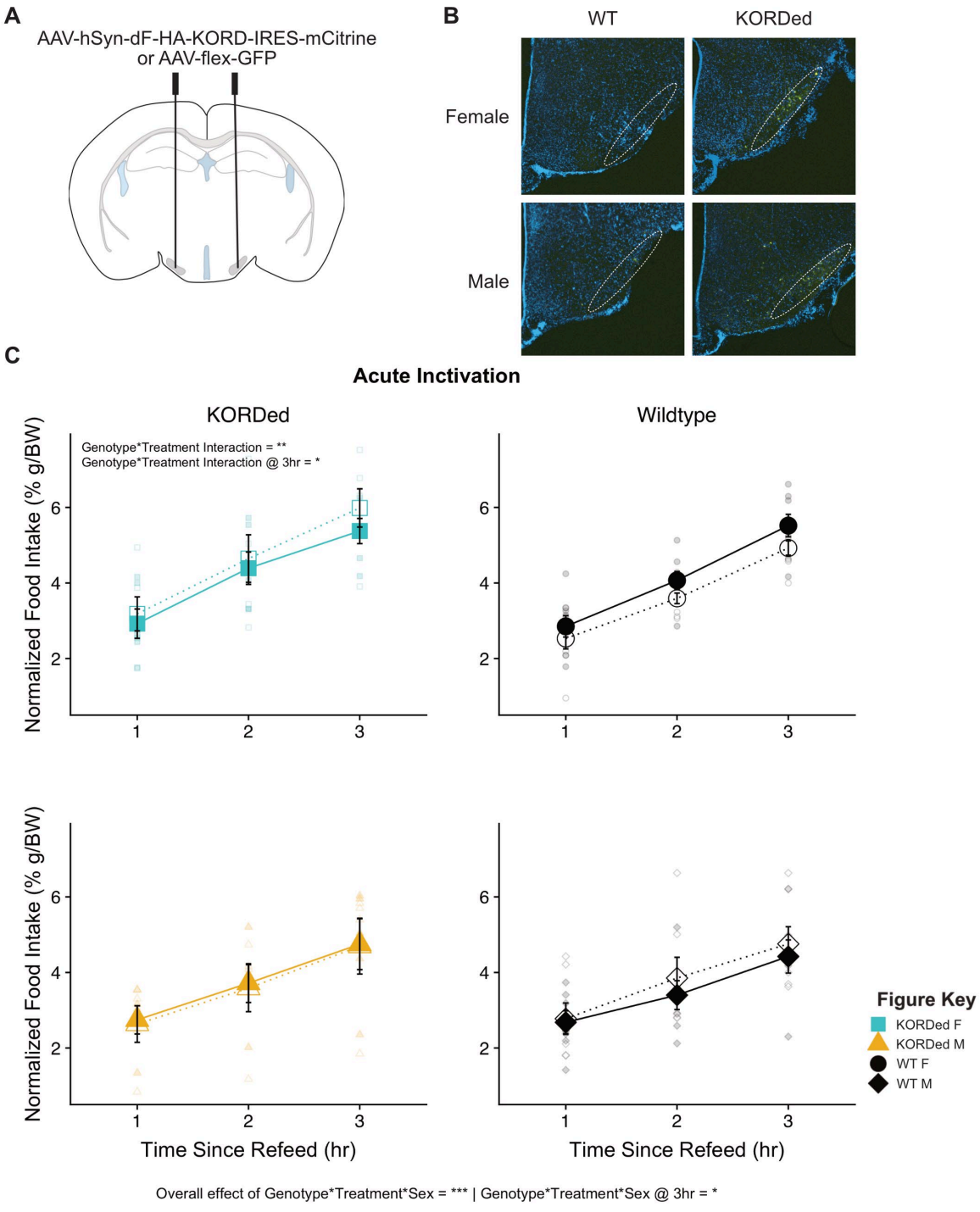

Figure 3-4. Transient inactivation of TN^{SST} neurons after overnight fast nominally decreases food intake in females. (A) Schematic of experimental paradigm. AAV viruses of KORD or GFP contained within FLEX cassettes were injected bilaterally into the TN of *SST-Cre* mice. (B) KORDed animals show KORD expression in the TN due to mCitrine conjugation. (C)

Inactivation of TN^{SST} neurons following fast show nominal decreases in food intake in females but not males (left column). SalB alone did not affect food intake (right column). Mean ± SEM, *p<0.05, **p<0.01

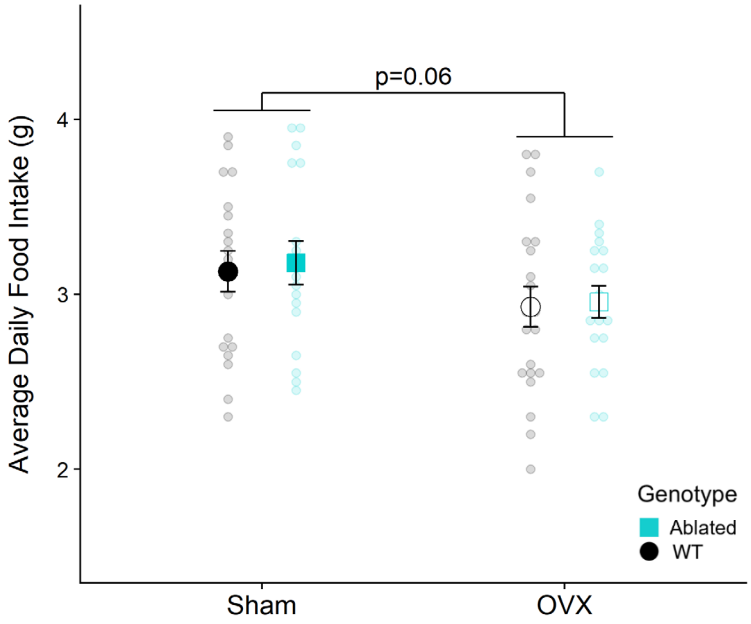

Figure 3-5. Ovariectomy decreases food intake regardless of TN^{SST} neuron ablation. 2x2 sham and ovariectomy study revealed a trending decrease in food intake following ovariectomy (open shapes) as compared to sham controls (filled shapes) regardless of ablation status. Ablation of TN^{SST} neurons did not decrease food intake in sham positive controls.

Experiment	Virus	Depositor & Procurement	Titer (vg/mL)	Volume (nL)	Citation
Caspase ablation	AAV2-flex-taCasp3-TEVp	Nirao Shah & Jim Wells, UNC Vector Core	1-8 x 10^{12}	200-250	(Yang et al., 2013)
Transient activation	AAV8-hSyn-hM3D(Gq)-mCherry	Brian Roth, Addgene viral prep # 50474-AAV8	$\geq$ 4 x 10^{12}	150-200	(Krashes et al., 2011)
Transient inactivation	AAV8-hSyn-dF-HA-KORD-IRES-mCitrine	Brain Roth, Addgene viral prep # 65417-AAV8	$\geq$ 7 x 10^{12}	150	(Vardy et al., 2015)
Fluorescent controls	AAV8-Syn-FLEX-Mac-GFP	Edward Boyden, Addgene plasmid #58852	1:5 dilution of stock	Matching volume to experimental animals	

Table 3-1: List of viral vectors used.

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Chapter 4

Adiposity Affects Modulation of Food Intake by the Tuberal Nucleus of the Hypothalamus

4.1 Introduction

The failure of the positive sham surgery control during the ovariectomy experiment (Figure 3-5) led to an exploration of why these data ran contrary to previous experiments (Figure 3-3C) and published studies (Luo et al., 2018). Given the interactions between metabolism and reproduction, namely the interactions apparent between adiposity, feeding, and reproduction (see Chapter 1), I began to investigate whether differences in body weight and fat mass contributed to these anomalous results.

The overarching question of this chapter is: are the effects of TN^{SST} neurons on feeding modulated by body mass and/or adiposity? To answer this question, I re-analyzed data from previous experiments for effects of body weight and correlations with adiposity. I then used transcriptomics bioinformatic analysis to confirm communication between TN^{SST} neurons and peripheral metabolic tissues before repeating ablation experiments following direct modulation of white adipose tissue. These converging lines of evidence and preliminary data from fat transplantation experiments point to the direct detection of estradiol and adipokine cues by TN^{SST} neurons to modulate feeding behavior.

4.2 Materials and Methods

Fat analyses

Mice (see Chapter 3.2) were analyzed for fat mass, lean mass, and free water composition by EchoMRI. Inguinal and white adipose tissue was collected post-mortem and drop-fixed in 4% paraformaldehyde (PFA) for at least 18 hours. Tissue was then washed in phosphate buffered saline (PBS) before being stored in PBS at 4°C until tissue analysis. For histological processing, tissue was placed in tissue processing cassettes and submerged in 70% ethanol before being embedded in paraffin, sectioned at 4 μM, and stained with hematoxylin & eosin (H&E) by the UCLA Translational Pathology Core Laboratory. Three regions of interest per tissue type per mouse were imaged by light microscopy at 20x

magnification. Adipocyte area was quantified using a pipeline in CellProfiler. Inclusion parameters were cell diameter of 100-300 pixel units, and a global threshold strategy with minimum cross-entropy were utilized.

Flow-Seq RNA sequencing & analyses

Female and male mice expressing the *Sst-Cre* driver transgene (stock no. 013044, $Sst^{tm2.1(cre)Zjh}$/J) maintained on the C57BL/6 background were injected with a Cre-depednent tdTomato virus (AAV2-FLEX-tdTomato, Edward Boyden, Addgene viral prep 28306-AAV2) as described in Chapter 3. Following at least two weeks of viral expression, TN^{SST} neurons were enriched and collected via the FlowSeq method (van Veen et al., 2020). Animals were sacrificed and the tuberal nucleus was microdissected (Figure 4-3A), dissociated using a papain-based enzymatic process (Worthington Biochemical), and then sorted by FACS using parameters to select for tdTomato signal. RNA was isolated from 500-2500 cells by RNeasy Micro kit (Qiagen). Cells were then submitted for bulk RNA sequencing. Single-end reads (~10 million unique reads per mouse) were assembled to the mouse transcriptome (version mm10) using kallisto (version 0.46.2). Differentially expressed genes and normalized read counts were identified using DEseq2 Galaxy Version 2.11.40.6+galaxy1. Volcano plots were produced by the custom R function “deseq_volcano_plot_gs()” available through the following package: http://github.com/jevanveen/ratplots. Raw reads of the RNA sequencing data were also examined for hypothalamus-peripheral tissue co-correlations across stomach, small intestine, skeletal muscle, visceral fat, and subcutaneous fat as hypothalamic reads using the GTEx database as previously described (Seldin et al., 2018).

Estrogen responsiveness visualization

Female mice expressing the *Esr1-Cre* driver transgene (stock no. 017911, B6N.129S6(Cg)-*Esr1*$^{tm1.1(cre)And}$/J from Lee et al., 2014) were stereotaxically injected with a novel

pan-estrogen receptor viral reporter (reportER, see Figure 4-2C for schematic). To ensure maximum viral expression, mice were perfused with 4% PFA prior to cryosectioning and inflorescent microscopy visualization of native fluorescent expression following DAPI counterstain.

Fat transplants

Donor fat was taken from various visceral fat depots of wildtype female C57BL/6 mice and implanted into female mice recently stereotaxically injected under standard surgical conditions (see Chapter 3). Four depots of 0.15-0.25g were placed subcutaneously on the dorsal surface through a single incision mid-back. Fat for each depot was divided into at least three individual pieces. The visceral-to-subcutaneous paradigm was used due to the deleterious effects of this graft (Tran et al., 2008). Food intake was assayed 2-3 weeks following transplantation to allow for sufficient angiogenesis (Gavrilova et al., 2000) and graft stabilization without endogenous fat depot compensation (Rooks et al., 2004). Upon sacrifice, grafts were excised and prepared for histological and RNA analyses.

4.3 Results

Body weight influences effect of TN^{SST} neuronal ablation on food intake

To investigate whether body weight influenced the lack of reproducibility found in Figure 3-5, starting body weight differences between the two experiments were assessed (Figure 4-1A). Intact animals in the ovariectomy study had a significantly higher body weight as compared to the original caspase ablation experiment ($F(1,11.64)=6.2614$, $p=0.01747$). Pooling all intact animals (Figure 4-1B) revealed TN^{SST} neuron ablation-mediated decreases in food intake in lighter animals (ablation status x estrous phase interaction $F(3,36)=5.0178$, $p=0.0043$), with a significant decrease occurring during the night of proestrus ($t(13.448)=-2.4619$, $p=0.02803$). In heavier animals, there was no interaction between ablation and estrous phase but instead an

overall effect of the estrous cycle ($F(3,16)=4.2098$, $p=0.0225$) and a trending effect of ablation to increase food intake overall ($F(1,7)=4.5509$, $p=0.0703$).

Analysis of covariance (ANCOVA) revealed an interesting relationship between estrous stage, with an overall interaction between weight and estrous phase ($F(1,150)=3.8529$, $p=0.0515$) and between weight and ablation ($F(1,150)=16.0734$, $p>0.0001$). Analyzing the relationship between body weight and food intake within each estrous stage (Figure 4-1C) revealed significant negative correlations between body weight and food intake in wildtype animals during the nights of proestrus ($r^2=0.3263$; $F(1,17)=8.235$, $p=0.01062$) and metestrus ($r^2=0.2801$; $F(1,16)=6.227$, $p=0.0239$), stages with days of heightened circulating estradiol levels (Figure 3-1; Handelsman et al., 2020). TN^{SST} neuron ablation uncoupled this relationship with body weight (proestrus: $r^2=0.00029$, $F(1,19)=0.05492$, $p=0.08172$; metestrus: $r^2=0.09921$, $F(1,19)=2.093$, $p=0.1643$).

Fat mass may influence effect of TN^{SST} neuronal ablation on food intake

Body weight is primarily contributed to by fat mass, lean mass, bone mass, and water weight. In order to assess which of these primarily modulates the body weight effect, body composition analyses were conducted. Due to vivarium constraints, fat mass analysis was a terminal procedure, occurring up to 8 weeks after food assays. Terminal fat mass (Figure 4-2A) of intact animals from the ovariectomy study was significantly higher than that of animals from the original caspase ablation experiment ($F(1,23)=12.2655$, $p=0.001918$). Post-mortem histological samples (Figure 4-2B) of subcutaneous inguinal and visceral gonadal white adipose tissue (iWAT and gWAT, respectively) both positively correlated with body mass at the beginning of the feeding experiment, with no interactions between body weight and TN^{SST} neuron ablation but overall effects of body weight for both iWAT ($F(1,22)=4.3346$, $p=0.04919$) and gWAT($F(1,18)=14.9872$, $p=0.001119$; Figure 4-2C). Body weight accounted for a larger

percentage of variation in visceral gWAT adipocyte size (r^2=0.4481; F(1,20)=16.24, p=0.0006557) than it did for subcutaneous iWAT (r^2=0.1623; F(1,24)=4.649, p=0.0413).

Transcriptomics analysis reveals TN^{SST} neuron responsivity to reproductive and metabolic cues

Transcriptomics analysis of TN microdissection and enrichment for TN^{SST} neurons (Figure 4-3A) revealed numerous differentially expressed genes between females and males, including the gene for estrogen receptor alpha, *Esr1* ($W\chi^2$=6.736, adj p = 2.356 x 10^{-8}; Figure 4-3B). Confirmation of estrogen-sensitivity and responsivity was achieved through the injection of reportER virus (Figure 4-3C). Neurons in the TN showed *Esr1* expression through *Cre*-linked production of FusionRed and active transcription through the ERE region via visualization of native GFP (Figure 4-3D). Further analysis also revealed expression of numerous adipokine receptors at high levels, including both adiponectin receptors and known resistin receptor *Cap1* (Avtanski et al., 2019; Lee et al., 2014b; Table 4-1).

In order to determine whether TN^{SST} neurons were communicating with peripheral adipose tissue, high-expressing genes from TN^{SST} neurons were subjected to cross-tissue co-correlational analysis through using the GTEx database (Seldin et al., 2018; Figure 4-4A). As previous data indicated that TN^{SST} neuron responsivity to metabolic cues might be localized to periods of higher circulating estradiol (Figure 4-1B&C), GTEx database individuals were assessed for hypothalamic progesterone receptor (*PGR*) expression as a proxy of hypothalamic estradiol levels. Due to the sequential nature in which estradiol and progesterone affect rodent sexual behavior (Boling and Blandau, 1939), it has been consistently shown that estradiol treatment (Shughrue et al., 1997) and across the estrous cycle (Sá and Fonseca, 2017) increases hypothalamic progesterone receptor through activation of nuclear estrogen receptors alpha and beta (Kudwa and Rissman, 2003; Moffatt et al., 1998; Temple et al., 2001) as well as membrane estrogen receptor(s) (Sá et al., 2014) in females and males (Temple et al., 2001). Individuals in the GTEx database showed a spectrum of hypothalamic *PGR* expression

regardless of Y chromosome presence ("sex"), with a large proportion of individuals showing very low levels as expected for a post-mortem database with a preponderance of older, post-menopausal and andropausal individuals (Figure 4-4B). Binning individuals into "low" and "high" hypothalamic *PGR* expression revealed weakly associated groups as per biweight midcorrelation (Langfelder and Horvath, 2008; bicor coefficient = 0.269, $p = 2.14 \times 10^{-97}$; Figure 4-4C). Co-correlation analysis within low and high *PGR* groups revealed alternate patterns of co-correlation, with humans in the low hypothalamic *PGR* (estradiol) group showing increased co-correlation with skeletal muscle and humans with high hypothalamic *PGR* (estradiol) showing larger co-correlation percentages from visceral and subcutaneous adipose (Figure 4-4D). Together, these data indicate that TN^{SST} can detect and respond to reproductive and metabolic cues, preferentially co-correlating with adipokine tissue when circulating estrogens are high.

Effect of TN^{SST} neuron ablation depends on fat mass specifically

To test the causal, directional relationship between fat adipokines and TN^{SST} neuron modulation of food intake, caspase ablation studies were repeated in the presence of fat transplantation. Initial experiments transplanting four 0.1 g depots on the dorsal subcutaneous surface did not show increased fat mass before or after food assay (Figure 4-5A). Transplant of four 0.2 g depots did persist, with ANOVA revealing a significant interaction between depot transplant size on resultant raw fat mass (starting $F(1,23)=18.0663$, $p=0.0003016$; ending $F(1,23)=7.8314$, $p=0.01021$; data not shown) and percent fat mass (starting $F(1,23)=16.8123$, $p=0.0004384$; ending $F(1,23)=5.7538$, $p=0.02495$). Average daily food intake was also significantly affected by mass of original transplant ($F(1,22)=70.5915$, $p=2.59 \times 10^{-8}$, data not shown). Thus, animals with 0.8 g of transplant were further analyzed. Transplanted animals revealed significantly increased body weight ($F(1,12)=6.9825$, $p=0.0214$; data not shown), raw fat mass ($F(1,12)=10.8539$, $p=0.006405$; data not shown), and percent fat mass ($F(1,12)=10.5184$, $p=0.007045$) and no interaction with TN^{SST} neuronal ablation in any case.

Fat transplant increased food intake in general ($F(1,11)=11.8713$, $p=0.005472$), and the effect of TN^{SST} neuronal ablation was affected by fat transplant ($F(1,11)=16.5335$, $p=0.001864$; Figure 4-5B). *Post-hoc* t-tests showed a trending effect of TN^{SST} neuronal ablation to decrease food intake in sham transplant animals ($t(4.8752)=-2.2283$, $p=0.0777$) but significantly increase food intake in animals receiving fat transplant ($t(4.1902)=4.1602$, $p=0.005766$), similar to the previous effect seen due to body weight in proestrus (Figure 4-1C). Fat transplant also shows an overall effect across estrous stage ($F(1,9)=8.4051$, $p=0.0176$) and a trending effect of transplant effect by ablation status ($F(1,9)=3.4991$, $p=0.0942$), but was underpowered to detect individual effects of phases. However, sham animals, but not animals receiving fat transplant, do appear to show a possible effect of TN^{SST} ablation decreasing food intake only during proestrus (Figure 4-5C).

4.4 Conclusions

Together, these data implicate body weight, and specifically fat mass, as a modulator of TN^{SST} neuronal function during periods of high estradiol. The significant increase in fat mass and body weight induced by the ovariectomy surgery (sham or OVX) resulted in a failure of the positive control (i.e., TN^{SST} neuronal ablation failed to decrease food intake). Analysis of intact animals revealed a role for TN^{SST} neuron integration of metabolic and reproductive cues: while wildtype animals exhibited a significant negative correlation between food intake and body weight during the fertile period of proestrus, this was uncoupled with TN^{SST} neuron ablation. This significant negative correlation in wildtype animals may be indicative of metabolic-reproductive trade-offs, whereby lighter animals without the metabolic reserves to support a successful pregnancy stave off the anorexigenic effects of estradiol and increase their food intake. In animals with sufficient metabolic resources, however, food intake is forsaken for perhaps mate-seeking behaviors.

While muscle, skeletal, free water, and fat mass all contribute to body weight, fat mass seems to be one of the crucial factors for modulating TN^{SST} neuronal response. Not only did adipocyte size (a proxy for fat mass) in the experimental mice significantly correlate with body weight, but TN^{SST} neurons were also found to be sensitive to adipokines and actively responsive to estradiol, and modulation of raw and percent body fat differentially modulated the effect of TN^{SST} neuron ablation. This paradigm also seems to be conserved across species. Analysis of mouse TN^{SST} neuronal transcriptome in the GTEx database demonstrated a similar role in humans, where hypothalamic expression of TN^{SST} genes seem to “pay attention” to adipokine cues preferentially when a proxy for hypothalamic estradiol levels are elevated.

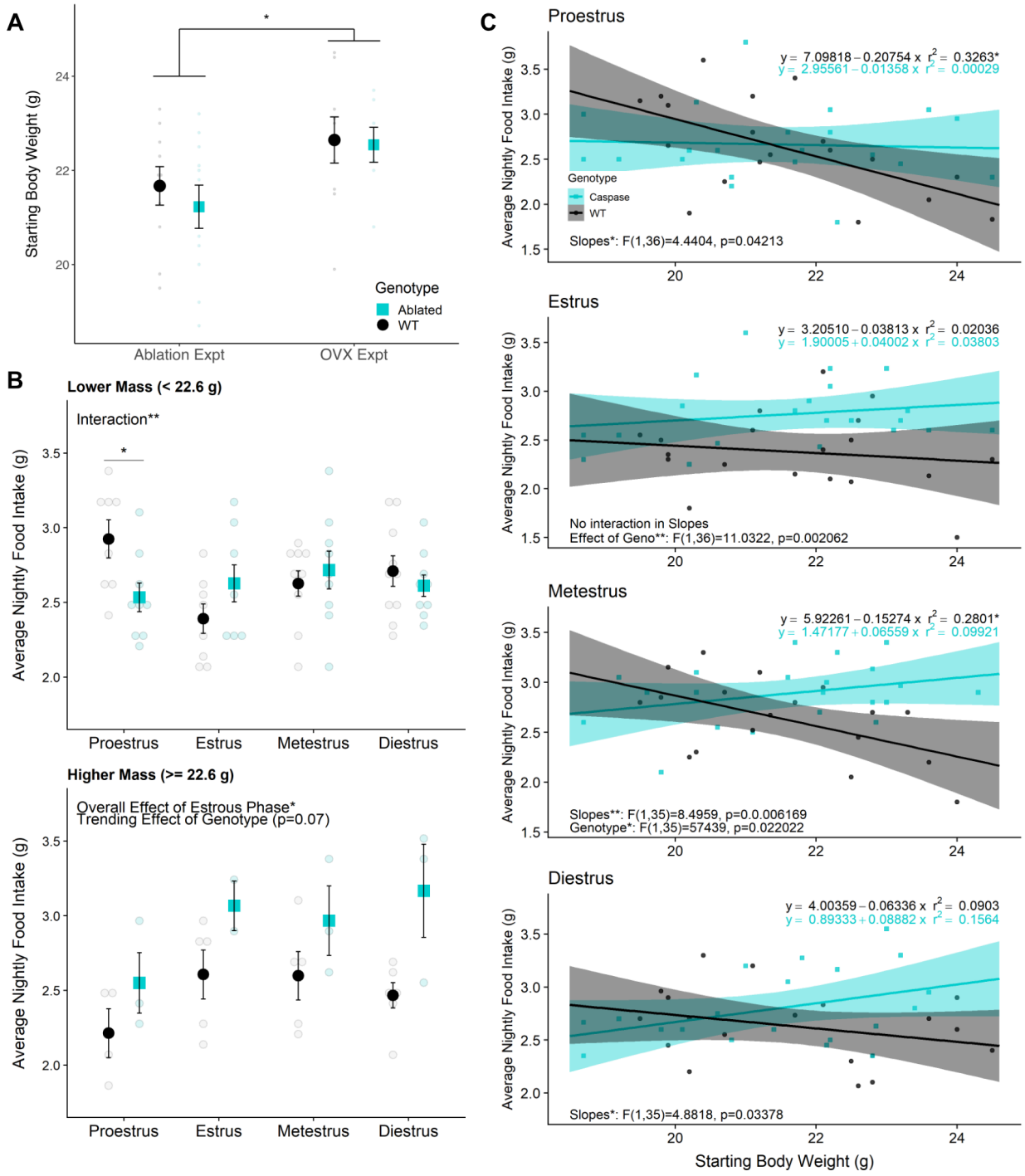

Figure 4-1. Body weight influences effect of TN^{SST} neuronal ablation on food intake in an estrous cycle-dependent manner. (A) Failure of positive control in the ovariectomy (OVX) experiment (Figure 3-5) is due to differences in body weight between the original (Ablation Expt, Figure 3-2) and OVX experiment (OVX Expt). (B) Examination of all intact animals from all

experiments indicates that TN^{SST} ablation in lower mass animals (< 22.6 g), but not higher mass animals (≥ 22.6 g), decreases food intake during the proestrus phase as previously shown (Figure 3-2). (C) ANCOVA analysis within each estrous stage across all intact animals reveals an interaction between beginning body weight and nightly food intake. Significant negative correlations in wildtype animals are seen in the high estradiol (Figure 3-1) phases of proestrus and diestrus but not in caspase ablated animals. Mean ± SEM, *p<0.05, **p<0.01

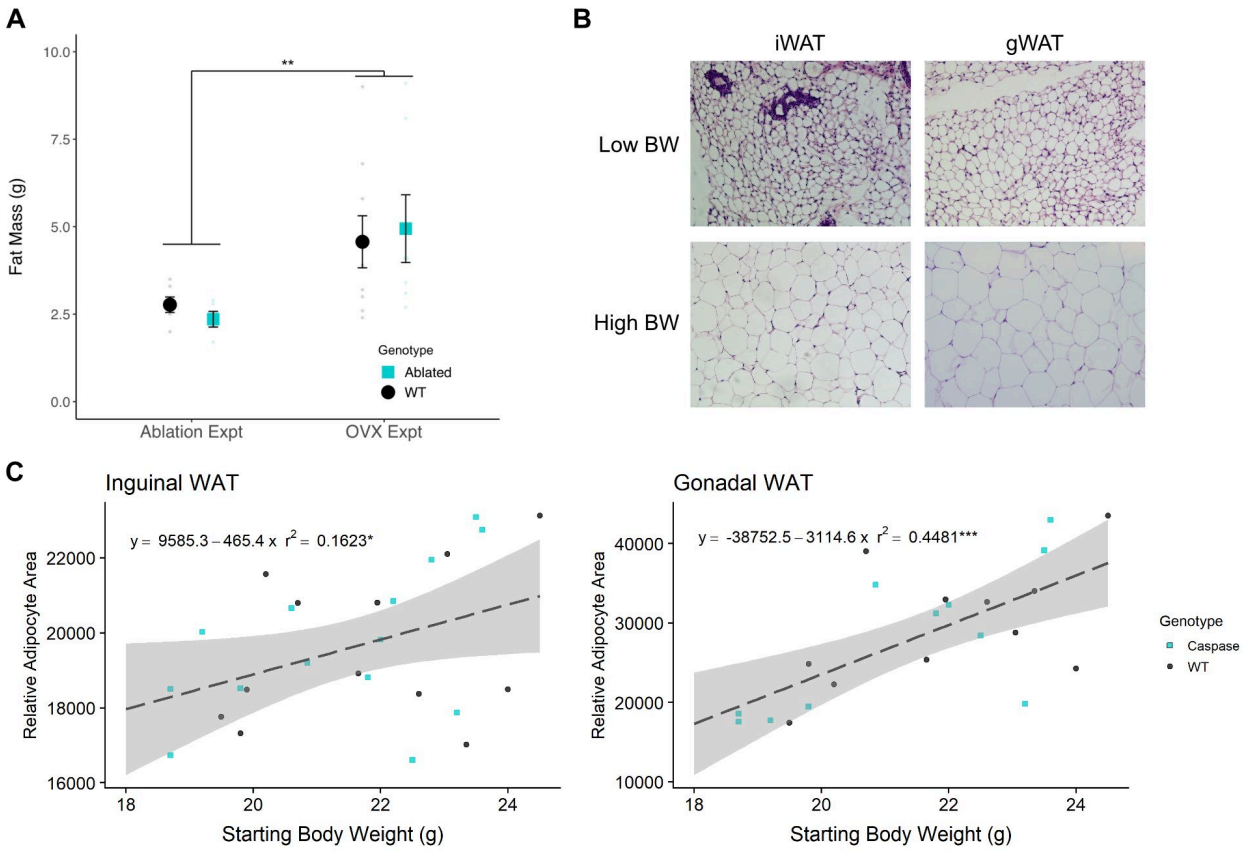

Figure 4-2. Metrics of adiposity significantly correlate with body weight regardless of TN^{SST} neuron ablation. (A) Terminal bodily fat mass is significantly increased in the ovariectomy experiment (OVX Expt) as compared to the original caspase ablation experiment (Ablation Expt). (B) Representative histology samples of inguinal and gonadal white adipose tissue (iWAT & gWAT, respectively) in high and low body weight (BW) animals. (C) Terminal iWAT (left) and gWAT (right) adipocyte size both positively correlate with starting body weight regardless of TN^{SST} neuron ablation (linear regression lines represent compiled data across ablation status). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001

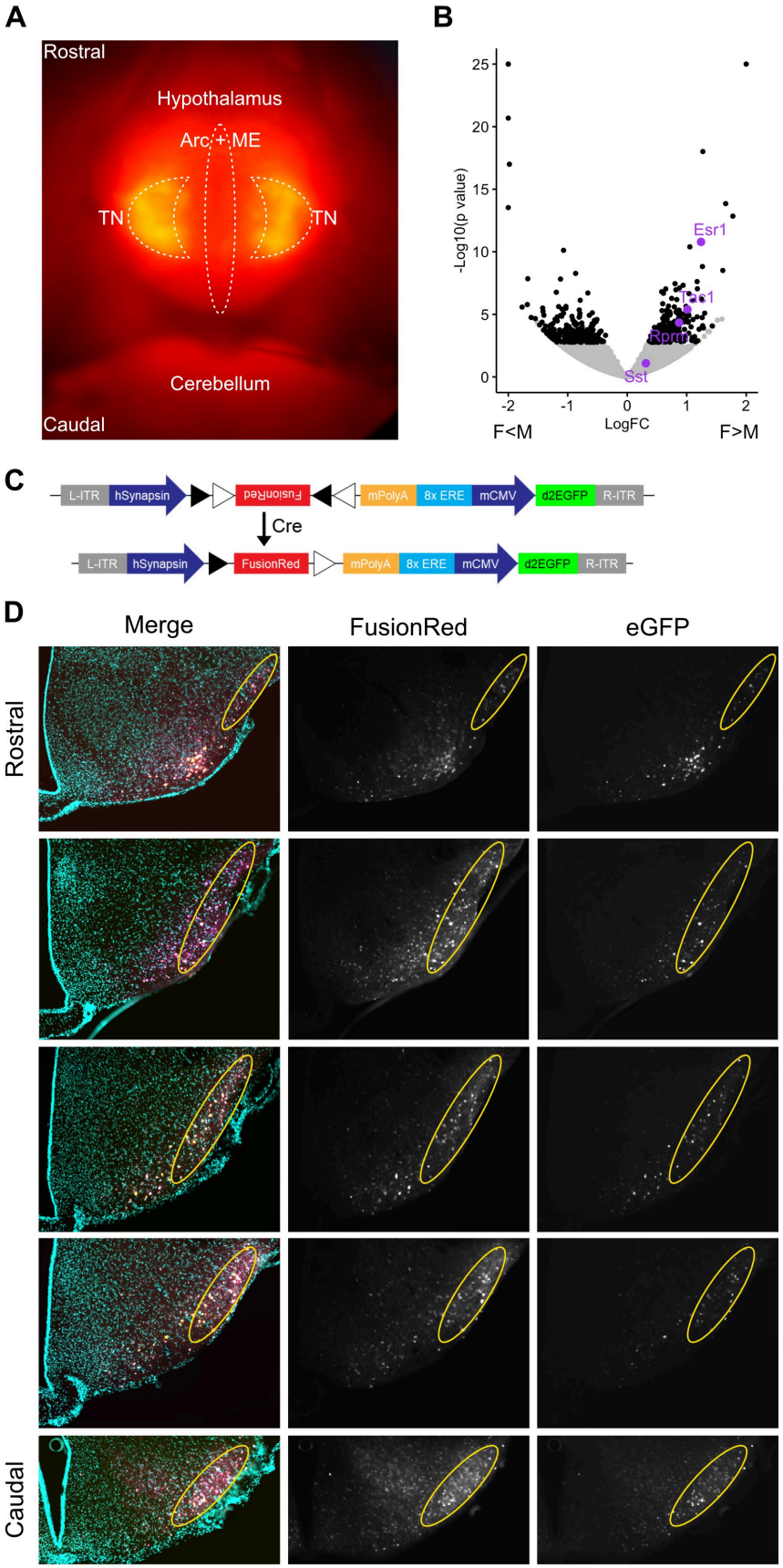
A
Rostral
Hypothalamus
Arc + ME
TN
TN
Cerebellum
Caudal
B
-Log10(p value)
LogFC
F<M
F>M
Esr1
Tac1
Rprm
Sst
C
L-ITR
hSynapsin
FusionRed
mPolyA
8x ERE
mCMV
d2EGFP
R-ITR
Cre
D
Merge
FusionRed
eGFP
Rostral
Caudal

Figure 4-3. TN^{SST} neurons sense and respond to estrogens. (A) Representative expression of bilateral tdTomato in TN^{SST} pre-dissection. Image is showing the ventral surface of the hypothalamus. (B) Transcriptomics analysis reveals numerous differentially expressed genes between females and males, including *Esr1*. Black dots indicate differential expression with adj $p < 0.05$. (C) Novel viral vector "reportER" independently reports Cre presence and transcription through the estrogen response element (ERE). FLEXed FusionRed gene under the human synapsin promoter is only expressed in the presence of Cre recombinase. Universal mouse cytomegalovirus (CMV) promoter drives expression of a two-hour destabilized green fluorescent protein (d2EGFP) when activated through the eight upstream EREs. A poly-A tail separates these two segments to prevent readthrough (D) ReportER-injected *Esr1-Cre* mice reveal *Esr1* expression (FusionRed) and active ERE-induced transcription (eGFP) throughout the entirety of the tuberal nucleus.

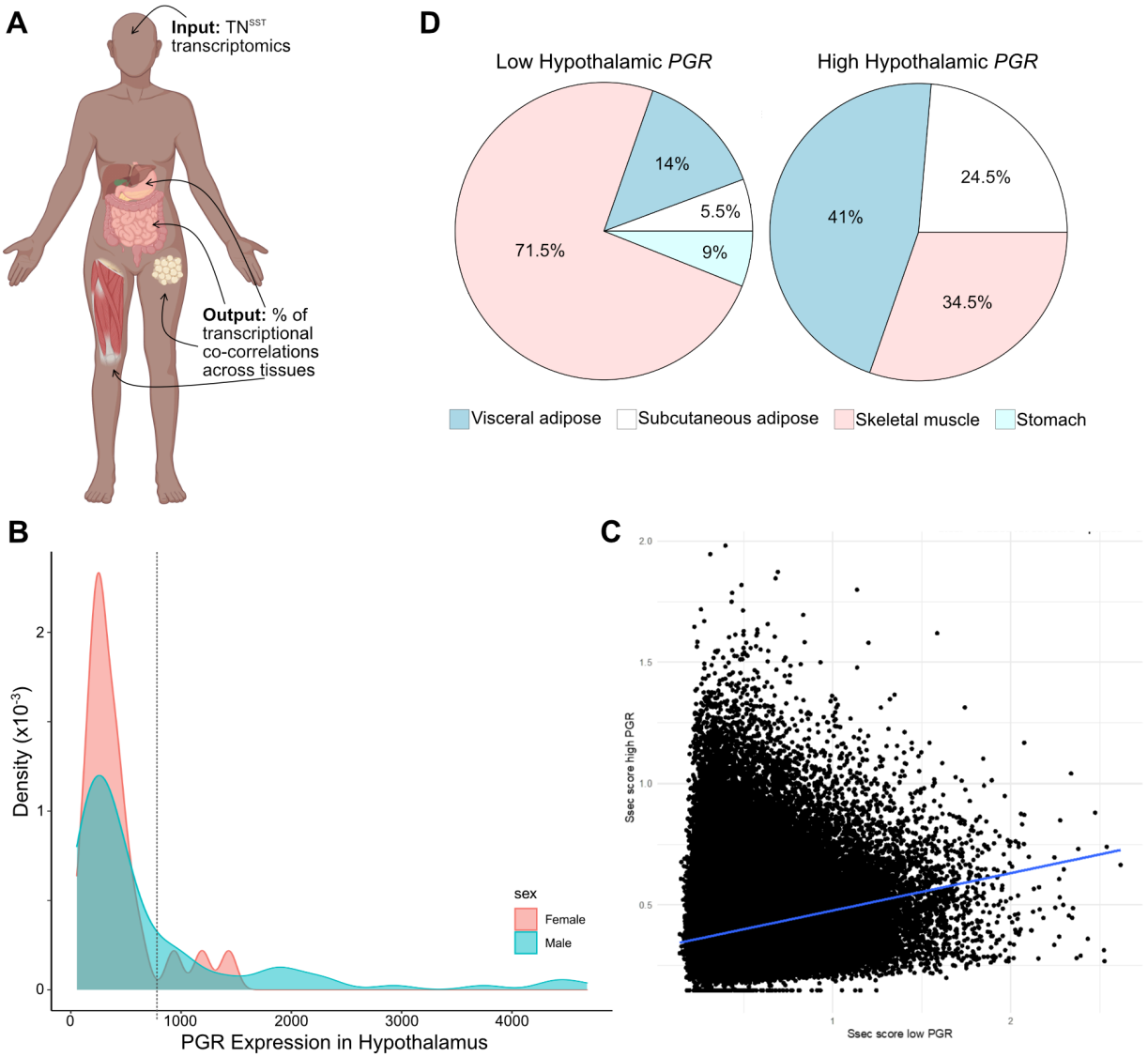

Figure 4-4. Highly expressed genes in TN^{SST} neurons preferentially co-correlate with adipocyte depots when hypothalamic *PGR* is high. (A) Graphical depiction of GTEx co-correlation procedure. Gene list isolated from TN^{SST} neurons were input as hypothalamic genes of interest and examined for co-correlations across muscle, subcutaneous adipose, visceral adipose, skeletal muscle, stomach, and small intestine. Created with BioRender.com. (B) Hypothalamic progesterone receptor (*PGR*) expression across the GTEx database. Sex is determined by Y chromosome presence, with “Female” denoting no Y chromosome and “Male” denoting Y chromosome. Low and high *PGR* individuals were binned according to the vertical

dashed line cutoff. (C) High and low *PGR* human groups were anti-correlated with each other. (D) Proportion (percent inside each slice) of pan-tissue enrichment co-correlations of TN^{SST} neuron genes with peripheral tissues as a function of *PGR* hypothalamic expression (proxy for estradiol levels).

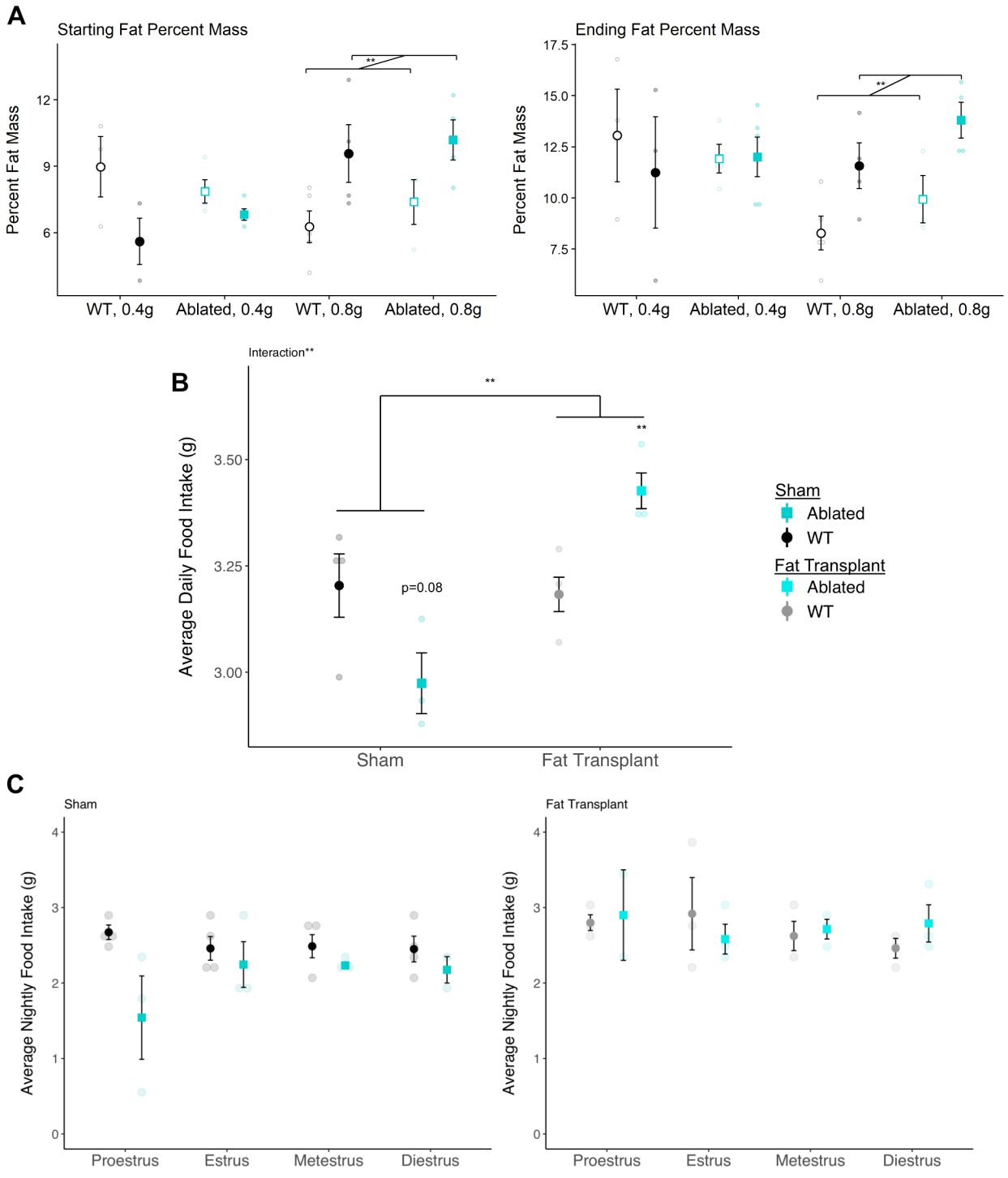

Figure 4-5. Fat transplantation modulates effect of TN^{SST} neuron ablation. (A) Fat transplant size affects resultant fat mass presence and longevity. Total transplantation of 0.4 g across 4 dorsal subcutaneous depots did not persist and exhibited compensatory reactions.

Total transplantation of 0.8 g across 4 dorsal subcutaneous depots persisted until and were maintained throughout food assay. Overall effects of transplant in starting ($p=0.0003$) and ending ($p=0.010$) fat mass are evident. (B) Fat transplant increases average daily food intake and increases food intake in ablated animals. (C) Effects of TN^{SST} neuron ablation across the estrous cycle are only seen in animals without excess adiposity. Mean ± SEM, $^{**}p<0.01$

Gene		Raw Basemean
Adipor1	Adiponectin receptor 1	571.26
Adipor2	Adiponectin receptor 2	560.99
Lepr	Leptin receptor	90.79
Cap1	Resistin receptor, Cyclase Associated Actin Cytoskeleton Regulatory Protein 1	2036.38
Ghsr	Ghrelin receptor	247.22
Sst	Somatostatin	4000.49

Table 4-1. Raw transcriptional reads of select metabolic receptors from isolated and enriched TN^{SST} neurons

4.5 References

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Chapter 5

Discussion

5.1 Main conclusions & limitations

The data presented in the previous chapters suggests that TN^{SST} neurons are a locus in the brain that mediates metabolic and reproductive tradeoffs. While activation of TN^{SST} neurons increases food intake across sexes, permanent inactivation by ablation during adulthood results in decreased food intake only in females (animals with ovaries) during the proestrus phase. This effect depends on body weight, as this effect is apparent only in lighter animals. Indeed, in wildtype mice, body weight inversely correlates with food intake on the night of proestrus, but TN^{SST} neuron ablation uncouples this effect. Further analysis reveals that white adipose tissue is a significant contributing factor to this effect. Not only does post-mortem adiposity and adipocyte size correlate with body weight in previous experiments, but ongoing fat transplantation studies suggest that TN^{SST} neuron ablation only decreases food intake in lean animals as compared to their fat transplanted counterparts. This interaction between cycling adipokines and gonadal hormones, namely estradiol, may be mediated by direct effects of these hormones on TN^{SST} neurons, as these cells show adipokine sensitivity, estradiol sensitivity and responsivity, and may utilize rising estradiol to increase said adipokine sensitivity.

In cycling rodents, fertile periods during the estrous cycle are accompanied by alterations to metabolic output, including a decrease in food intake (Asarian and Geary, 2002, 2013; Brobeck et al., 1947; Eckel, 2011), increase in locomotion (Brobeck et al., 1947; Kent et al., 1991; Sanchez-Alavez et al., 2011; Steiner et al., 1982), and increased core body temperature (Kent et al., 1991; Sanchez-Alavez et al., 2011). Colloquial hypotheses postulate that this functions to suppress energy intake and promote active mate-seeking behavior and sexual receptivity. This dissertation identifies TN^{SST} neurons as possible mediators of such a trade-off, actively promoting energy intake during fertile periods when metabolic reserves may be insufficient to support reproduction (Figure 5-1).

Ongoing fat transplantation studies will increase the statistical power to definitively confirm the contributions of white adipose tissue to this phenomenon. The current data are underpowered as per power analysis (target sample size = 8-9 animals/group). However, the trending effects and already significant interaction between fat transplantation and TN^{SST} neuron ablation on the effects of daily food intake are promising (Figure 4-5B). This study is potentially confounded by total body mass, as the fat transplantation also seems to have affected total body weight. This confound is limited by analysis of percent body fat (Figure 4-5A). Furthermore, the average body weight of all groups is below the 22.6 g threshold identified in Figure 4-1B, placing all animals in the “low” body mass group and providing further support for the adipose-specificity of this effect.

The effects of fat transplantation and neuron ablation across estrous cycle are similarly underpowered. In the proposed reproduction-metabolism tradeoff paradigm, we would expect to see an effect of estrous cycle (and a decrease of food intake in proestrus) in fat transplanted wildtype animals but not lean shams. In fat animals, ablation should not alter this result. However, in lean animals, TN^{SST} neuronal absence would result in a decrease in food intake during proestrus, diverging from their wildtype counterparts. While preliminary data in Figure 4-5C looks promising in lean sham controls, there is no indication of an estrous cycle effect in any fat transplanted animals. This may indicate the contribution of other metabolic cues to this phenomenon. Indeed, bioinformatics data revealed a considerable proportion of co-correlations between TN^{SST} neuronal transcripts and skeletal muscle (71.5% and 34.5% in the low and high *PGR* conditions, respectively; Figure 4-4D). Future studies should not neglect the possible effects of lean mass, specifically skeletal muscle, as a metabolic contributor.

Regardless of metabolic contributor, the aforementioned paradigm provides a plausible explanation for the varied effects of estradiol on food intake in mice. While endogenous fluctuations and experimental manipulations of estradiol consistently reveal that estrogens

decrease food intake in rats and guinea pigs (Asarian and Geary, 2002, 2013; Clegg et al., 2007; Eckel, 2011), the mouse literature is less definitive (Eckel, 2011; Geary et al., 2001; Naaz et al., 2002; Petersen, 1976; Witte et al., 2010). As stated in Chapter 2, the more consistent phenotype in mice is a decrease in energy expenditure following estradiol depletion (Correa et al., 2015; Musatov et al., 2007; Xu et al., 2011). In light of this dissertation research, the effects of estradiol on feeding in mice as observed by both endogenous estrous cycle fluctuations and ovariectomy manipulation could be confounded by body mass and adiposity. Thus, factors like age at time of experiment/ovariectomy and time from ovariectomy to estradiol replacement might present confounds based on changes to fat and/or lean mass due to experimental condition.

How circulating estrogen levels contribute to this circuit also requires further investigation. The human GTEx data shows that the proportion of co-correlations between TN^{SST} genes and that in adipose tissue grows from 19.5% to 65.5% based on hypothalamic estradiol levels as proxied by progesterone receptor expression (Figure 4-4D). This suggests that higher estrogen levels may increase communication between TN^{SST} neurons and white adipocyte depots. While this could be due to the actions of circulating estrogens on white adipose tissue itself (reviewed in Hevener et al., 2015 and Palmer and Clegg, 2015), it is also possible that estrogens directly act on TN^{SST} neurons to increase their sensitivity and/or responsivity to adipokines. Indeed, TN^{SST} neurons do seem to exhibit estrogen sensitivity and responsivity (Figure 4-3). Transcriptomic profiling and/or quantitative polymerase chain reaction of TN^{SST} neurons under different levels of circulating estrogens would be needed to confirm the possible tuning effect.

The proxy of hypothalamic estradiol by *PGR* expression is an imperfect one, as gene expression can be affected by other means. However, the strong correlations between estradiol and progesterone receptor (see Chapter 4) alongside the large constituency of individuals with

low hypothalamic *PGR* as would be expected due to a large proportion of people in andropause and/or menopause at the time of death, indicates that this proxy holds merit. This proxy could be strengthened through use of an array of established estradiol-responsive genes as in gene set enrichment analyses. Regardless of proxy used, stratifying GTEx data based on presumed estradiol levels is confounded by white adipocyte production of estradiol. This means that the "high estradiol" group may have an increased proportion of individuals with higher adiposity at time of death. This can be potentially corrected via the use of the imperfect measure of BMI provided by GTEx. Even with this consideration, approximation of estradiol levels still provides correlational evidence of hypothalamic TN^{SST} neurons communication with peripheral tissues in response to circulating estradiol levels, regardless of source.

The paradigm presented in Figure 5-1 postulates that because activation of TN^{SST} neurons increases food intake (Figure 3-2C), estradiol may lead to increased activation of TN^{SST} neurons during low body weight conditions. Studies investigating the electrophysiological responses of TN^{SST} neurons to estradiol treatment should be examined in the future. Alternatively, estradiol might be detected elsewhere in the brain and impact TN^{SST} neuronal modulation of feeding through circuit integration. Indeed, TN^{SST} neurons have been found to project to many estrogen-sensitive nodes or nodes receiving direct input from estrogen-responsive regions, including the bed nucleus of the stria terminalis, parabrachial nucleus, and central amygdala (Luo et al., 2018). This circuit-wide integration of estradiol is a known mechanism of action for the gonadal hormone, with estrogens acting on many circuit nodes to coordinate behavioral output in a variety of cases, including reward/addiction (Becker and Chartoff, 2019) and thermoregulation (Zhang et al., 2021). It is therefore probable that the effects of estradiol on feeding function similarly, as the anorexigenic effects of estradiol have already been localized to numerous feeding nodes such as the hypothalamic arcuate nucleus

(Roepke et al., 2007, 2010; Santollo et al., 2011; Stincic et al., 2018b, 2018a) and the nucleus of the solitary tract of the brainstem (Asarian and Geary, 2006; Maske et al., 2017).

Few studies have reported TN expression of estrogen receptor, though examination of early atlases reveals estrogen sensitivity in this region (Pfaff and Keiner, 1973; Simerly et al., 1990). Interestingly, preliminary studies from the lab have shown that available commercial estrogen receptor antibodies may be insufficient to detect estrogen receptor in this region. Attempts to detect estrogen receptor alpha did not reveal immunoreactivity in this region (data not presented). Combined with older hormone concentrating studies, the bioinformatics expression and reportER data (Figure 4-3B&D) suggest that perhaps ERα in TN^{SST} neurons is below the immunohistochemistry detection limit or else TN^{SST} neurons express an *Esr1* splice variant (overview in Taylor et al., 2010) which cannot be detected by traditional immunohistochemistry methods but that still affects genomic signaling through estrogen response element binding and cofactor recruitment. More research investigating this possibility is needed.

In all, this dissertation adds to the growing literature interrogating the contributions of TN^{SST} neurons to feeding behavior. While the main effects of TN^{SST} neuron modulation of feeding were first reported in 2018 (Luo et al.) and then reconfirmed in 2019 (Mickelsen et al.), this research was underpowered to detect a sex difference and reveal the population's role in metabolic and reproductive integration. Indeed, the original ablation experiment (Figure 3-3C) reveals an overall effect of ablation ($F(1,38)=7.3353$, $p=0.0101$) and, when the sexes are pooled together, the females carry the effect ($t(39.86)=-2.2713$, $p=0.0286$). Also divergent from previous literature is the failure of transient chemogenetic inactivation to robustly decrease food intake following fast (Luo et al., 2018). Given the reproductive and metabolic conditions that must converge in order to see an effect of TN^{SST} neuron ablation, it is unsurprising that the effect of transient inactivation was weak, at best, in my hands (Figure 3-4). Due to the use of

older (and therefore heavier/higher adiposity) mice and the difficulty in planning fast-refeed experiments during proestrus, stratification of inactivating chemogenetic data by estrous phase and body weight was underpowered to detect any effect. Future experiments wishing to recapitulate the effect of transient TN^{SST} neuron inactivation might use estradiol depletion and replacement in conjunction with body composition analysis to reconfirm the effect. Interestingly, I may not have been the only one unable to recapitulate the effects of fast on TN^{SST} neurons. A follow-up paper from the same lab as Luo et al., 2018 were unable to show an increase in activation of TN^{SST} neurons following fast as was previously reported (Mohammad et al., 2021). Similarly, the switch from using all sexes (Luo et al., 2018) to only males (Mohammad et al., 2021) may be due to the specificity of the effect in females and inability to replicate previous results. The model of reproductive and metabolic integration presented here may account for these discrepancies and may also possibly point to a specific role of TN^{SST} neurons in animals with ovaries as compared to animals with testes.

Alternatively, the dissimilar roles for TN^{SST} neurons seen in Mohammad et al., 2021 and this dissertation may be due to distinct neuronal cell types/subpopulations within this region. In a sequencing analysis of the lateral hypothalamic area, TN^{SST} neurons were not only identified but also possibly revealed two distinct subsets (Mickelsen et al., 2019). The subset hypothesis is corroborated by convergences between previous data and data presented in this dissertation. In Luo et al., 2018, only a subset of TN^{SST} neurons were sensitive to the orexigenic stomach hormone ghrelin. And in our GTEx data, co-correlations between hypothalamic TN^{SST} neuron transcripts and the stomach transcriptome only appeared relevant when circulating estradiol is low (Figure 4-4D). Thus, the TN^{SST} neurons that integrate metabolic and reproductive cues and those that contribute to food context learning (Mohammad et al., 2021) may be present in all animals to differing degrees and/or engaged in different contexts.

5.2 Outstanding questions

The largest outstanding question is what adipokine factors are being detected by TN^{SST} neurons to modulate this effect. Using *Ghsr* as a baseline (given that a subset of TN^{SST} neurons are known to express ghrelin receptor; Luo et al., 2018), the transcriptomics data in Table 4-1 point to adiponectin and resistin as candidates given their receptor expression levels. The merits of adiponectin as a candidate have already been discussed in Chapter 1. Resistin, a relatively novel adipokine by comparison, seems to be a prime candidate as well. Resistin exhibits numerous interactions with the hypothalamic-pituitary-gonadal axis (Mathew et al., 2018; Nogueiras et al., 2003; Tsatsanis et al., 2015) and has been shown to decrease during the fertile periods of the mouse estrous cycle (Gui et al., 2004). Additionally, its expression is correlated with increased adiposity (Yang et al., 2012). Even more convincing, resistin not only decreases food intake (Nogueiras et al., 2009), but also seems to communicate nutritional status, as levels were affected by chronic food deprivation (Nogueiras et al., 2003). It should be noted, however, that resistin's interaction with the *Cap1* receptor has mainly been found peripherally in both humans (Lee et al., 2014) and mice (Avtanski et al., 2019). Thus, this role for resistin in the central nervous system would be novel.

Finally, the question of what TN^{SST} neurons are doing in males remains to be resolved. It is plausible that the sex difference seen in Figure 3-3C is due to differences in adiposity between female and male mice, but this is yet to be determined. Outside research has located these neurons within a food contextual learning paradigm in males only (Mohammad et al., 2021), but possible differences in circuitry, basal cellular activity, and sex hormone responsivity have yet to be explored.

5.3 Summary

This dissertation research identifies a novel role for tuberal somatostatin neurons in the integration of metabolic and reproductive cues to alter feeding output in animals with ovaries (Figure 5-1). This context-dependency was uncovered by using appropriate positive controls (Figure 3-5) and highly powered experimentation (Figure 3-3C), highlighting the need for such rigor and specificity in current studies investigating the effects of the biologies of sex.

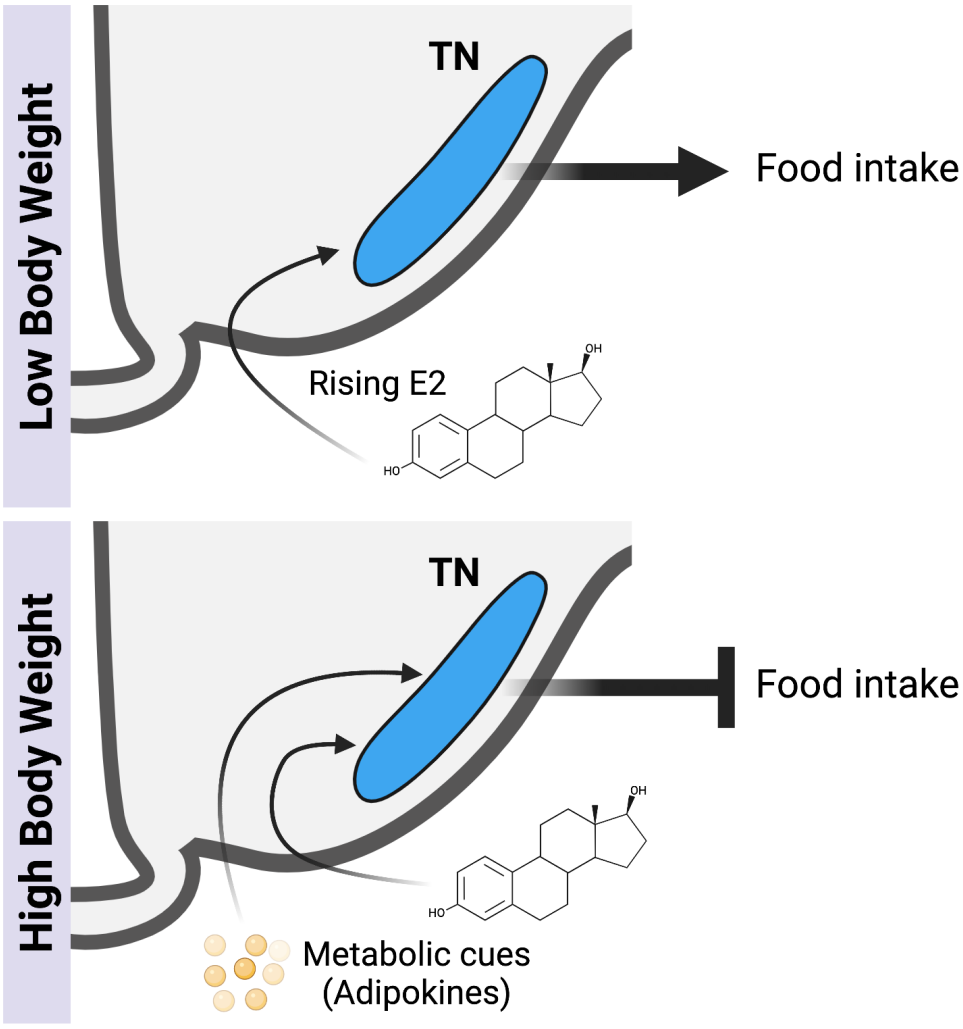

Figure 5-1. Graphical abstract of TN^{SST} neuronal integration of metabolic and reproductive cues to affect food intake. In low body weight (and perhaps low adiposity) animals, rising estradiol (E2) levels may be directly activate TN^{SST} neurons to induce increases in food intake. In higher body weight animals, TN^{SST} detect and integrate both estradiol and metabolic cues, including adipokines, to no longer activate to increase food intake. Created with BioRender.com.

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Chapter 6

“Sex” is Not a Coherent Biological Variable – A Sex Variables Framework

6.1 Introduction

The Genotype-Tissue Expression (GTEx) portal is a robust resource for exploring gene expression. In my research on the neural control of metabolic processes, I wanted to use the post-mortem human gene expressions available in GTEx to identify how peripheral metabolic tissue may communicate with my neuronal population of interest. Early on, my collaborator and I were finding promising results – but we soon came across a hiccup. My data from mouse studies indicated that these neurons were not simply detecting metabolic cues, but possibly mediated trade-offs between reproductive and metabolic processes. Specifically, it seemed that my cells of interest were behaving differently under varying levels of circulating estrogens. I needed to stratify my GTEx co-correlation results by gonadal hormone levels, but the database contains no data regarding circulating hormone levels. The next best thing we had to work with was “sex.”

The GTEx database defines “sex” as Y chromosome presence. While Y chromosome presence can provide important information, for my purposes, this was unhelpful. This metric, in and of itself, is not “sex.” This is because the canonical categories of sex – female and male – are complex and multifactorial. They contain a plethora of physiologies which result in varying phenotypes (for examples, see Table 6-1). Thus, while ideas of “sex” and reproduction often go hand in hand, utilizing one single metric of a multifactorial category can lead to obfuscation of scientific processes. In other words, assumed concordance withing sex categories (in this case, that Y chromosome presence correlate with low circulating estradiol levels, etc.), incorrectly assumes a binary and discrete set of aligned physiologies (Richardson, 2021). Given that GTEx data is based on snapshots of individuals’ physiologies at the time of death – snapshots that capture particular moments of phenomena that cycle across days, weeks, months, and lifetimes – simple stratification by “sex” defined in this manner left me with data uninterpretable for my research needs.

The pervasiveness of simplified (often binary) “sex” categorization presents serious scientific problems. For years, researchers have supported a non-reductive model of sex (Ainsworth, 2015) and a research paradigm that urges contextual understandings (Richardson, 2021) and attention to physiologies that contribute to sex phenotypes (Becker et al., 2005; McCarthy and Arnold, 2011; McCarthy et al., 2012). Despite this, research science and databases alike tend to define “sex” by one single variable. This is a long-known limitation of binning with a binary sex framework, a pervasive hypersimplification that prematurely reduces multiple physiologies into a single discrete binary (Waters, 2004) variable. This truncation, and assumed coherence among physiologies within each category, distorts our ability to accurately describe, investigate, and understand the world around us (DiMarco et al., 2022).

When I devised a method of stratifying the GTEx data by a proxy for circulating estrogens, the resulting data unmasked a relationship previously obscured by the heuristic of “sex” (Figure 6-1). It led me to wonder: how would our science change if we instead observe and analyze specific physiologies of interest? What if, instead of the simplified binary of “female” versus “male,” we collected and analyzed factors like hormonal milieu and receptor responsivity, sex chromosome complement, and/or sex-specific environment? What if we designed studies, collected data, and analyzed results using a framework that recognizes the multidimensionality of what is so often collapsed into “sex”?

In the interest of more accurate, transparent, and responsible science, my collaborators and I urge fellow researchers to take these questions seriously and move away from the hypersimplified “sex” heuristic. To facilitate this shift, we offer a multidimensional **sex variables framework** with principles that can be applied in research across biological sciences.

6.2 From sex omission, to Sex as a Biological Variable, to Sex Variables

In a 2009 study, Annaliese Beery and Irving Zucker identified an alarming trend in the biomedical sciences: overwhelmingly, published research either omitted female subjects or

neglected to report sex at all (Beery and Zucker, 2011). This vast oversight has huge implications for understanding the diversity of experiences and preclinical studies. As such, biomedical researchers and funding agencies rushed to reassess both experimental design and reporting methods. This finding helped spur a global effort (White et al., 2021) to rectify this omission, including the adoption of an policy requiring all funded projects to include “sex as a biological variable” (SABV, NIH, 2015).

Efforts like the SABV policy have afforded much necessary progress. Individuals and organism types that are wrongfully omitted due to incorrect, gendered (Shansky, 2019) assumptions ought to and must be studied. This does not mean that we should not keep moving forward. Unfortunately, the implementation of sex-related analyses have been uneven and requires further refinement (Arnegard et al., 2020). While analysis of grant applications from similar international initiatives demonstrated an increase in considerations of sex in submissions (Haverfield and Tannenbaum, 2021), a 2020 study found that while some fields did increase inclusion of female subjects, few studies presented analyses involving sex (Woitowich et al., 2020). Furthermore, even papers that do report and analyze by sex were found to draw conclusions unsupported by statistical analyses and thus led to data misinterpretation and misreporting (Garcia-Sifuentes and Maney, 2021).

The groundbreaking and important SABV policy may have also unintentionally reified a binary sex categorization. In an effort to fulfil SABV, much research has focused on reporting sex differences between cohorts of females and males (Garcia-Sifuentes and Maney, 2021). Because sex is so routinely treated as a coherent, binary category, when these sex differences are found, we automatically attribute observed difference to “sex.” This presumed coherence coalesces with gendered assumptions to influence how and when scientists test sexed physiologies (Shansky, 2019), strangling our research questions and limiting our understanding. Such a caveat can be found in the case study of drug dosage and pharmacokinetics. Even though body composition (e.g., adiposity, muscle mass, etc.) is a predominant influence on drug

reaction (Anderson, 2008; Richardson, 2021), differences in drug efficacy and dosage in human cohorts are often attributed to “sex category” as opposed to investigating actual underlying physiologies. The SABV frame effectively promotes this kind of binary sex category (Clayton, 2018; Joel and Fausto-Sterling, 2016a) data analysis which ultimately obfuscates relevant physiological phenomena.

The **sex variables framework** we propose here builds upon the progress already made to advance more accurate and responsible science (DiMarco et al., 2022; Miyagi et al., 2021; Richardson, 2021). Our framework not only recognizes that physiologies we assign to sex (which we term “canonical sex variables;” Table 1) can interact, synergize, counteract, or simply coexist with one another and change over the course of a lifetime, but also allows researchers to expand the definition of what constitutes a “sexed physiology” to avoid the condensation of multifactorial and malleable sex down to an oversimplified heuristic. Together, this helps free researchers from assumptions of within-sex consistency to allow for the multidimensional investigation of physiologies. With a multiplicity of canonical and non-canonical sex variables in mind, researchers would directly assess individual physiologies relevant to their research question instead of relying on the hypersimplified female/male sex heuristic. This would allow researchers to situate sex variables within the appropriate context and promote greater etiological understanding. This paradigm shift enables researchers to appreciate and heed the variability and multidimensionality among and beyond “females” and “males.”

6.3 Sex Variables in Practice

What would implementing a sex variables framework take? Just as SABV was not a call to study sex differences in all cases (Clayton, 2018), our proposal is not a blanket demand for every sex variable be considered in every scientific study. Rather, the sex variables approach encourages a shift in how scientists approach question synthesis, research design, and data analysis & interpretation.

Experimental Design: From Sex to Physiology

The sex variables framework recognizes that the canonical sex categories of “female” and “male” can be a useful starting point for scientific exploration. Heuristics are a necessary part of science. The problem is allowing them to stand as an analytic endpoint. Early in the research process, it can be useful to check for differences between “females” and “males” or use binary coherent sex as a covariate. However, once sex differences are found, a sex variables framework would prevent biological essentialization through prompting the investigation of candidate physiologies (including co-correlates such as body composition mentioned above) across contexts, not simply within them. For instance, the continued experimentation of estradiol actions primarily in “females” and those of testosterone primarily in “males” precludes analyses that might reveal variation among members of each category. What if the actions of estradiol, a hormone present at varying levels in all individuals, differ by context (Kurth et al., 2014; Massa et al., 2017; Temple et al., 2001)? Interpretations from these kinds of studies may help us invaluable knowledge of interacting physiologies. A sex variables framework frees us from preconceived notions of what “belongs” in a sex category and instead appreciates the malleability and intricacies of biological systems.

On the other hand, a sex variables frame recognizes that canonical sex variables may be relevant even when no significant difference between “females” and “males” is found. We know that sex differences in the brain can mask phenotypic differences (De Vries, 2004); there are multiple physiological ways to achieve the same final behavioral output. Thus, if a sex variable is demonstrated to affect a phenotype relevant to the experimental question, the absence of a sex difference may be shaped by variation apparent sex categories (Diver et al., 2003; Machida et al., 1981).

A sex variables model also urges consideration of reproductive context (e.g., reproductive experience or reproductive capacity) in all subjects when relevant. Simplified models of sex connote immutability of related variables even though physiologies within each

sex category change over time and environmental condition. Even when just looking at hormonal milieu, an individual will experience changes in hormonal production across different reproductive life stages. And genetic and environmental differences produce a great deal of variation even within these life stages. In humans, parental behavior can decrease testosterone levels (Gettler et al., 2011), and in fish, social interactions can alter the hypothalamic-pituitary-gonadal axis (and thus testicular function; White et al., 2002).

In moving away from a binary sex frame, a sex variables framework decentralizes the single variable of “sex” and necessitates interrogation of what categorical stratification is actually relevant for the question at hand. This may lead us to revise our sex categorization models or even conclude that those canonical categories are irrelevant to the phenomenon of interest. For example, plainfin midshipmen have two “male” morphs – the territorial, signing type 1 and the sneaker type 2 – each displaying vastly different behavioral phenotypes and reproductive strategies but deemed “male” solely due to their sperm-production capacities. In questions regarding reproductive strategy, most would agree that it makes more sense to separate the morphs rather than binning them together. But on the other hand, if the scientific question were investigating the regulation of body mass, perhaps type 2 sneaker males and females ought to both be examined first together and later contextually. A sex variables framework supports scientific stratification based on relevant phenotypes instead of a single sex variable, such as gametic production.

Importantly, a sex variables framework allows for expansive scientific creativity. Many things can alter reproductive capacity and other phenotypes we associate with sex. In honeybees, enriched food (royal jelly) determines which honeybee will be queen (the only member of the hive capable of producing viable eggs)(Kucharski et al., 2008; Shuel and Dixon, 1960). Temperature, and even climate change (Janzen, 1994), can alter reproductive development in some reptiles and fish (2004). In rodents, parental treatment in the form of pup anogenital licking can alter adult sexual behavior (Cameron et al., 2008; Moore, 1992). And in

humans, gendered experiences can impact brain epigenetics (Cortes et al., 2019) and architecture (Jordan-Young and Rumiati, 2012). A sex variables framework could free researchers from our current presumption of discrete, binary sex to produce more expansive, accurate knowledge. When we liberate ourselves from the constraints of a simplified, binary sex model, we become free to follow observations wherever they lead us (Fausto-Sterling, 2000) – even when they defy the conventional knowledge deemed "the biology of sex."

Data Collection and Analysis

A sex variables framework encourages more specific data collection (National Academies of Sciences Engineering and Medicine, 2020), particularly for non-inbred organisms. Underspecified data leads researchers to attribute various patterns to "sex" without identifying relevant physiological processes (Joel and Fausto-Sterling, 2016b; see Figure 6-1 for example). If a dataset utilizes only one variable to denote sex (e.g., Y chromosome presence), use of that dataset is limited by assumed coherence to the "default." Collecting karyotypes, blood serum samples, and full medical history would be ideal when relevant and feasible, but even survey methods could be expanded easily for human studies. With a few well-poised questions (see examples Figure 6-2), the usability of the data would be greatly expanded. Even in cases of inbred organisms, collection or suspected relevant tissue samples and serums would be useful to collect when the involvement of sex variables is suspected.

Data analysis under this frame would analyze based on experimental question and relevant sex variables only, forgoing the sex heuristic and utilizing relevant physiologies. However, because multiple sex variables can and do interact (Chen et al., 2012), multivariate analysis can also be utilize to dissociate the contributions of independent physiologies. In an ideal sex variables framework, studies would use large-scale data procurement and multivariate analyses to forego the sex heuristic. This is unfortunately untenable for all studies under current funding structures. We therefore call on funding agencies to take into consideration the

necessities of scientific studies to pinpoint specific etiologies for research concerning “sex” as science continues to strive for the improved accuracy that a sex variables framework furnishes.

6.4 Sex Variables in Context

This proposal exists in a long lineage of biological discovery and progress. Researchers like Art Arnold have long argued for the decentralization of the hormonal milieu (Arnold et al., 2004), and that research methodologies should not end with “sex differences” but instead include efforts towards etiological discovery (Becker et al., 2005; McCarthy et al., 2012), particularly as it relates to the differing contributions of gonadal hormones and sex chromosome complement. Indeed, research paradigms such as the Four Core Genotypes model (De Vries et al., 2002) have led to groundbreaking understandings of ways that seemingly concordant physiologies (e.g., XY-testes, XX-ovaries) can interact (Chen et al., 2013, 2015) and interfere (Chen et al., 2012) with one another. However, in this lineage, sex variables are primarily limited to gonadal hormone actions and sex chromosome complement effects. We argue that phenotypes and physiologies that contribute to the binary sex heuristic are even more diverse, varied, and malleable than this, while also not adhering to coherency within the binned heuristic.

Much like Sarah Richardson’s “sex contextualism” (2021), our sex variables framework aims for improved transparency, rigor, and precision (DiMarco et al., 2022; Miyagi et al., 2021) by resisting the inappropriate truncation of physiologies and codification of binary dualism in scientific research. What a sex variables framework adds to this conversation is the scope of such a reconsideration. A sex variables framework prompts researchers to explore sexed physiologies as contextual and multidimensional while also interrogating assumptions of what “sex” actually entails. What other, non-canonical physiologies (Massa and Correa, 2020) might be at play? Why shouldn’t the effects of sexed physiologies be tested outside of their typically-associated sex category (e.g., estradiol tested in animals with testes)? A sex variables lens removes ambiguation through specificity of language (Miyagi et al., 2021), expands scientific

capacities through a focus on relevant physiologies (DiMarco et al., 2022), and questions current frameworks of coherent internal sex. As a result, sex variables research has the potential to be more applicable across research contexts, studies, and species.

6.5 Sex Variables for a More Responsible Science

It will take considerable time, effort, and creativity to actualize this kind of paradigm shift. It will not be easy, but it is – in our view - necessary. Widespread reproducibility issues and consistent debates on the effects (Takahashi et al., 2020) (or not, as in Shattuck-Heidorn et al., 2021) of sex in various models signal an urgent need for a more nuanced and specific approach. We believe that a sex variables model will help produce more rigorous science with better understandings of relevant physiologies across all contexts.

Crucially, shifting our research design and data collection to include a variety of sex variables will foster research inclusive of and relevant to all individuals, of particular importance for demographics minoritized by our current research paradigms. Adherence to the binary sex model and SABV not only homogenizes data, but also results in criteria that exclude and marginalize all those who do not fit our stringent and discrete definitions, with dire consequences. Medical professionals subject intersex people (including infants and children) to unnecessary surgeries without consent while denying trans people access to appropriate medical care, all for the sake of maintaining preconceived notions of a population neatly divided into “female” and “male” categories (Davis et al., 2015). A sex variables framework helps decentralize this harmful scientific practice.

The switch to sex variables research may also help disambiguate scientific understandings of sex for the general public. While researchers acknowledge the nuance, complexity, and multifactorial nature of “sex,” public representations still tend to rely upon stringent binary classifications of a bioessentialist nature. This inconsistency results in misinterpretation and misapplication of our work (John, 2021), ultimately resulting in attempts to

deny rights and resources (Krishnakumar, 2021) to trans, intersex, and other marginalized people on the basis of an overly-simplified binary model of “biological” sex that the scientific community has long rebuked. Employing a nuanced sex variables framework in both our research process and science communication can help align the public with the well-established scientific understanding that sex is not a stagnant categorization, but instead a malleable (Machida et al., 1981), responsive (Gettler et al., 2011; White et al., 2002), and ever-changing (Casas et al., 2016) set of interacting physiologies contributing to incredible organism diversity.

6.6 A Steppingstone Towards a Better Future

We urge the scientific community to embrace a sex variables framework as a path toward more accurate and responsible science. This framework both acknowledges and challenges how our understanding of sex as a single, internally consistent variable has been shaped by an adherence to a model that fails to describe the complexity and nuance of nature. It frees us from notions that impede our science and reproduce harmful marginalization.

However, we also envision a science freed from these preconceptions, one where our ideas about sex (and indeed, gender) no longer limit our understanding of chromosomes, gonadal hormones, environment, etc. Where these variables are no longer *sexed* and instead viewed as physiologies that exist to be explored, altered, and adapted to produce the immense variability and individuality. We hope “sex variables” provides a useful framework for this moment on the way to that future.

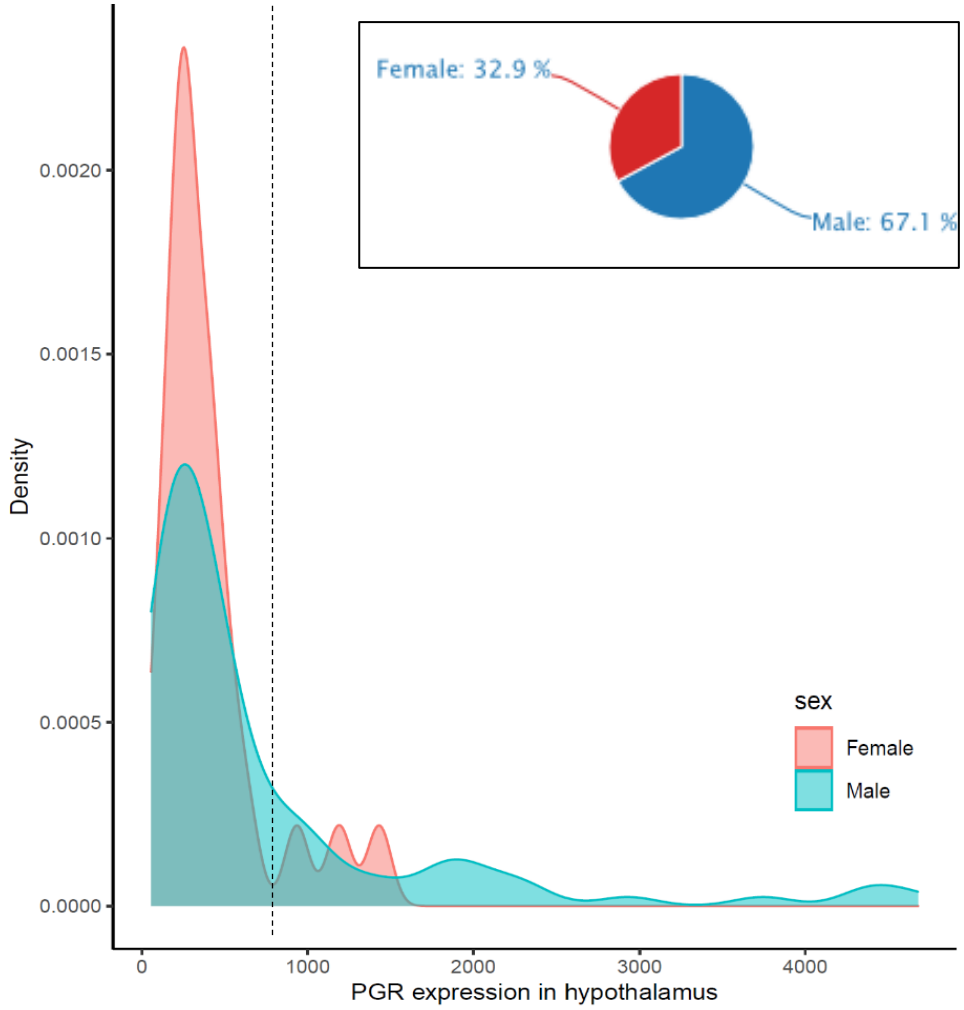

Figure 6-1. When Concordant Binary Sex Fails: A GTEx Case Study. Physiologically-based questions demand accurate stratification methods, something that the binary heuristic of sex can no longer provide. (Inset) GTEx version 8 currently reports 32.9% “female” and 67.1% “male” donor tissue as defined by Y chromosome presence. (Graph) Progesterone receptor (*PGR*) expression in the hypothalamus is a known downstream target of estrogen receptor activation. Using hypothalamic *PGR* expression as a proxy for circulating estradiol levels, it is clear that segregation by sex (Y chromosome presence) is an insufficient method for analysis for experimental questions involving hormonal physiologies. Instead, low and high “estradiol” individuals can be separated and analyzed at the dotted line. *PGR* graph was created with the assistance of Marcus Seldin, PhD.

1. What is your gender? _____
2. What is your gender modality(Ashley, 2021)? Cis Trans
3. Do you, to your knowledge, have any intersex traits? Yes No
    a. If yes, please describe: _____
    b. Alternative models for collection of intersex data by InterACT
4. What age did you begin puberty? _____
5. What is your current age? _____
6. If applicable, when was the first day of your last period? _____
7. If you are cycling, is your cycle regular? Yes No
    a. If no, please describe: _____
8. Are you on any hormones or hormone blockers? Yes No
    a. If yes, please name them: _____
9. Are you on any other medications that alter hormones? Yes No
    a. If yes, please name them: _____
10. Have you ever been pregnant? Yes No
    a. If yes, how many times? _____
    b. If yes, how many births? _____
11. Are you currently breastfeeding? Yes No
12. Have you been sexually active in the last 2 years? Yes No
13. Have you undergone menopause/andropause? Yes No Don't know In midst
14. What time of day is it? _____

Figure 6-2. Sample Human Sex Variables Survey

Canonical Sex Variables	Possible Phenotypes
Chromosomes	Mammals: XO, XX, XY, XXY, etc. Birds: ZZ, ZW, etc. *Drosophila*: X chromosome ratio
Gametes	Eggs & sperm, function notwithstanding
Gonads	Ovaries, testes, ovotestes
Gonadal hormone levels	Production of androgens, estrogens, and progestogens
Gonadal hormone receptor functionality	Receptor (in)sensitivities, etc.
Internal genitalia	Development of Müllerian and/or development of Wolffian ducts (or neither)
External genitalia	Phallus size, ano-genital distance
Other, post-pubertal sex-associated phenotypes	Tissue development (e.g., breast, muscle) Body & facial hair location and quantity Plumage coloration

Table 6-1. List of canonical sex variables and a non-exhaustive list of their possible phenotypes.

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