## Title

Selective sexual differentiation of neurone populations may contribute to sex-specific outputs of the ventromedial nucleus of the hypothalamus



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**Title: Selective sexual differentiation of neuron populations may contribute to sex-specific outputs of the ventromedial nucleus of the hypothalamus**

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Running Head:
Selective sexual differentiation of the ventromedial hypothalamus

**ABSTRACT**

Sex differences among neurons in the ventrolateral region of the ventromedial hypothalamic nucleus (VMHvl) allow for the display of a diversity of sex-typical behaviors and physiological responses, ranging from mating behavior to metabolism. Here we review recent studies that interrogate the relationship between sex-typical responses and changes in cellular phenotypes. We discuss technologies that increase the resolution of molecular profiling or targeting of cell populations, including single cell transcriptional profiling and conditional viral genetic approaches to manipulate neuron survival or activity. Overall, emerging studies indicate that sex-typical functions of the VMH may be mediated by phenotypically distinct and sexually differentiated neuron populations within the VMHvl. Future studies in this and other brain regions could exploit cell-type-specific tools to reveal the cell populations and molecular mediators that modulate sex-typical responses. Further, cell-type-specific analyses of the effects of sexually differentiating factors, including sex hormones, can test the hypothesis that distinct cell types within a single brain region vary with respect to sexual differentiation.

## INTRODUCTION

Sex differences in the brain include molecular, cytoarchitectural, or connectivity features of cells and brain regions that are nonneutral between males and females. These sex differences are embedded within neural circuits and could provide the functional basis for sex-typical behaviors and physiological responses (Figure 1)[1,2]. The origins of these sex differences are not fully understood, but can be the result of genetic, hormonal, or environmental factors acting in adulthood or during development (reviewed in [3]). Some of the best studied sex differences in the brain appear to arise from the effects of sex hormones, mainly testosterone or its metabolite estradiol. *Activational* effects of sex hormones arise in adulthood and are reversible. In contrast, *organizational* effects of sex hormones are programmed during a short perinatal time window, or critical period, and are permanent. For example, perinatal estradiol can alter cellular survival and lead to sex differences in neuron number in the sexually dimorphic

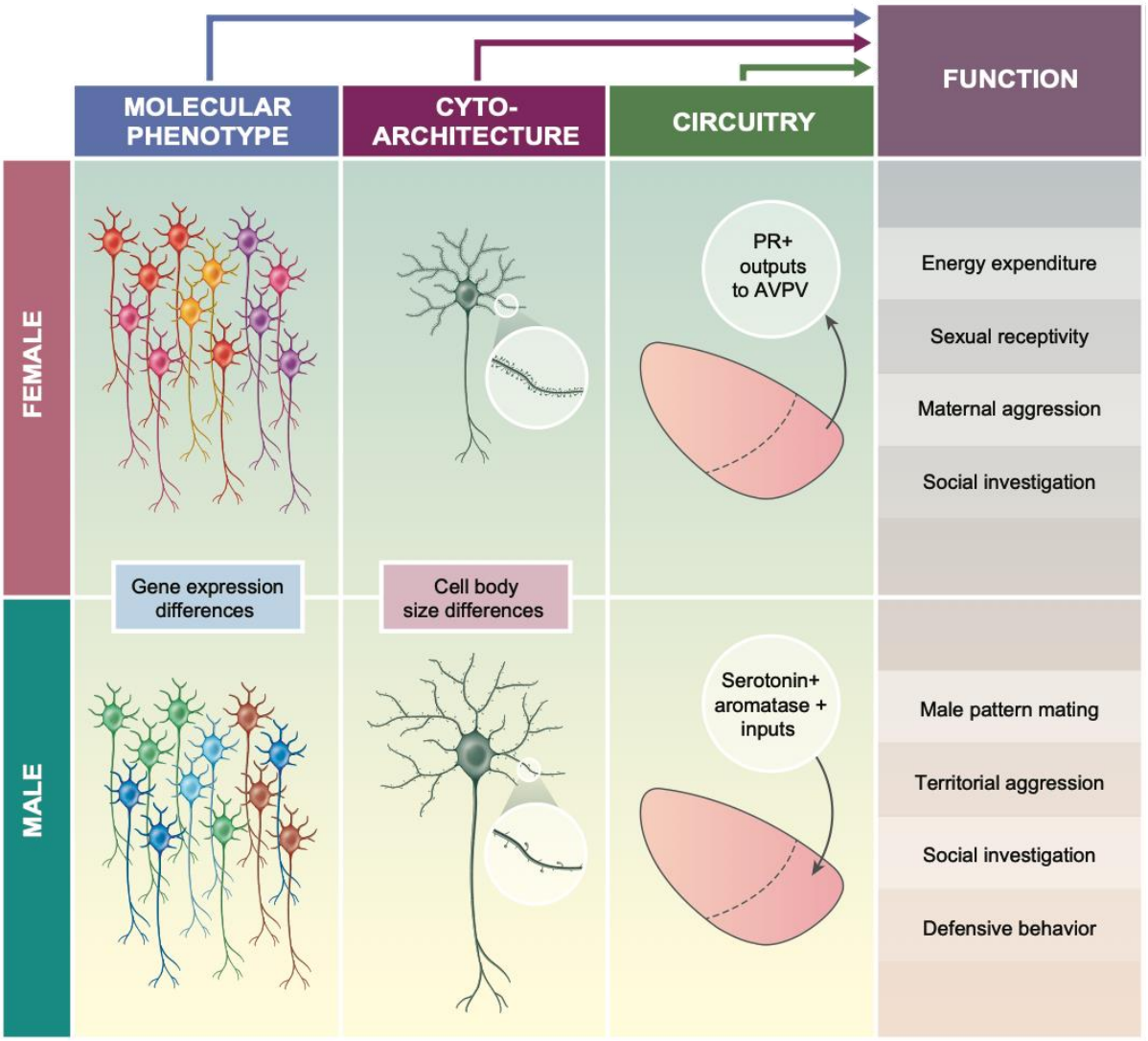


**Figure 1.** Schematic depiction of three types of sex differences in the VMH. Sex differences in molecular phenotype (gene expression), cytoarchitecture (cell body size, synaptic density in response to estradiol), and circuit connectivity (afferent and efferent projections) are thought to contribute to sex-typical functions, including behavioral and physiological outputs.

nucleus of the preoptic area of the hypothalamus (SDN-POA) and the anteroventral periventricular nucleus (AVPV) in rodents[4-9]. However, other hypothalamic regions exhibit sex differences in gene expression or morphology rather than cell number. In the VMHvl, perinatal estradiol establishes permanent sex differences in gene expression[4,5]. This sexual differentiation of cellular phenotype extends to glial cells, including astrocytes[10] and microglia[11], and opens the door to more subtle differences between male and female brains.

Earlier reviews have highlighted some of the challenges in characterizing sex differences in the brain: (1) sex differences rarely present as binary sexual dimorphisms but rather as quantitative differences[2], and (2) while some cells and brain regions tend to be more strongly differentiated within each sex, the actual profile of masculinization and feminization depends on the hormonal and non-hormonal conditions of an individual[12-14]. These findings suggest that traditional methods of profiling sex differences in the brain, such as bulk tissue analysis, are unlikely to discriminate patterns of sexual differentiation specific to individual cells, and instead capture the average patterns of sexual differentiation within a brain region. Furthermore, bulk tissue-level analysis may be biased towards the characteristics of one or a few dominant cell populations and can miss those characteristics that are discordant among cell types and cell subpopulations[15].

Recent technologies, such as single cell RNA sequencing (scRNA-seq) and viral genetic tools, have been used delineate which sexually differentiated cell populations contribute to the display of sex-typical responses in rodents. scRNA-seq tools isolate transcriptomes from single cells and examine transcriptional signatures among cell types (Figure 2A). Viral genetic tools include adeno-associated viruses that express genetically engineered proteins in a manner that is dependent on Cre recombinase (Figure 2B). This conditional gene expression allows for selective expression of genetic tools when delivered to mice that express Cre under the control of cell-type-specific gene markers. Here we discuss studies that induce cell-autonomous cell death using virally delivered Caspase-3 or bidirectionally manipulate neural activity using chemogenetic or optogenetic approaches. Briefly, chemogenetics refers to the use of synthetic G-protein coupled receptors that modulate neuronal excitability in response to an exogenous small molecule ligand, clozapine-N-oxide. Similarly, optogenetics refers to light-sensitive ion channels and implanted optical fibers to deliver light. Together, these studies reveal cell-type-specific effects on behavior that can differ between the sexes. Because these sex differences in function are restricted within and unique to distinct cell subpopulations, we suggest that sexual differentiation can have differential effects on cell populations. We propose the term “selective sexual differentiation” to describe heterogeneous responses to sexual differentiation factors, such as sex hormones, and suggest that these could underlie the sex-specificity and heterogeneity of functions mediated by the VMH. Finally, we discuss the potential and limitations of cell-type-specific analyses to advance our understanding of sex differences in the brain.

## SEX-SPECIFIC FUNCTIONS REGULATED BY THE VMH

The VMH is a sexually differentiated structure that regulates a wide range of physiological processes, from sexual receptivity and mating behaviors, to physical activity and metabolic processes. Manipulations of molecularly defined neuron populations in the VMHvl have revealed sex-specific regulation of mounting behavior[16-19], sexual receptivity[19-23], territorial aggression[16-18,24,25], maternal behavior[26-29], physical activity[30,31], and temperature[32,33]. The VMHvl also regulates defensive social behaviors[34] but an effect of sex has not been established. Similarly, SF-1 neurons in the central and dorsomedial regions of the VMH (VMHc and VMHdm, respectively) control escape and defensive behaviors in both males and females[35-37]. Additionally, VMH neurons are sensitive to metabolic signals, including leptin and glucose, similarly in both sexes[38-41]. These studies suggest that many, but not all, functions mediated by the VMH occur in a sex-biased manner.

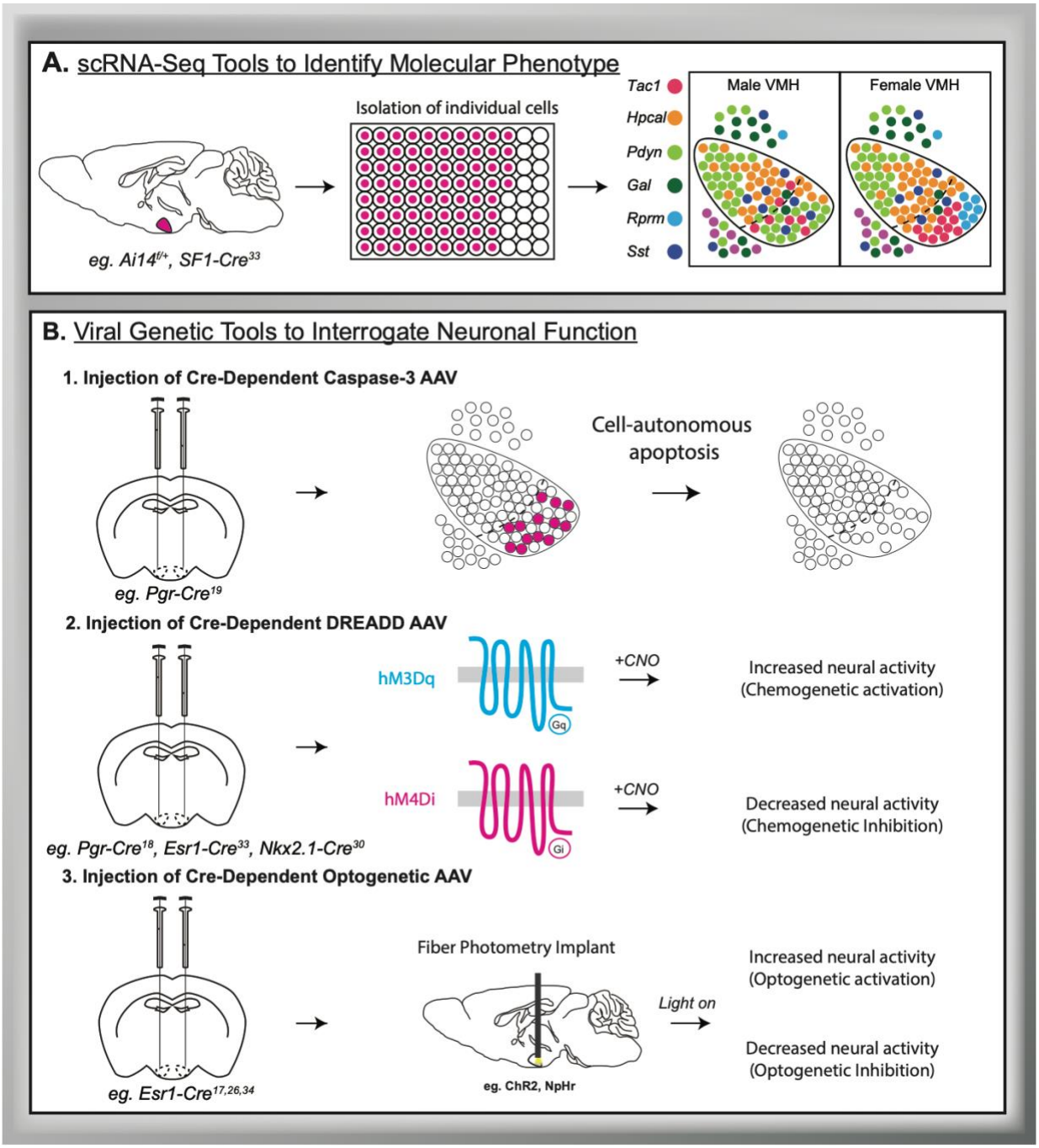


**Figure 2.** Single cell RNA sequencing (scRNA-Seq) and viral genetic tools can help identify the molecular phenotype of sexually differentiated neuronal populations within the VMH and determine their contribution to VMH-mediated functions. **A.** Pipeline for scRNA-Seq of VMH neurons as reported in[33]. Briefly, fluorescently labeled VMH neurons are isolated. A single-cell suspension allows for the transcriptome of individual cells to be analyzed. This approach revealed 6 molecularly distinct populations of neurons in the VMH. Figure adapted from[33]. **B.** Viral genetic tools that manipulate neuronal survival or activity patterns can be used to determine cellular function. *Top row*, injection of Cre-dependent Caspase-3 AAV virus allows for cell-autonomous apoptosis (cell death) of specific neurons within the VMH. *Middle row*, injection of Cre-dependent DREADD AAV (eg. hM3Dq, hM4Di) allows for chemogenetic activation or inhibition of neuronal activity within VMH neurons following delivery of clozapine-N-oxide (CNO). *Bottom row*, injection of Cre-dependent optogenetic AAV (eg. ChR2, NpHr) allows for optogenetic activation or inhibition of VMH neurons via fiber photometry.

Sex hormone effects during development and puberty are the most robust mechanisms that drive sexual differentiation in the brain. Therefore, sex differences are most likely to manifest within populations of sex hormone-responsive neurons, and the VMH is rich in sex hormone receptor expression. In adult mice, neurons co-expressing estrogen receptor α (ERα) and progesterone receptor (PR) localize to VMHvl[19,42] and are estimated to constitute 40-50% of cells in the VMHvl[17,19]. Furthermore, ERα is expressed at significantly higher levels in the VMHvl of females than males in rats[43-45] and mice[46]. This sexually differentiated expression of ERα has been demonstrated in rats to arise during the early postnatal period (by postnatal day 10, P10) and persist into adulthood[45]. Neurons expressing the other ER subtype, estrogen receptor β (ERβ), are also localized to the VMHvl and largely overlap with ERα+ neurons, although ERβ expression is much lower than that of ERα in both mice[47] and rats[43]. ERβ is also expressed at higher levels in females than males in neonatal (P5) rats; however this sex difference becomes marginal by adulthood and is not observed in mice[43,47]. Finally, androgen receptor (AR)-expressing neurons are localized throughout the VMH, with expression observed in mice in the early neonatal period (P1-P4). However, ARs do not show sex differences in expression in rats or mice[48-50]. Instead, AR neurons are thought to contribute to sex differences in the VMH through the regulation of aromatase expression and activity. In rats, testosterone treatment increases aromatase activity more in males than females, while mutations that render males unresponsive to androgens contribute to decreased aromatase activity and demasculinization of the VMH[51,52].

*Mating behavior (sexual receptivity and male-pattern mating behavior)*

Subsets of VMHvl neurons contribute to the circuitry underlying female sexual receptivity and male pattern mating behavior. In females, the VMH is the final integration site for the hypothalamic and limbic circuits that underlie the expression of lordosis[53]. Female rodents are sexually receptive at specific times in their estrus cycle, starting with the evening of proestrus and ending with the morning of estrus, in a manner that is dependent on estradiol and progesterone[53]. RNAi-mediated silencing of ERα in the murine VMHvl impairs the display of receptive behavior, and instead triggers vigorous rejection of the male[54]. Unlike ERα, ERβ does not seem to play a major role in female sexual behavior, as ERβ-null mice show normal reproductive behavior[55].

Gene targeting studies have identified several receptor and neuropeptide-encoding genes in the VMHvl that are necessary for female mating behavior. *Cckar,* the cholecystokinin A receptor, is expressed by the majority of ERα+/PR+ neurons in the VMHvl of female mice and is induced by estrogen signaling[20,26]. *Cckar*-null females[56] show specific deficits in sexual receptivity, but show wild-type maternal behaviors and estrus cycles[20]. In contrast, *Cckar* is only very weakly expressed in males, and *Cckar*-null males show wild-type male-pattern mating behaviors[19,20]. The oxytocin receptor (*Otr*) is also necessary for estrogen-induced sexual receptivity behavior, as infusion of *Otr* antisense oligos into the VMH leads to reduced lordosis frequency and intensity without affecting locomotor activity following an estrogen priming paradigm in rats[21]. The gene encoding the precursor of the neuropeptide enkephalin (*Penk*) also contributes to lordosis behavior, as disruption of *Penk* function by intrahypothalamic antisense oligonucleotide injection decreases lordosis behavior without affecting locomotor behavior in rats[22]. Interestingly, although *Penk* is induced by estrogen signaling in adult guinea pigs, single cell RNA-seq and co-expression studies in mice find that only very few enkephalin-immunoreactive cells in the murine VMHvl co-express ERα or PR[33,57]. Lastly, subsets of neurons in the rat VMHvl encoding the neuropeptide substance P project to the dorsal midbrain central grey[58], and injection of substance P to this target region facilitates lordosis behavior[23]. The gene encoding the substance P precursor (*Tac1*) is colocalizes with ERα in the rodent VMHvl[33]. However, a direct effect of *Tac1* disruption on lordosis behavior remains to be shown. Together, these studies suggest that genes expressed by sex hormone-responsive cells, or

induced by activational effects of sex hormones, have been linked to female-specific receptive behaviors.

In contrast to females, studies using genetic ablation, optogenetic or chemogenetic manipulation, and neural activity pattern recordings of VMHvl ERα+ or PR+ neurons have shown that these cells play a less robust role in male-pattern mating behavior[16]. Inducing death of PR+ neurons, using Cre-dependent Caspase-3 delivered to VMHvl of male mice expressing Cre under the Pgr promoter (*Pgr-Cre*), leads to reduced mounting, intromission, and reduced intromission length[19]. Optogenetic activation of neurons expressing Cre under the *Esr1* promoter (*Esr1*-Cre) is not sufficient to increase mounting frequency towards females, although it does increase mounting duration in male mice[17]. Furthermore, unlike in females where mating-related VMHvl neurons increase *in vivo* spiking activity during and throughout a male encounter, VMHvl neurons in male mice show only a transient increase in *in vivo* spiking activity with a female encounter, and this is extinguished during progression of the behavior[59]. Similarly, optogenetic inhibition of *Esr1*-Cre neurons in male mice is insufficient to affect frequency, duration, or halt ongoing mounting of females[17]. Of note, residual neuronal activity in the optogenetic experiments may have impeded complete behavioral manipulation, accounting for some of these discrepancies[17]. Indeed, chemogenetic inhibition of VMHvl neurons in male *Pgr-Cre* mice reduces the quantity and duration of intromission events[18]. Nevertheless, optogenetic inhibition of *Esr1*-Cre neurons in the medial preoptic area (mPOA) is sufficient to inhibit mounting in both sexes, suggesting a nuanced role for the VMHvl in male-pattern mating behavior[16,60].

*Social behaviors (aggression and social investigation)*

VMHvl neurons also contribute to aspects of various social behaviors, including defensive behavior, social investigation, and aggression. Optogenetic activation of *Esr1*-Cre neurons in the murine VMHvl can elicit either aggressive or defensive behaviors in males, while optogenetic inhibition of this same population compromises social defense[34]. Subsets of neurons in the murine VMHvl of males also show increased *in vivo* spiking activity during the initiation of an aggressive behavior towards a male intruder, and these neurons are normally inhibited during female encounters[59]. In contrast, optogenetic activation of these VMHvl neurons in males induces a rapid, time-bounded attack towards any intruders, including conspecific females[59]. Parallel findings from *Esr1*-Cre and *Pgr*-Cre mice show that these VMHvl neurons are necessary and sufficient to induce and maintain male intruder aggression[17-19]. Furthermore, intruder aggression can be elicited by chemogenetic activation of *Pgr-Cre* neurons in male mice even in the absence of testicular hormones[18]. In addition, the social behavior elicited by this population is scalable, such that a larger population of optogenetically or chemogenetically activated cells preferentially induces aggressive behavior, while a weaker activation of cells (via manipulation of the optogenetic or chemogenetic parameters) preferentially induces mounting and social investigative behavior towards both sexes[17,18].

In contrast to males, activation of any-sized population of *Pgr-Cre* or *Esr1*-Cre VMHvl neurons in female mice generally fails to elicit aggressive behavior. Instead, activation of these neurons induces social investigation and occasional mounting[17,18]. However, the genetic background and reproductive state of the female can sometimes facilitate the expression of aggressive behavior following optogenetic activation of *Esr1*-Cre neurons, such as in the case of Swiss Webster mice or naturally aggressive lactating C57BL/6 mice[26]. In these females, the neurons that are active during the aggressive behavior are localized medially within the VMHvl and are functionally distinct from VMHvl neurons mediating mating behaviors[26].

Finally, aggressive behavior can also be modulated by neurons projecting to the VMHvl. Optogenetic activation of lateral septum-VMHvl projections selectively inhibits an ongoing attack behavior in male mice, without affecting mounting behavior[61]. VMHvl neurons also receive synaptic inputs from GABAergic subparaventricular zone neurons, as well as neurons in the

central VMH, as part of a larger circuitry that induces circadian phase-dependent regulation of VMH-mediated aggressive behaviors in mice[62]. Together, these studies suggest that social behaviors elicited by sex hormone-sensitive neurons in the VMHvl are modulated by both intrinsic factors and external circuitry.

*Metabolism (energy expenditure and glucose sensing)*

Neurons within the VMHvl also have important roles in regulating aspects of metabolism, including energy expenditure and glucose sensing, and many of these show sex-specific characteristics. Loss of ERα in the VMH by gene knockout or silencing impairs estrogen-dependent wheel running and ambulatory movement, metabolic rate, and brown adipose tissue (BAT) thermogenesis in female rats and mice[30,31,63]. The effects on movement in mice appear to be selectively mediated by a subset of ERα neurons that co-express *Tac1*. Indeed, developmental loss of ERα/*Tac1* neurons leads to a reduction in movement without affecting BAT thermogenesis[30]. In rats, movement is regulated by *Otr* [44,64], but co-expression of *Otr* and *Tac1* has not been analyzed.

We recently characterized a second population of ER$\alpha_+$ neurons in the murine VMHvl that is largely distinct from the *Tac1* population and expresses the p53-induced gene reprimo (*Rprm).* Interestingly, siRNA-mediated knockdown of *Rprm* leads to a baseline increase in core body temperature in female mice[33]. This suggests that *Rprm*$_+$/ER$\alpha_+$ neurons also contribute to sex-specific regulation of energy expenditure by modulating thermogenesis. While the roles of the *Tac1*$_+$ and *Rprm*$_+$ neurons have not been adequately interrogated in males, it is interesting to note that chemogenetic activation of ERα neurons in the VMHvl is sufficient to induce increased movement and energy expenditure in both males and females, suggesting that while the molecular pathways needed to elicit these aspects of metabolism may be enriched in females, synthetic activation of the circuitry can yield equivalent behavioral and physiological outputs in males and females (Kammel, van Veen, Correa unpublished).

Sex differences in glucose homeostasis are mediated in part by estrogen-sensitive glucose sensing neurons in the VMHvl. Glucose-excited (GE) and glucose-inhibited (GI) neurons, as well as a subclass of GI neurons that adapt to low extracellular glucose levels, are distributed throughout the murine VMH and work together to control glucose homeostasis[40,41]. The majority of glucose-sensing neurons within the VMHvl are GE, which increase their activity pattern with higher extracellular glucose levels as measured by patch-clamp electrophysiology[40,65,66]. While the percentage of GE neurons in the VMHvl is similar between male and female mice, males have a greater percentage of GI neurons that do not adapt to low extracellular glucose[40]. Furthermore, GI neurons in the male VMHvl show increased excitability in low levels of glucose, which suggests an inherent sex difference in glucose-elicited activity[40]. Finally, both types of GI neurons show activity blunting in response to estradiol[40]. Together, these findings suggest that female VMH neurons have a reduced ability to detect low glucose, and that this response can be further blunted by cyclic increases in estradiol[40]. One type of GI neuron was recently identified by expression of the pituitary adenylate cyclase-activating peptide (PACAP), and chemogenetic activation of this subset of VMH neurons was sufficient to inhibit insulin secretion and increase plasma glucose[65]. The sex difference in GI neuron activity could therefore underlie the clinical observations that women have weaker counterregulatory responses to hypoglycemia than men[67].

Finally, developmental ablation of glucokinase (*Gck*), the major glucose-sensing enzyme, in murine VMH neurons leads to sex differences in fat mass, glucagon secretion, and autonomic nervous activity, with females showing increased gonadal, inguinal, and total fat mass, decreased hypoglycemia-induced glucagon secretion, and reduced parasympathetic and sympathetic nerve activity compared to males[68]. These findings further suggest that VMH *Gck* is required for hypoglycemia counter-regulation in females but not males[68]. Ultimately, sex

differences in the VMH contribute to the differential recruitment of circuitry underlying metabolic homeostasis.

## SELECTIVE SEXUAL DIFFERENTIATION OF VMH NEURONS

*Sexual differentiation of molecular phenotype*

Selective sexual differentiation among sex hormone-sensitive neurons could allow for the diverse set of sex-specific physiological processes mediated by the VMHvl. Males and females share qualitatively similar pools of molecularly defined neurons[2]. Recently, we used single cell RNA sequencing to characterize the transcriptomes of VMH neurons from male and female P10 mice of the C57BL6 background on a cell-by-cell basis. We identified 6 clusters of glutamatergic neurons that were found in both males and females[33]. The top most differentially expressed gene within each of the clusters was *Tac1*, somatostatin (*Sst*), *Rprm*, prodynorphin (*Pdyn*), hippocalcin-like protein 1 (*Hpcal1*), and galanin (*Gal*), respectively. To determine if some of these genes identified subsets of estrogen-sensitive neurons, we co-localized ERα with each cluster marker. In the female VMHvl, the majority of ERα immunoreactivity was restricted to the two subpopulations identified by *Tac1* and *Rprm*, and both *Tac1* and *Rprm* were expressed at higher levels in the VMHvl of female compared to male mice. In the male VMHvl, we detected ERα immunoreactivity in *Pdyn*+ neurons, along with male-biased expression of *Pdyn*. Together, these findings suggest that the molecular signature of estrogen-responsive neurons in the VMHvl is heterogeneous and quantitatively different between males and females.

Non-hormonal mechanisms, such as the sex chromosome complement, can also contribute to neuronal sexual differentiation, either by supplementing or opposing hormonal sex differences[69]. In particular, X chromosome genes regulate aspects of metabolism, including food intake and adiposity[70]. However, we did not find evidence that the sex chromosome complement was contributing to the sex differences in *Tac1*, *Rprm*, or *Pdyn* expression in the murine VMHvl[33]. Rather, gonadal sex was critical for determining the sex difference in *Tac1* and *Rprm* expression, suggesting that these patterns are established during development and maintained into adulthood. Finally, testicular hormones were required in adulthood for maintenance of the sex difference in *Pdyn* expression, as this difference was eliminated by castration. It was previously shown that half of the genes that exhibit sex differences in the VMHvl are regulated by adult effects of sex hormones in mice[20]. Therefore, while we cannot rule out the possibility that sex chromosome-linked genes may regulate other aspects of VMH neurons, the combined organizational and activational effects of gonadal hormones are likely to account for the majority of the sex differences in the molecular signature among estrogen-responsive neurons in the VMHvl.

*Sexual differentiation of VMH architecture*

Sex hormones and estrus cycle phase can induce sex differences in cell morphology and cytoarchitecture within the VMH. In rats, the volume of the VMH is greater in males than females across all subregions, suggesting organizational effects of sex hormones on aspects of VMH architecture[71]. However, due to a gain in VMH volume from diestrus to proestrus, this sex difference is smaller when comparing males to proestrus females than to diestrus females, indicating additional activational effects[71]. Male rats that are unresponsive to androgens also show significantly smaller VMHvl volumes compared to wild-type males[51]. Differences in VMH volume are primarily accounted for by the larger neuronal soma sizes and neuropil volume in males than females rather than reflecting relative differences in neuronal numbers between males and females within VMH subdivisions[51,71]. In particular, the male rat VMHvl receives more axodendritic contacts than the female VMHvl, and this sex difference in the synaptic pattern is reversible with neonatal castration in males or testosterone treatment in females[72]. Greater numbers of both serotonergic and aromatase-positive projections to the VMH in male rats and mice contribute to this sex difference in VMH innervation[73,74]. Finally, spine density is

differentially regulated by sex hormones in males and females, with estradiol decreasing spine density in the male VMH but increasing spine density in the female VMH, though the mechanism underlying this dichotomy is unknown (reviewed in [75]).

Sex differences in efferent projection patterns from hormone sensitive neurons in the VMH could also contribute to the display of sexually differentiated behaviors, in particular because subpopulations of ERα+ neurons along the anterior-posterior axis of the VMHvl differ in their projection targets. One study mapped efferent projections from murine *Esr1*-Cre neurons in the VMHvl and surrounding regions and found that caudally positioned *Esr1*-Cre neurons project rostrally to the amygdala and other areas of the hypothalamus, while rostrally positioned *Esr1*-Cre neurons project caudally to premotor brain areas[76]. However, no consistent differences between the sexes were detected[76]. In contrast, another study mapping efferent projections specifically from the VMHvl found major projections from murine *Pgr-Cre* neurons in the VMHvl to the AVPV in females that were largely absent in males[19]. Additional studies are required to determine if efferents are sexually dimorphic overall or differentially partitioned among subpopulations. Additionally, the behavioral or physiological significance of sex-specific projection patters will require functional circuit tracing studies in both sexes.

## POTENTIAL AND LIMITATIONS OF UNICELLULAR ANALYSIS

Classically, sex differences are thought to initiate with the presence or absence of the Y-linked *Sry* gene, which leads to differentiation of the gonads through the activation of the testis or ovary differentiation pathways. Subsequent sex differences in gonadal hormones then lead to masculinization or feminization of other tissues. However, a large body of evidence has revealed cell autonomous sex differences in tissues all over the body[3]. The advent of single cell profiling technology provides the exciting opportunity to dissect sex differences at the level of single cells or cell populations in any tissue, including specific regions of the brain.

Transcriptional profiling studies with single cell resolution often include samples from both male and female mice. However, few studies are designed to detect sex differences or distinguish between sex differences in gene expression or cell composition within a tissue. A recent study examined sex differences in the various cell lineages of the developing mouse gonad[77]. Even during the differentiation of testes or ovaries, the cells of the gonad upregulate hundreds of genes equally in both sexes and the majority of sex differences are restricted to the lineage that expresses *Sry*. Thus, it is not surprising that scRNA-seq within the brain also finds limited sex differences in gene expression. In the medial amygdala, GABAergic neurons exhibit sex differences in gene expression, whereas gene expression in glutamatergic neurons is neutral with respect to sex[78]. Of the 40-60 genes that are differentially expressed in GABAergic neurons, at least two were confirmed by histological analysis; however, it is unclear how many are due to sexual differentiation of this neuron population compared to activational effects of hormone signaling. We analyzed VMH neurons from P10 male and female mice by scRNA-seq, before activational effects of sex steroids are induced. Many transcripts were enriched in males or females but these were expressed throughout the VMH and were not robust when analyzed by histology[33]. Instead, spatial analyses revealed robust sex differences in a few transcripts within the VMHvl sub-region. Importantly, manipulation of single transcripts, e.g. *Tac1* or *Rprm*, seems to alter the function of the VMHvl[30,33], suggesting that wholesale differences in molecular signature are not required for sex differences in cell, neuron, or circuit function.

From these studies, it seems that sex differences in gene expression are restricted to certain cell populations and may involve a limited number of key transcripts. Therefore, sex differences in a few genes may lead to sex-specific functions without sex differences in the overall molecular signature of cells. However, the ability to detect these key differences that underlie sex differences in physiology will largely depend on the sensitivity of our methods to interrogate gene expression within sub-regions or individual cell populations. We argue that single cell resolution studies coupled with validation and functional manipulations will be crucial

in our efforts to pinpoint the biological underpinnings of sex differences in physiology and behavior.

## CONCLUDING REMARKS

Single cell resolution studies have revealed surprising cellular heterogeneity in a wide variety of tissues. In the VMH, it appears that the cells that mediate these sex-specific functions may be specialized, sexually differentiated, and intermingled with cells that are neutral with respect to sex. We propose that sexual differentiation contributes to the cellular heterogeneity within a tissue or brain region and leads to sex differences in some, but not all, cell populations. Some of this heterogeneity can be attributed to sex hormone receptor status, allowing for selective effects on cells that express androgen or estrogen receptors during the critical period of sexual differentiation. However, the studies reviewed here point to additional heterogeneity within hormone-sensitive cell types. Specifically, neurons that express ER$\alpha$ within the VMHvl form distinct subpopulations as identified by differences in molecular phenotype and activity patterns during VMH-mediated behaviors. Further studies will be necessary to determine if this molecular and functional specialization is established prior to or as a result of organizational effects of sex hormones.

It is possible that sexual differentiation builds upon pre-existing heterogeneity within hormone-sensitive cell populations to induce phenotypic diversity among cell types and between the sexes. In this framework, masculinization or feminization of a brain region is not uniform for all cell types, but rather reflects a cell-type-specific response to sex hormones during the critical period of sexual differentiation. Accordingly, we propose the use of unicellular analysis tools to identify sex differences within individual cell populations comprising a sexually dimorphic brain region. This high-resolution understanding of sex differences in the brain can be combined with hormone manipulations early in life to understand cell-type specific sexual differentiation and cell-type-specific manipulations of gene function in adulthood to pinpoint the key molecular mediators of sex-specific behaviors and physiologies.


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