

# Genetic and Neural Circuit Approaches to Studying Sex Differences

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## Introduction

Much of our knowledge about the cellular and molecular differences between the sexes in the mammalian brain has been obtained through studies of the hormonal regulation of the differentiation and function of neural circuits underlying innate, sex-typical behaviors and physiology in rodents. Recent studies have employed modern circuit mapping and manipulation methodologies to identify causal relationships between specific brain areas, cell types, and neural projections and the display of sexual behavior, aggression, and parenting (Fig. 1) (Chen and Hong, 2018; Li and Dulac, 2018). In this chapter, I will provide a brief overview of genetic tools and approaches used to dissect the role of gonadal hormone receptors in mediating these sex-typical behaviors. Strategies for characterizing sex differences in neural activity and behavior will be discussed, with an emphasis on understanding the neural substrates that underlie such differences. The goal is to provide neuroscientists with the tools and knowledge to identify in their own research system causal factors underlying sex differences.

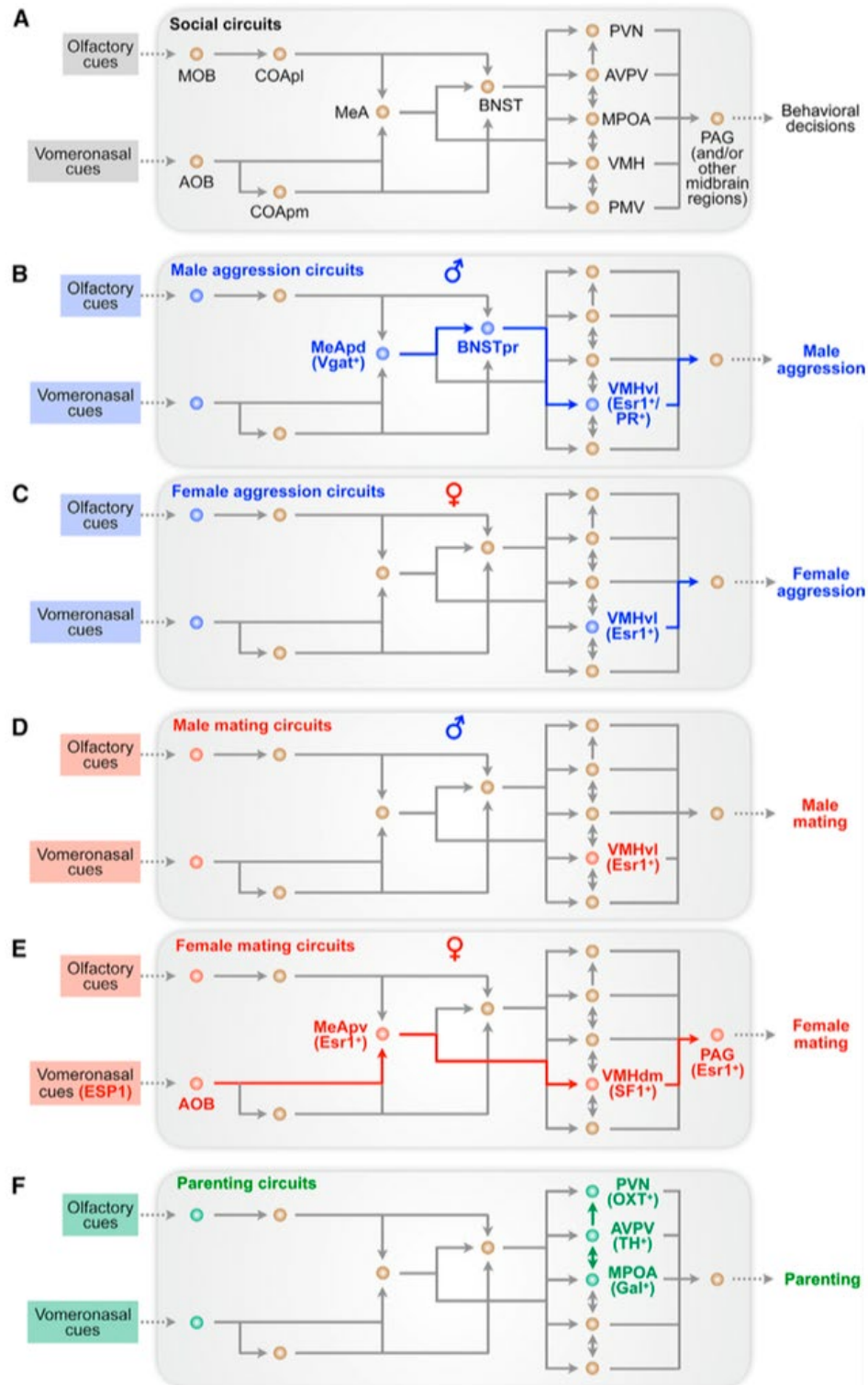
## Sexual Differentiation of the Brain Is Regulated by Gonadal Hormones

The neural circuitry that controls innate social behaviors develops under the control of gonadal hormones (Phoenix et al., 1959; Simerly, 2002; Arnold, 2009; McCarthy et al., 2009; Wu and Shah, 2011). Male mice undergo a surge of testosterone at birth that subsides within hours (Motelica-Heino et al., 1988; Corbier et al., 1992). This circulating testosterone is converted to estradiol directly in the brain by aromatase (MacLusky and Naftolin, 1981; Amateau et al., 2004). Estradiol is the primary endogenous estrogen, although estrone and estriol also bind estrogen receptors (ERs); here, we primarily use the general term “estrogen” for simplicity. Pharmacological and genetic experiments have demonstrated that this brain-derived perinatal estrogen is the primary driver of sexual differentiation of the rodent brain and permanently establishes sex-typical differences in the structure and function of neural circuitry that mediates sex-specific behaviors in the adult (Honda et al., 1998; Rissman et al., 1999; McCarthy, 2008; Wu and Shah, 2011). Females given estradiol at birth display male-typical fighting behavior as adults, with no additional hormone supplementation (Wu et al., 2009). This sensitivity to estradiol is lost by the second postnatal week (Gerall et al., 1967; Motelica-Heino et al., 1993; Toda et al., 2001). Although sex differences in neural circuitry are specified during this postnatal critical period, sex-typical behaviors are not displayed until

puberty, when the male testes produce testosterone and female ovaries make estrogens and progesterone. These hormones are acutely required in adult life: gonadectomy abolishes mating and aggression, but circuit structure remains intact and behaviors can be restored by giving exogenous hormones. Although testosterone is the primary driver of adult male-typical behaviors, estradiol alone can restore some mating and territorial behaviors (Södersten, 1975; Kimura and Hagiwara, 1985; Cross and Roselli, 1999; Bakker et al., 2004). Therefore, estrogen acts both to modulate postnatal male-typical circuit development and to “activate” circuits for sex-typical behaviors in adulthood. Although estrogen is the primary driver of sexual differentiation in rodents, both estrogen and testosterone signaling are required for full masculinization of adult rodent behaviors.

In addition to its masculinizing effects on behavior, perinatal estrogen is known to give rise to anatomical and molecular sex differences. Many excellent reviews have summarized findings on cellular and neuroanatomical sex differences, including in cell number, neural projections, and spine number (Simerly, 2002; McCarthy, 2008; Forger, 2009; McCarthy et al., 2009; Bao et al., 2011; Yang and Shah, 2014). Notably, males have more neurons than females in select reproduction-related brain areas: the principal region of the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA) of the hypothalamus, and the posterodorsal medial amygdala (MeApd). In contrast, females have more neurons than males in the anteroventral periventricular nucleus (AVPV) (Hines et al., 1992; Morris et al., 2004; Forger, 2009; Wu et al., 2009; Scott et al., 2015). Sex differences in the BNST have also been reported in humans (Allen and Gorski, 1990; Raznahan et al., 2015). These sex differences in cell number are caused by perinatal estradiol, which promotes both cell survival in male-biased brain areas as well as cell death in the AVPV. These regions could therefore influence sex differences in autonomic and physiological functions, as well as in reward circuitry, via projections to the hypothalamus, lateral septum, parabrachial nucleus, ventral tegmental area (VTA), and amygdala (Simerly and Swanson, 1988; Canteras et al., 1992, 1994; Hutton et al., 1998; Dong et al., 2001; Dong and Swanson, 2004).

In humans, brain masculinization occurs largely through testosterone signaling rather than estrogen. Human males with mutations in *CYP19A1*, the gene for aromatase, cannot synthesize estrogen and yet present as normal males. Men with aromatase deficiency experience sustained linear growth rather than a pubertal growth spurt and epiphyseal closure,



**Figure 1.** Current understanding of circuits and brain regions in males and females implicated in different social behaviors. **A**, Overview of key social behavioral circuits and regions. **B**, **C**, Circuits involved in male (**B**) and female (**C**) aggression. **D**, **E**, Circuits involved in male (**D**) and female (**E**) mating. **F**, Circuits involved in parenting in males and females. MOB, main olfactory bulb; CO-Apl/pm, posterolateral and posteromedial cortical amygdala; BNSTpr, principal nucleus of the bed nucleus of the stria terminalis; PVN, paraventricular hypothalamic nucleus; VMHvl/dm, ventrolateral and dorsomedial subregions of the ventromedial hypothalamic nucleus; PMV, ventral premammillary hypothalamic nucleus. In **B–F**, some of the nodes and connections are hypothetical. Colored nodes and connections represent circuits with direct experimental evidence for the corresponding behavior. Reprinted with permission from Chen and Hong (2018), Fig. 2. Copyright 2018, Elsevier.

demonstrating that estrogen is required in males for proper skeletal maturation (Grumbach and Auchus, 1999). In contrast, androgen receptor (AR) function is essential for phenotypic and behavioral masculinization of human males. Patients with an XY karyotype and a complete loss of AR function have complete androgen insensitivity syndrome, present as women, and have female-typical brain morphology (Van Hemmen et al., 2017). Humans also experience developmental testosterone surges, which, although consistent with the scaling of natal development, are much more prolonged than those in rodents. The testes begin to secrete testosterone around week 7 of gestation, reaching maximal levels between weeks 8 and 24 (Reyes et al., 1974; Hines, 2006). Human brain at midgestation is similar to mouse brain at birth with regard to the staging of cortical development (Willsey et al., 2013; Workman et al., 2013). The timing of developmental hormone surges is thus somewhat conserved between rodents and humans: the midgestation testosterone surge in humans is concordant with the perinatal surge in mice and rats. Human males also experience an additional surge in infancy that peaks between months 1 and 3 (Winter et al., 1976; Hrabovszky and Hutson, 2002). Female ovaries are also known to be active during infancy, but the levels of estradiol are variable, and the time course of its secretion is not well described (Winter et al., 1976; Chellakooty et al., 2003; Thompson et al., 2010).

## Sex Chromosome Influences on the Brain

Sex chromosomes also contribute to sexual differentiation of the brain, both directly through their own genetic content, and indirectly through regulation of gonadal development (Arnold, 2004; Cox et al., 2014; Arnold et al., 2016; Bramble et al., 2017). Sex chromosome aneuploidies are some of the most common genetic disorders in humans, affecting nearly 1 in 400 live births (Lenroot et al., 2009). These disorders are associated with cognitive and behavioral symptoms, particularly in social skills and motor abilities (Hong and Reiss, 2014). Notably, language and spatial abilities appear to correlate with sex chromosome dosage. Females with X monosomy show normal or increased verbal and lexical abilities and visuospatial deficits, whereas individuals with sex chromosome polysomy have language impairments that increase with the number of chromosomes, while their spatial skills are often enhanced (Crespi, 2008; Lenroot et al., 2009; Hong and Reiss, 2014). Brain imaging studies have identified a relationship between sex chromosome dosage and brain volume

(Lenroot et al., 2009) and highlight specific chromosomal effects in cortical (Lin et al., 2015) and subcortical (Raznahan et al., 2015; Reardon et al., 2016) brain areas. Mouse models of sex chromosome aneuploidies have been used to discern the effects of sex chromosomes on specific behaviors, including social behaviors, anxiety, feeding, and nociception (Cox et al., 2014). The most widely used model is that of the “four core genotypes.” This system employs two modified alleles of the testis-determining Sry gene: one in which Sry has been deleted from the Y chromosome, resulting in genetic males that resemble females, and another in which Sry has been inserted on an autosome to generate XX animals that develop testes (De Vries et al., 2002). Comparison of these mutants with wild-type XX and XY animals thereby permits the dissociation of sex chromosome complement from gonadal development.

## Location of Hormone-Receptor-Expressing Neurons in Rodents

Gonadal hormones such as estrogen and testosterone exert many of their effects via their cognate steroid hormone receptors (SRs): nuclear receptor transcription factors that can recruit chromatin remodeling machinery to activate or repress gene expression. All four gonadal hormone receptors (AR, progesterone receptor [PR], ER $\alpha$ , and ER $\beta$ ) are expressed most abundantly in limbic and hypothalamic areas. These areas regulate innate reproductive behaviors, including the BNST, MPOA, MeA, and the ventrolateral nucleus of the ventromedial hypothalamus (VMHvl) (Shughrue et al., 1997; McAbee and DonCarlos, 1999; Mitra et al., 2003; Shah et al., 2004; Quadros et al., 2007; Yang et al., 2013; Mahfouz et al., 2016). All receptors but ER $\beta$  are expressed in the arcuate nucleus, which regulates homeostasis, including feeding and energy balance (Andermann and Lowell, 2017). Extensive analysis of ER $\alpha$  ER $\beta$ , and PR expression describes signaling throughout cortex and in midbrain areas, such as the VTA, substantia nigra (SNc), periaqueductal gray (PAG), and dorsal raphe (Shughrue et al., 1997; Mitra et al., 2003; Creutz and Kritzer, 2004; Quesada et al., 2007; Quadros et al., 2008; Purves-Tyson et al., 2012). Thus, sex differences in reward processing and reward-seeking behavior may be controlled by the effects of SR function in VTA- and SNc-associated dopaminergic pathways, whereas SR expression in the PAG may underlie sex differences in pain processing and analgesia.

Similarly, serotonergic projections from raphe nuclei have ramifications throughout the brain: the

widespread effects of such neuromodulation may underlie sex differences in fear and anxiety behaviors as well as stress sensitivity and the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Goel and Bale, 2009; Bangasser and Wicks, 2017). AR is also expressed in the cortex, particularly in primary visual cortex and prefrontal cortex (Nuñez et al., 2003). Cortical ER $\alpha$  expression has been reported in both deep and superficial layers, whereas ER $\beta$  is expressed more broadly throughout the cortex and has been specifically implicated in parvalbumin neuron function (Shughrue et al., 1997; Kritzer, 2002; Clemens et al., 2018). The four gonadal hormone receptors are present in the suprachiasmatic nucleus of the hypothalamus, which regulates circadian rhythm, allowing gonadal hormones to directly influence daily fluctuations in adrenal output, sleep, and mood (Kruijver et al., 2003). Finally, ER $\alpha$ , ER $\beta$  and AR are found in astrocytes and endothelial cells (Kruijver et al., 2002, 2003), and ER $\beta$  has anti-inflammatory effects in microglia (Saijo et al., 2011). The explosion of single-cell RNA-sequencing (RNA-seq) analyses will undoubtedly reveal more populations that express SRs, simultaneously detailing the cell-surface molecules, channels, neurotransmitters, and transcription factors that impart their neuronal identity.

For researchers who wish to assess the expression of these receptors in their own populations of interest, the antibodies for ER $\alpha$  and AR are of high quality and effective when used on frozen or vibratome sections. The Tollkuhn lab uses the rabbit polyclonal antibody 06-935 (EMD Millipore, Hayward, CA) at a 1:10 K dilution to stain for ER $\alpha$  and the ab52615 rabbit monoclonal antibody (Abcam, Cambridge, MA) at 1:500 to detect AR. Unfortunately, there is currently no commercially available antibody for ER $\beta$  that gives consistent and reproducible immunostaining (Nelson et al., 2017).

## Genetic Tools for Steroid Hormone Receptors

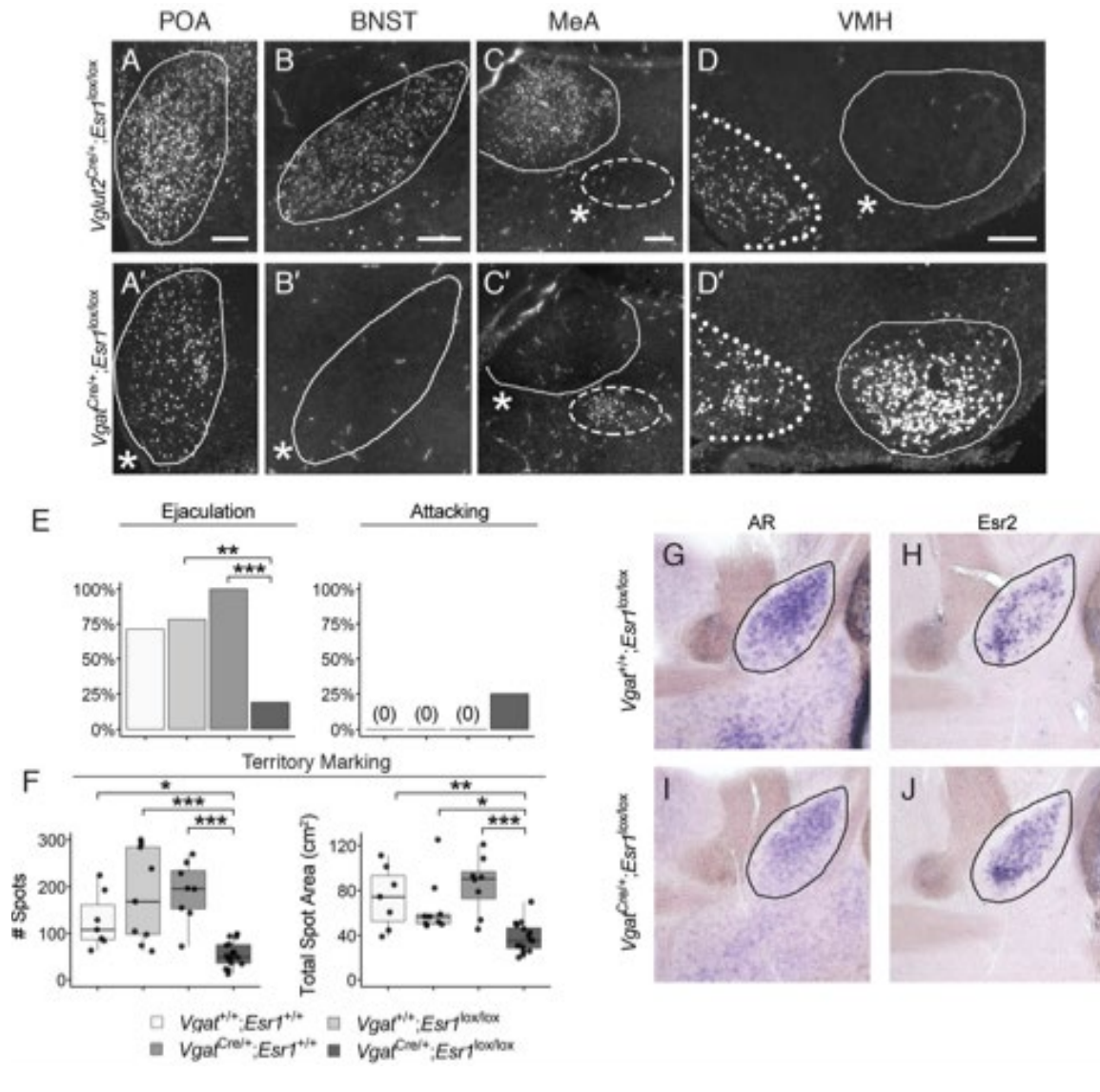
Mutant mouse alleles exist for all four SRs, making it possible to test the contribution of an individual receptor to sex differences in behavior phenotypes. Consequently, the requirements of ER $\alpha$  and AR for male-typical behaviors have been extensively characterized (Rissman et al., 1999; Juntti et al., 2008, 2010; Zuloaga et al., 2008). Male mice mutant for AR also show decreased spatial memory and increased anxiety (Zuloaga et al., 2008; Juntti et al., 2010). In contrast to ER $\alpha$ , ER $\beta$  does not appear to be necessary for the display of mating and aggression.

Rather, ER $\beta$  null males show increased levels of aggression and altered social investigation (Handa et al., 2012a). Estrogen is anxiolytic, and studies support a role for both ERs in modulating anxiety and the HPA axis (Handa et al., 2012b; Handa and Weiser, 2014). Conditional alleles for gonadal hormone receptors have been generated, thereby permitting the deletion of these receptors with a variety of cell-type-specific Cre lines. Recent studies from the Herbison and Tollkuhn labs have deleted *Esr1*, the gene for ER $\alpha$ , from either excitatory or inhibitory neurons using vGlut2-Cre and vGAT-Cre driver lines, respectively. Females lacking ER $\alpha$  in excitatory neurons show altered hypothalamic-pituitary-gonadal axis function, early puberty onset, and infertility (Cheong et al., 2015). Surprisingly, loss of *Esr1* in vGlut2-positive neurons does not affect male-typical behaviors, but deletion of *Esr1* in GABAergic neurons dysmasculinizes male sexual and territorial behaviors. The expression of *Ar* and *Esr2* (ER- $\beta$ ) is also feminized: there is less *Ar* in the BNST of mutant males and more *Esr2* (Fig. 2) (Wu and Tollkuhn, 2017).

Deleting SRs in specific classes of neurons is thus an ideal strategy for dissecting the contribution of developmental and adult hormone signaling to sex differences in multiple behavioral paradigms, including reward, stress, and addiction. There is now a vast toolkit of genetic tools available for conditional gene deletion in specific subclasses of neurons defined by the expression of specific neuropeptides, transcription factors, or other identity markers (Huang and Zeng, 2013; Daigle et al., 2018). Tamoxifen-inducible Cre drivers can be used to delete receptors either at distinct developmental time points, such as immediately before puberty, or in adulthood to test the acute requirement for a receptor after postnatal sexual differentiation of the brain has occurred.

Cre drivers have been generated for PR, ER $\alpha$ , and ER $\beta$  (Yang et al., 2013; Lee et al., 2014; Cacioppo et al., 2016; Daigle et al., 2018). These tools enable visualization of inputs and outputs of SR-expressing neurons through the use of Cre-inducible tracers. Hashikawa et al. combined anterograde Cre-dependent Synaptophysin-mCherry and retrograde CTB (cholera toxin B) to resolve two functionally distinct subdivisions of ER $\alpha$  neurons in the posterior VMHvl. The more lateral posterior VMH (VMHpvl) was activated by mating and projected strongly to the AVPV, whereas the more medial posterior VMH (VMHpvlm) neurons were active during bouts of aggression and projected to the PAG





**Figure 2.** Deletion of ER- $\alpha$  (*Esr1*) in inhibitory neurons dysmasculinizes behavior and gene expression. Representative images of ER- $\alpha$  immunostaining in postnatal day (P) 0 pups lacking *Esr1* in excitatory (**A–D**) and inhibitory (**A'–D'**) neurons. Solid lines outline the MPOA, BNST, MeApd, and VMHvl; dashed lines outline the MeApv; and dotted lines outline the arcuate. Regions with significantly decreased ER- $\alpha$  expression are denoted with an asterisk just outside the lower left corner of the outlines. ER- $\alpha$  expression is virtually absent in the MeApv and VMHvl of *Vglut2-Cre* mutants (**C,D**) and in the BNST and MeApd of *Vgat-Cre* mutants (**B',C'**). *vGAT-Cre*; *Esr1*<sup>lox/lox</sup> males show altered sexual behavior (**E**); 25% attack females in a mating assay. *vGAT-Cre*; *Esr1*<sup>lox/lox</sup> males display a feminized pattern of territorial urine marking (**F**). Compared with controls (**G,H**), *vGAT-Cre*; *Esr1*<sup>lox/lox</sup> males have decreased *Ar* (**I**) and increased *Esr2* (**J**) in the BNST. **K, L**, Average quantified pixel intensity from  $n = 4$  animals. Box plots denote median and first and third quartiles. Whiskers denote  $1.5 \times$  interquartile range;  $**p < 0.01$ ;  $***p < 0.005$ ; Fisher's  $2 \times 4$  contingency table followed by *post hoc* Fisher's  $2 \times 2$  contingency table with Bonferroni correction (**A–E**).  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.005$ ; Kruskal–Wallis omnibus test followed by Dunn's *post hoc* test for multiple comparisons with one control (**E,F**);  $*p < 0.05$ ; Mann–Whitney U test (**K,L**). Adapted with permission from Wu and Tollkuhn (2017), Figs. 1, 4, 5. Copyright 2017, Elsevier.

(Hashikawa et al., 2016). Wei and colleagues utilized the same *Esr1-Cre* driver to optogenetically induce male-typical mounting behavior and maternal pup retrieval in both female and male mice during POA stimulation (Wei et al., 2018). These functional studies show that the circuitry that regulates sex-typical behaviors is largely shared.

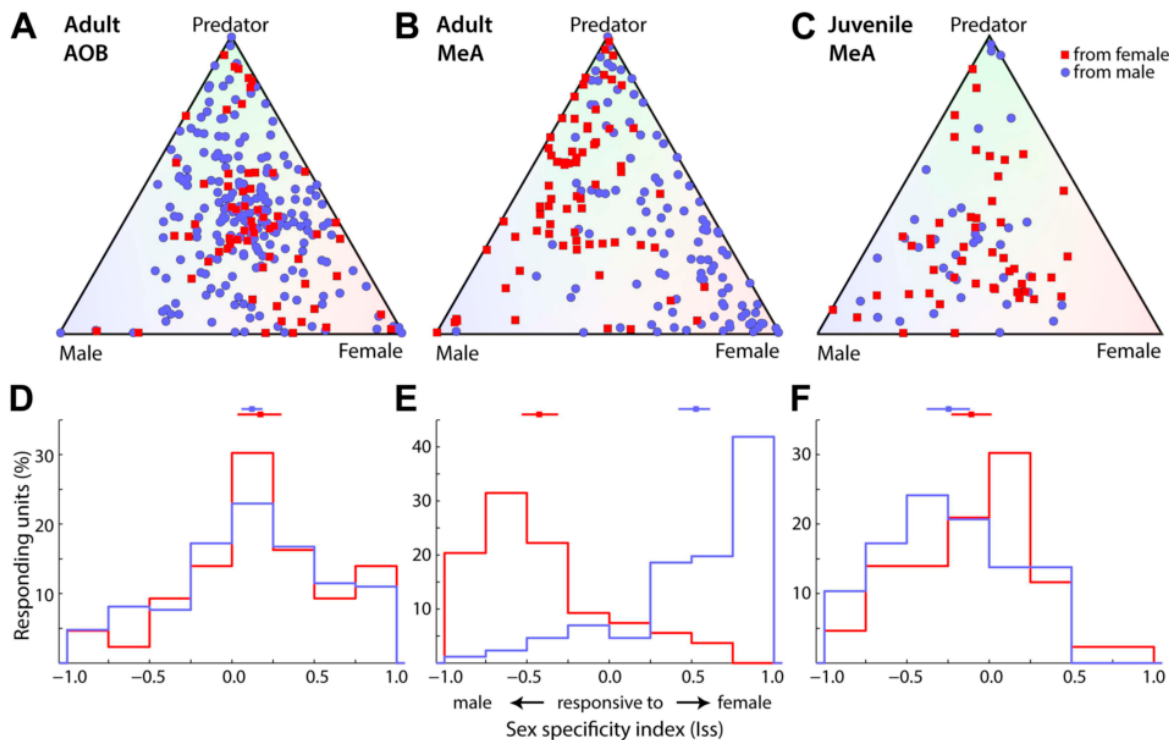
## Sex Differences in Neural Activity

True sexual dimorphism in vertebrate behavioral responses can be achieved by two strategies: quantitative differences in the number or strength of projections from one brain area to another, and sex differences in activated populations of neurons within an anatomically identical output. Both strategies

are used in female sexual behavior. The first strategy is seen in the PR/ER $\alpha$  neurons in the VMHvl, which receive inputs from the MeApd and send much stronger projections to the AVPV of females compared with males (Yang et al., 2013). Intriguingly, a parallel adjacent pathway from the posteroventral MeA (MeApv) to the dorsal VMH appears to employ the second strategy. The sex pheromone exocrine-secreted peptide 1 (ESP1) is secreted from the lacrimal glands of males and acts to facilitate sexual receptivity in females through the vomeronasal receptor V2Rp5 (Haga et al., 2010). As with other pheromones, neurons in the vomeronasal organ send projections to the accessory olfactory bulb (AOB) and then to the MeA, BNST, and cortical amygdala (Stowers and Logan, 2010). ESP1 activates distinct ensembles of neurons in the MeApv of females and males, resulting in sex-specific outputs from a common circuit: female-active neurons project to the VMHvl, and male-active neurons project to the POA. The MeApv-to-VMHvl projection promotes the display of a sexually receptive lordosis posture in females via activation of ER $\alpha$

neurons in the PAG. Importantly, the projections themselves are the same in the two sexes; the sex difference lies in the populations of cells that respond to ESP1 (Ishii et al., 2017).

The MeA appears to be the primary source of sex differences in neural processing of olfactory information. Using extracellular recordings in anesthetized animals, Bergan and coworkers detected neurons in the MeA that selectively fire in response to odors from the opposite sex (Fig. 3). This selectivity was not seen one synapse upstream in the AOB, nor was it apparent in male mice mutant for aromatase, or in juveniles (Bergan et al., 2014). More recent studies have assessed neural activity in mice engaged in innate sex-typical behaviors, using genetically encoded calcium indicators (in this case, GCaMP) with fiber photometry or gradient index lenses to visualize deep brain areas (Li and Dulac, 2018). However, few studies have performed such experiments in both sexes (Li et al., 2017; Kohl et al., 2018; Wei et al., 2018).



**Figure 3.** Sexual dimorphism of adult MeA responses. **A**, Responses of AOB neurons to vomeronasal stimuli in adult male (210 units) and female (64 units) mice. **B**, Responses of MeA neurons to vomeronasal stimuli in adult male (106 units) and female (91 units) mice. **C**, Responses of MeA neurons to vomeronasal stimuli in juvenile male (37 units) and female (50 units) mice. Units shown in panels **A–C** are classified according to the sex of the animal recorded. Blue circles, Units recorded from male mice. Red squares, Data recorded from female mice. **D–F**, Sex-specificity histograms shown for all units recorded from male (blue) and female (red) animals in the adult AOB (**D**), adult MeA (**E**), and juvenile MeA (**F**). Red and blue horizontal lines (above) indicate the mean and 95% confidence interval (bootstrap CI) for the mean for each distribution. Data collected from males versus females were different only in the adult MeA (adult AOB,  $p = 0.26$ ; adult MeA,  $p < 0.00001$ ; juvenile MeA,  $p = 0.18$ ; permutation tests). Reprinted with permission from Bergan et al. (2014), Fig. 4. Copyright 2014, The Authors.

## Summary

The results above suggest that brain wiring in females and males is largely identical outside of key populations that regulate fertility or female sexual receptivity. This work is consistent with earlier studies demonstrating that manipulation of adult testosterone levels or blocking pheromone perception can induce male-typical levels of mounting behavior in females (Baum et al., 1974; Kimchi et al., 2007; Yang and Shah, 2014). Therefore, the potential to display a given behavior is almost universal, but the ability or motivation to do so varies depending on internal state and environmental context. Future studies delineating the second- and third-order projections of hormone-responsive neurons are likely to reveal the neuronal populations that underlie diverse sex differences in physiology and behavior.

## Acknowledgments

Parts of this chapter were excerpted with permission from a previously published article: Manoli and Tollkuhn (2018) Gene regulatory mechanisms underlying sex differences in brain development and psychiatric disease. *Ann N Y Acad Sci* 1420:26–45. Copyright 2018, New York Academy of Sciences.

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