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Signatures of sex: sex differences in gene expression in the vertebrate brain

Bruno Gegenhuber,

Cold Spring Harbor Laboratory, Watson School of Biological Sciences

Jessica Tollkuhn

Cold Spring Harbor Laboratory

Abstract

Women and men differ in disease prevalence, symptoms, and progression rates for many psychiatric and neurological disorders. As more pre-clinical studies include both sexes in experimental design, an increasing number of sex differences in physiology and behavior have been reported. In the brain, sex-typical behaviors are thought to result from sex-specific patterns of neural activity in response to the same sensory stimulus or context. These differential firing patterns likely arise as a consequence of underlying anatomic or molecular sex differences. Accordingly, gene expression in the brains of females and males has been extensively investigated, with the goal of identifying biological pathways that specify or modulate sex differences in brain function. However, there is surprisingly little consensus on sex-biased genes across studies and only a handful of robust candidates have been pursued in follow-up experiments. Furthermore, it is not known how or when sex-biased gene expression originates, as few studies have been performed in the developing brain. Here we integrate molecular genetic and neural circuit perspectives to provide a conceptual framework of how sex differences in gene expression can arise in the brain. We detail mechanisms of gene regulation by steroid hormones, highlight landmark studies in rodents and humans, identify emerging themes, and offer recommendations for future research.

Graphical Abstract

gegenhu@cshl.edu.

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Two scenarios by which persistent sex differences in gene expression can arise in the developing brain: long-term changes in chromatin state lead to increased levels of transcription of a given gene, or developmental programming of cell types results in an increased number of cells expressing that gene. Without single-cell resolution, these outcomes cannot be identified.

Introduction

We wish to remind readers that “sex” refers to biological and physiological characteristics, whereas “gender” is defined by societal and cultural expectations of behaviors, roles, and preferences associated with a given sex. Although many scientific publications report “gender differences” in laboratory model organisms, this is factually incorrect, as these species do not have genders. In neuroscience research, sex difference findings are increasing, particularly in studies assessing behavior. These observations have likely been spurred by the National Institutes of Health (NIH) requirement to consider “Sex as a Biological Variable” in grant applications since 2014, which has led to increased inclusion of females. Before 2014, sex differences were mainly studied in the context of innate behaviors displayed exclusively or primarily by one sex, such as territorial aggression, reproductive behavior, and parental care (Beach 1965; Numan and Insel 2006; Balthazart, Arnold, and Adkins-Regan 2010; McCarthy et al. 2012). These behaviors are largely regulated by limbic brain areas that integrate external sensory cues released from conspecifics with internal physiological state and past experience. For example, female mice are sexually receptive to males upon priming with estradiol and progesterone, whereas male mice are not normally receptive, even when given the same hormone treatment (Blaustein 2008; Edwards and Burge, 1971). The use of molecular genetic approaches and manipulation of neural activity has facilitated the functional dissection of these pathways from olfactory input to behavioral output, (reviewed in (P. Chen and Hong 2018; Y. Li and Dulac 2018)), providing a prototype

for understanding the neural circuit basis of sex differences in behavior. However, there have been relatively fewer studies on the molecular mechanisms giving rise to sex differences in circuit function. Such differences are likely a consequence of gene expression programs that act in early life to specify neuronal identity and connectivity, as well as additional adult programs that acutely respond to physiological state or social context. Since the advent of microarray technology, there has been a persistent effort to identify sex-biased genes in the brain, with some notable successes. However, the contribution of individual sex-biased candidate genes to sex differences in behavior or human disease remains largely obscure.

In this review, we examine the molecular mechanisms that define sex differences in the brain. We aggregate findings from transcriptomic analyses comparing females and males, with a focus on understanding molecular sex differences during early life, puberty, and adulthood. We first review sexual differentiation of the rodent brain and the contributions of sex chromosomes and steroid hormones. We then describe how steroid hormones act to regulate gene expression and neural activity and where these events are known to occur in the brain. We propose that hormones exert three modes of action in the brain: direct regulation of gene expression through nuclear receptors, developmental programming of cell types, and modulation of neural activity. Finally, we assess the recent literature to identify individual sex-biased genes that have been reported in multiple publications and are therefore optimal candidates for functional investigation.

TYPES OF SEX DIFFERENCES

Before considering sex differences in gene expression, we will first discuss how sex differences fall into one of three general categories, as described in (McCarthy et al. 2012): qualitative, quantitative, and latent. Sex dimorphism, or a qualitative sex difference, describes a trait that is predominantly distinct between the sexes, such as mating behavior, or the increased size of vocal control brain areas in male songbirds (McCarthy et al. 2012). There are minimal anatomic dimorphisms between mammalian female and male brains, and these dimorphisms relate to reproductive physiology and behavior (Morris, Jordan, and Breedlove 2004; Bao and Swaab 2011; Kim et al. 2017). Sexual dimorphism in gene expression in the brain has been observed for genes on sex chromosomes (J. Xu, Burgoyne, and Arnold 2002; Armoskus et al. 2014).

Besides the reproductive organs, most sex differences in the body are quantitative, in which the distribution differs between the two sexes but largely overlaps, as is the case with height or brain volume (Maney 2016; Ritchie et al. 2018), as well as many physiological traits such as stress (Bangasser and Wicks 2017), opioid sensitivity (Averitt et al. 2019), and immune response (Klein and Flanagan 2016). What can manifest as a sex difference in a specific behavior, neural response, or gene expression may in fact originate from underlying differences in physiology. For example, progesterone receptor is expressed more highly in female hypothalamus compared to males, but this sex difference is most pronounced during phases of the estrous cycle when the level of estrogens peaks.

Finally, sex differences can be latent and only appear in response to specific contexts such as altered caloric intake, social interaction, or stress. Early life stress permanently reduces parvalbumin-positive cells in the orbitofrontal cortex of females but not males (Goodwill et

al. 2018). To date, there are few confirmed examples of individual genes exhibiting a context-specific sex bias, as it is technically and financially challenging to obtain sufficient sample sizes for multiple group comparisons (Walker, Goetz, and Gore 2014; Hodes et al. 2015; Labonté et al. 2017; Marrocco et al. 2017). However, such studies are likely to increase as sequencing costs continue to decline, and the inclusion of both sexes in experimental paradigms becomes the norm.

SEX CHROMOSOMES

Several reviews have described the contribution of sex chromosomes to the development of sex differences throughout the body (Deng et al. 2014; Hughes and Page 2015; Snell and Turner 2018) and within the brain (D. S. Hong and Reiss 2014; Grgurevic et al. 2016; Arnold 2017; Rosenfeld 2017). In vertebrates, a general theme in sex determination is *diversity* – a variety of mechanisms allow sex differences to manifest as a spectrum of behavioral and physiological traits. In the classic pathway of mammalian sex determination, males are heterogametic with one X and one Y chromosome, while females possess two X chromosomes. The testis-determining gene *Sry* resides on the Y chromosome and activates *Sox9* expression to direct differentiation of the bipotential gonad into testes. In the absence of *Sry*, the combined expression of *Wnt4*, *Rspo*, and *Foxl2* directs ovary formation (Kashimada and Koopman 2010). However, there are many exceptions to this rule (Bachtrog et al. 2014; Graves 2016). For example, in many species of reptiles, sex is determined by the temperature at which the egg is incubated (Crews 2003). *Sry* is not absolutely required for maleness. Even some mammalian species do not have a Y chromosome (Rosenfeld 2017). Within mammals, there are large species differences in gene content of the sex chromosomes. Of note, the mouse Y chromosome contains about 10 times more genes than the Y chromosome in primates, including humans (Hughes and Page 2015)).

To achieve equal expression of genes on the X chromosome between females and males, female mammals undergo X-inactivation, an epigenetic process by which transcription from one X chromosome is silenced (Lyon 1961; E. G. Schulz and Heard 2013). This process is random and occurs early in development so that every female is a mosaic of cells containing an inactive X inherited from the mother or father. Consequently, females can be buffered from the deleterious effects of mutations in X-linked genes. Many of such mutations contribute to diseases that manifest more severely in males, including many forms of X-linked mental retardation (Skuse 2005; Deng et al. 2014). However, many genes escape X-inactivation, and this escape can vary between tissues (Andergassen et al. 2017; Tukiainen et al. 2017). Quantification of allele-specific expression (ASE) shows that between 10–50% of transcripts of an escaping gene are produced from the inactive allele (Tukiainen et al. 2017). ASE also occurs on autosomes and is thought to increase gene expression diversity within an organism, as with true monoallelic expression of olfactory receptors and the T-cell receptor and immunoglobulin loci of the adaptive immune system (Keverne 2009). Initial reports of sex differences in ASE in the brain were later shown to be a statistical artifact, a common error in the early days of RNA-seq experiments (Gregg et al. 2010; DeVeale, van der Kooy, and Babak 2012; Kelsey and Bartolomei 2012). However, more recent work has highlighted the widespread occurrence of ASE in the brain, which could increase gene expression plasticity, leading to phenotypic diversity (Huang et al. 2017; Huang, Bennett, and Gregg

2018). With the emergence of brain single-cell RNA-seq datasets, improved statistical methods, and genetic tools for cell-type isolation (Sidebar 1), sex differences in autosomal gene ASE relevant to brain function may be identified.

Studying mice and humans with sex chromosome aneuploidies has provided insight into how sex chromosome dosage affects sex differences in gene expression, brain structure, and behavior (Lin et al. 2015; Raznahan et al. 2015; Arnold et al. 2016; Reardon et al. 2016; Raznahan et al. 2018). It is likely that sex differences in gene dosage partially underlie sex differences in gene expression in the brain. However, there are few, if any, examples of individual genes on the sex chromosomes that show a causal relationship to established sex differences in physiology and behavior. Likely candidates are the histone lysine demethylases *Kdm5c* and *Kdm6a*, which both escape X-inactivation, as well as *Kdm5d*, which is located on the Y chromosome. Determining whether these ubiquitously-expressed enzymes modulate the sex-specific expression of individual genes remains a central challenge in the field (Manoli and Tollkuhn 2018; Snell and Turner 2018).

STEROID HORMONE REGULATION OF SEX DIFFERENCES IN THE BRAIN

Gonadal steroid hormones direct sexual differentiation of the brain—Although sex chromosomes largely define biological sex, steroid hormones are the paramount regulators of sex differences in the vertebrate brain. Cholesterol is the precursor to all steroid hormones. Here we discuss testosterone, which is primarily produced by the testes, and progesterone and estrogens (estradiol, estriol and estrone), which are primarily synthesized in the ovaries. Steroidogenesis also occurs in the placenta, and in both sexes, the adrenal cortex and brain. Many locally-derived “neurosteroids” influence brain development and function (Do Rego et al. 2009; London 2016; Belelli et al. 2018). Classic experiments have demonstrated that manipulation of hormone levels can profoundly alter sex-typical reproductive and territorial behaviors. Crucially, the introduction of exogenous testosterone into females in early life attenuates sexual receptivity in adult females, while increasing the frequency and intensity of male-type mounting behaviors (Phoenix et al. 1959). This observation led to the Organization and Activation Hypothesis – a central tenet in neuroendocrinology stating that early exposure to steroid hormones during a critical period establishes sex differences in brain function that are activated by hormonal exposure during pubertal maturation (Arnold 2009; K. M. Schulz, Molenda-Figueira, and Sisk 2009).

In rodents, the sensitive period for sexual differentiation of the brain occurs perinatally. At birth, the testes of male mice secrete testosterone at almost pubertal levels, yet this rapid surge subsides within hours (Motelica-Heino et al. 1988; Corbier, Edwards, and Roffi 1992; Clarkson and Herbison 2016) (Figure 1A). Strikingly, in rodents, testosterone directs sexual differentiation of the brain following its conversion to estradiol, the primary endogenous estrogen. The P450 enzyme aromatase converts testosterone to estradiol in discrete populations of neurons in the brain (Lephart 1996; Naftolin, Horvath, and Balthazart 2001; Wu et al. 2009). Genetic and pharmacological manipulations have demonstrated that perinatal estradiol, rather than testosterone, masculinizes brain development (McCarthy 2008), although testosterone signaling via androgen receptors is required in adulthood to

produce male-typical levels of mating and territorial behaviors (Wu et al. 2009; Juntti et al. 2010).

Testosterone is the principal regulator of sexual differentiation of the primate brain. The testes become active around week 7 of human gestation and secrete testosterone until around week 24, after which they become largely quiescent until birth (Figure 1B). Similar to rodents, there is no corresponding hormonal activity of the ovaries at this time, although fetuses of both sexes are exposed to estrogens and progesterone through the maternal circulation. Testosterone levels rise again during infancy then drop until the onset of puberty. Behavioral studies in children demonstrate an association between mid-gestation testosterone level and male-typical toy preferences and social behaviors (Hines 2006; Hines et al. 2016). Both testosterone surges coincide with major developmental events in the brain: the prolonged fetal bolus overlaps with the peak of neurogenesis and the onset of neuronal migration, whereas the secondary infant testosterone surge occurs during intense synaptogenesis and myelination (Figure 1B). How testosterone intersects with these events to direct male-typical brain development remains an open question. It is clear, however, that testosterone and other steroid hormones modulate gene expression, suggesting hormone-responsive transcriptional programs are involved in defining sex differences throughout the brain.

Steroid hormone receptor expression in the brain—To understand how sex differences in gene expression arise, it is essential to know which brain regions and cell types express hormone receptors. Steroid hormone receptors are nuclear transcription factors that directly bind DNA to regulate gene expression: progesterone binds to progesterone receptor (PR/*Pgr*), testosterone to androgen receptor (AR/*Ar*) and estrogens to estrogen receptors alpha and beta (ER α /*Esr1*, ER β /*Esr2*). Steroid hormones in the circulation pass through cell membranes to bind their receptors, which then undergo conformational changes to enable associations with transcriptional machinery and DNA and subsequently affect gene expression. Differential hormone levels can therefore lead to sex differences in gene regulation. Importantly, while analysis of postmortem human brain tissue has revealed the expression of hormone receptors and aromatase in adulthood, it is still not known when and where these factors initiate gene expression during brain development. The BrainSpan database reports almost undetectable levels (RPKM <1) for the four gonadal hormone receptors, which is likely due to the low transcript levels of these genes in the brain as well as their sparse expression pattern.

In contrast, hormone receptor expression has been well-characterized in the developing and adult rodent brain (reviewed in (Stincic, Rønnekleiv, and Kelly 2018; Manoli and Tollkuhn 2018)). In vertebrates, all four receptors are highly expressed in limbic brain regions such as the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), medial pre-optic area of the hypothalamus (MPOA) and the ventromedial hypothalamus (VMH) (Figure 2). These regions are core components of a circuit that responds to chemosensory cues from conspecifics to regulate innate reproductive and territorial behaviors and is often referred to as the vomeronasal network or social behavior network (Newman 1999; O'Connell and Hofmann 2011; Y. Li and Dulac 2018). These receptors are also found in the hippocampus and areas that mediate motivated behaviors (Manoli and Tollkuhn 2018), as well as specific

cortical layers and regions. While ER α expression is most abundant in the hypothalamus, it appears that ER β is the primary estrogen receptor in cortex (Shughrue, Lane, and Merchenthaler 1997; Kritzer 2002; González et al. 2007; Clemens et al. 2019). ER β mutant animals have deficits in cortical development consistent with a role for ER β in cell survival and migration, potentially in response to maternal estrogens (L. Wang et al. 2003; Fan et al. 2010; Panda et al. 2018).

How do the sexes differ in hormone receptor-expressing neurons? As with sex differences in behavior, these receptors are best-characterized in the context of the social behavior circuit. Females express higher levels of ER α and ER β in the MeA, posterior BNST (BNSTp), MPOA and ventrolateral VMH (VMHvl), while males possess more *Ar*⁺ neurons in the BNSTp and MPOA. PR is higher in the female VMHvl and the male BNSTp (Figure 2). In the VMHvl, nearly 100% of *Esr1*⁺ cells express PR (Hashikawa et al. 2018). Exposure to perinatal estradiol is necessary and sufficient for these molecular dimorphisms, which persist into adulthood. Intriguingly, the sex-biased populations in the BNST and posterodorsal MeA (MeApd) are almost entirely GABAergic inhibitory neurons, many of which extend long-range projections (Canteras, Simerly, and Swanson 1995; Dong, Petrovich, and Swanson 2001; Dong and Swanson 2004; Gu, Cornea, and Simerly 2003; Wu and Tollkuhn 2017). For example, the male AVPV receives more GABAergic innervation from the BNST compared to the female AVPV (Hutton, Gu, and Simerly 1998; Polston, Gu, and Simerly 2004). There are strong reciprocal connections between the MeApd, BNSTp and MPOA. Stimulation of inhibitory *Vgat*⁺ neurons in the MeApd is sufficient to drive aggressive behavior in males through downstream excitatory *Esr1*⁺ neurons in the VMHvl (W. Hong, Kim, and Anderson 2014). *Esr1*⁺ neurons in the female VMHvl also control aggression as well as both sexual receptivity and mounting (Hashikawa et al. 2017; Hashikawa et al. 2018). Male-evoked neuronal activity in this region increases during estrous, allowing females to tune behavioral response according to hormonal status (Nomoto and Lima, 2015). Many neurons in this circuit express the LIM homeobox protein LHX6, which also specifies medial ganglionic eminence (MGE)-derived cortical inhibitory neurons (Zhao et al. 2008; Flandin et al. 2011; Vogt et al. 2014). The development and sexual differentiation of this circuit remains an active area of investigation (Tobet 2002; Garcia-Lopez et al 2008; Sokolowski and Corbin 2012; Abellán, Desfilis, and Medina 2013; Puelles, Paxinos, and Watson 2013; Huilgol and Tole 2016; Studer et al. 2017; Turano, Osborne, and Schwarz 2018). However, there is still little known about the identity and function of hormone-responsive neurons in other brain regions, such as cortex. In the next section, we review known mechanisms by which nuclear hormone receptors regulate gene expression and how they may apply to the brain.

GENE REGULATION BY STEROID HORMONE RECEPTORS

The genomic targets of steroid hormone receptors in brain have yet to be determined, due to the technical challenges of working with sparse populations in heterogeneous brain tissue. Therefore our understanding of the mechanisms utilized by these receptors derives from human cancer cell lines and peripheral mouse tissues, such as liver and uterus, from which sufficient material can be collected for chromatin immunoprecipitation sequencing (ChIP-seq) (Métivier et al. 2003; Carroll et al. 2005; Hurtado et al. 2011; Hewitt et al. 2012; Gertz

et al. 2013; Gordon et al. 2014; Droog, Mensink, and Zwart 2016; Palierne et al. 2016; Swinstead et al. 2016; Holding, Cullen, and Markowitz 2018). In this review, we focus on gene regulation by ER α , as this receptor has been studied extensively in multiple biological contexts and is considered the master regulator of sexual differentiation of the rodent brain.

Receptor activation and site-selection—Nuclear hormone receptors are activated by hormone binding to their ligand-binding domain (LBD), which facilitates receptor dimerization, tethering to other transcription factors, and direct DNA binding (Brzozowski et al. 1997; Maggi 2011). Extracellular molecules, such as growth factors, cytokines, and peptide hormones, can also activate these receptors through ligand-independent signaling pathways (Benesch and Picard 2015). For instance, dopamine has been shown to activate ER α in cell culture models (Power et al. 1991; Gangolli, Conneely, and O'Malley 1997). Typically, these signals activate intracellular kinases, such as ERK, AKT, or PKA, which then phosphorylate serine or tyrosine residues in the ER α activation function 1 (AF1) domain to release the receptor from its inhibitory complex with heat shock protein 90 (HSP90).

Following ligand-binding, steroid hormone receptors associate with coactivators and core transcriptional machinery and bind directly to specific sequence motifs at gene regulatory elements – ERs bind the estrogen response element (ERE) and AR binds the androgen response element (ARE) (Figure 3A). Historically, investigators wishing to identify hormone-responsive genes have performed bioinformatic searches for EREs/AREs in the vicinity of gene promoters. However, we now know from genome-wide approaches that less than 10% of receptor binding sites in the genome are found at promoters and that receptors are frequently recruited to DNA by other cell-type-specific transcription factors that associate with their own canonical binding motifs (Figure 3A). Specificity protein 1 (SP1) and activator protein-1 (AP-1), which consists of a FOS/JUN heterodimer, can also tether nuclear hormone receptors to the genome (Safe and Kim 2008). Since *Fos* is rapidly transcribed in response to neuronal firing, it is possible that neural activity influences estradiol regulation of gene expression. Hormone receptors may also regulate transcription independent of direct DNA binding by controlling the phosphorylation of cAMP binding protein (CREB) – a factor that regulates *Fos* and other genes in response to neural activity (Shaywitz and Greenberg 1999; Fix et al. 2004; Boulware et al. 2005; Nguyen, Yao, and Pike 2009). In primary hippocampal culture and striatal neurons, ER α localized to the postsynaptic membrane signals through group 1 or 2 metabotropic glutamate receptors (mGluRs) to promote or inhibit CREB phosphorylation, respectively (Figure 3B) (Boulware et al. 2005; Grove-Strawser, Boulware, and Mermelstein 2010).

Hormone receptor binding is determined by chromatin state—Binding site selection depends on chromatin accessibility, which is established by cell-fate-specifying transcription factors throughout development. One class of such factors, known as pioneer factors (PFs), bind and open chromatin, allowing other transcription factors, including hormone receptors, to associate with DNA (Zaret and Carroll 2011). Numerous PFs belonging to the forkhead box (FOX), GATA, and homeobox (HOX) families have been identified for ER α . For instance, FOXA1 has been shown to regulate ER α binding in human

breast cancer (Hurtado et al. 2011; Gertz et al. 2013), endometrial cancer (Droog et al. 2016), and liver cancer (Z. Li et al. 2012). The FOXA1 motif is enriched in mouse aorta and liver ER α binding sites, although to a lesser degree than in breast cancer due to lower expression (Gordon et al. 2014; Palierne et al. 2016). In mouse uterus, homeobox (HOX) transcription factor motifs are enriched in ER α sites (Hewitt et al. 2012), and in osteoblasts, the GATA4 motif is enriched (Miranda-Carboni et al. 2011). Because the cell-type-specific expression of PFs often dictates the cell-type-specific binding and gene targets of hormone receptors, it is possible that brain-specific or neuron type-specific PFs control hormone response element accessibility and thereby determine the transcriptional and epigenetic programs elicited by the perinatal hormone surge (Gertz et al. 2013; Droog, Mensink, and Zwart 2016). Depending on the co-expression of ER α PFs, developmental programming by estradiol could affect the expression of different combinations of genes in individual cells across sex.

Gene expression levels are determined not just by the presence of the requisite transcription factors and coregulators but by epigenomic processes such as DNA methylation, post-translational modifications to histones, and non-coding RNAs. It has been proposed that perinatal estradiol directs sexual differentiation of the rodent brain by irreversibly modifying chromatin to produce either a constitutive sex difference in gene expression or a sex-specific transcriptional response to a later signaling cue (McCarthy et al. 2009; Bale et al. 2010; Dulac 2010; Gagnidze, Weil, and Pfaff 2010; Anthony P. Auger, Jessen, and Edelman 2011; Nugent and McCarthy 2011) (Figure 4A). These persistent sex differences in gene expression could then give rise to sex differences in circuit wiring and associated behavioral responses. However, to date there are few examples of sex differences in the epigenome at specific loci, even near genes that have sex-biased expression from early life to adulthood. Reports of sex differences in DNA methylation in the brain have assessed either individual methylated cytosines at promoters (Kurian, Olesen, and Auger 2010; Schwarz, Nugent, and McCarthy 2010; Westberry, Trout, and Wilson 2010) or reported percent methylation across the entire genome (Ghahramani et al. 2014; Nugent et al. 2015). Genome-wide analysis of DNA methylation and histone modifications at multiple developmental time points combined with an understanding of the perinatal brain transcriptome will likely reveal regulatory strategies that define sex differences in gene expression.

ESTROGENS REGULATES CELL SURVIVAL TO DEFINE SEXUALLY DIMORPHIC BRAIN AREAS

Although there are few examples of direct gene targets of hormone receptors in the brain, there is ample evidence that perinatal estradiol programs sex differences in the brain by promoting cell survival or cell death in the first week of life (Figure 4B) (Nancy G. Forger, Strahan, and Castillo-Ruiz 2016). Consequently, four regions of the rodent brain are consistently described as truly sexually dimorphic, containing more cells in one sex, and they are all implicated in reproductive behaviors. The AVPV, MPOA, BNSTp, and MeApd (Figure 4) all possess more cells, occupy a larger regional volume in one sex than the other, and exhibit sex differences in neural projection patterns. The AVPV regulates the release of luteinizing hormone (LH) from the pituitary and subsequent ovulation in females and has more cells in females compared to males. Many of these neurons express tyrosine

hydroxylase (TH), an enzyme that is necessary for dopamine synthesis, and TH(+) neuron number is higher in mothers than in virgin females or males (Simerly et al. 1985; Scott et al. 2015). Conversely, the MPOA is larger in males, and male rats in particular have a pronounced sexually dimorphic nucleus (SDN) of the POA – the first sexually dimorphic brain region to be identified in mammals (Gorski et al. 1978). The MeApd and BNSTp are also larger in males (Hines, Allen, and Gorski 1992; Raznahan et al. 2015), and a sex difference in BNSTp volume has been described in humans (Allen and Gorski 1990). A post-mortem study of cisgender men, cisgender women, and transgender women found that BNSTp volume is similar in cisgender and transgender women, which is approximately 50% of the volume in cisgender men (Zhou et al. 1995). BNSTp size was not influenced by adult hormone status or sexual preference. Although this study was performed with very small sample sizes (42 total subjects), this result is consistent with core gender identity arising in early life (Hines 2010; Bao and Swaab 2011) as a consequence of developmental hormone signaling.

The role of steroid hormones in promoting postnatal neuronal survival was first observed in the spinal nucleus of the bulbocavernosus (SNB), which innervates penile muscles and contains more motor neurons in males (N. G. Forger et al. 1992). Females typically lose up to 70% of SNB motor neurons, and females treated with testosterone during the perinatal period showed decreased cell death and male-typical cell numbers at P10 (Nordeen et al. 1985). The BNSTp, MPOA and MeApd all contain increased numbers of dying cells in postnatal females compared to males, and perinatal estradiol treatment masculinizes cell number in these regions (Wu et al. 2009). Abolishing programmed cell death by genetic deletion of the pro-apoptotic gene *Bax* leads to similar cell numbers in the BNSTp of females and males (Forger et al. 2004). Some of these cells express aromatase, as adult females have fewer aromatase-expressing neurons in the BNSTp than males (Wu et al. 2009). In males, the BNSTp aromatase-expressing cell population controls mating, aggression, and sexual preference, whereas in females, it is not involved in mating or maternal aggression (Bayless et al. 2019). Conversely, in the AVPV, there is increased cell death in males, accompanied by decreased expression of the cell survival signal *Bcl-2* (Tsukahara, Kakeyama, and Toyofuku 2006), suggesting the existence of region-specific responses to perinatal estradiol signaling (Krishnan et al. 2009). Intriguingly, similar patterns of sex-specific cell death are seen in the developing brains of female *Drosophila* and hermaphroditic *C. elegans*, implying conserved mechanisms for generating sex-typical neural circuitry across certain branches of the animal kingdom (Conradt and Horvitz 1999; Kimura et al. 2005; Peden et al. 2007; Schwartz and Horvitz 2007; Sanders and Arbeitman 2008).

What is the cell identity of the surviving neurons that give rise to sexual dimorphism in the mammalian brain? The Dulac lab recently made unprecedented progress toward understanding the cellular repertoire of the POA. By combining scRNA-seq with multiplexed error-robust fluorescent in situ hybridization (MERFISH), Moffit, Bambah-Mukku and colleagues created a molecular and functional cell atlas of the POA (Moffitt et al. 2018). They obtained gene expression information for ~31,000 POA cells and integrated this information with spatial distribution of 155 marker genes in ~1 million POA cells to identify 70 neuronal populations. The authors went on to co-label with *Fos* to determine

which of these populations were active during mating, aggression, and parenting. One aromatase-expressing cluster of inhibitory neurons was enriched in males and was activated in males, but not females, during all three behaviors. This cluster is also marked by the expression of *Moxd1* (Tsuneoka et al. 2017) and *Cplx3*, a complexin family member that can regulate phasic exocytosis of neurotransmitters (Mortensen et al. 2016). Taken together, these results point strongly to the conclusion that the SDN-POA is defined by the coexpression of these two markers with hormone receptors and aromatase. It will be interesting to determine when the expression of *Cplx3/Moxd1* arises during development and whether neurons expressing these markers play a causal role in male-typical behaviors. This work exemplifies how sex differences in gene expression in the adult brain can originate from the survival or death of distinct classes of neurons (Figure 4B). It is clear that the most robust examples of sex differences in gene expression are male-biased genes in the rodent BNST and POA, regions where there are more cells in males compared to females.

THE MATURATION OF SEX DIFFERENCES IN THE BRAIN DURING PUBERTY AND ADOLESCENCE

Puberty is considered a second critical period for sexual differentiation of the brain, in which developmentally-programmed circuits undergo additional refinement and maturation (K. M. Schulz and Sisk 2016). Puberty refers to hormonal changes required for reproduction, while adolescence in humans is defined as the maturation of social and cognitive skills that can occur independently of gonadal hormones. Although many sex differences are established during the postnatal sensitive period, behaviors regulated by sexually dimorphic circuitry (mating, aggression, social preference, parental care) are not displayed until after puberty occurs and adult patterns of circulating hormones are established. Accordingly, sex-specific neuronal firing in the MeA in response to opposite-sex odors does not occur in pre-pubertal animals (Bergan, Ben-Shaul, and Dulac 2014).

During adolescence, the brain undergoes significant synaptic pruning which extends into the twenties in humans and P35–45 in rodents (Figure 1). Connectivity within the mesolimbic reward pathway continues to develop and is modulated by hormone signaling (reviewed in (Walker et al. 2017; Delevich, Thomas, and Wilbrecht 2019)). This pathway intersects with social behavior circuitry to modulate sex differences in motivated behaviors such as parenting, sexual behavior, threat avoidance, and intake of drugs of abuse (Becker 2009; O'Connell and Hofmann 2011; Gee et al. 2018). During this time, the brain is uniquely vulnerable to stress, and many psychiatric diseases first show symptoms (Paus, Keshavan, and Giedd 2008; Kessler et al. 2007). Early puberty can exacerbate the onset and symptoms of these conditions. Indeed, there are sex differences in the trajectory of puberty itself, with puberty onset occurring first in girls and at increasingly earlier ages in recent years (Sisk and Foster 2004; Piekarski et al. 2017).

It is thought that pubertal hormones end the plasticity of the juvenile brain, locking-in learned skills and impeding the ability to acquire new ones. Songbird research has demonstrated a direct relationship between testosterone level and plasticity associated with vocal learning. Many male songbirds acquire their unique song during a juvenile critical period in which both sensory experience and sensorimotor learning must occur (Brainard

and Doupe 2002). Similar to the role of perinatal estradiol in rodent territorial behaviors, testosterone in early life is required for the appropriate onset of singing, whereas high levels of pubertal testosterone are required to crystallize song structure into a permanent stereotyped adult song. Between these two developmental windows, a low level of gonadal hormones is required for song learning. Exogenous administration of testosterone during this quiescent period impairs song learning, resulting in abnormal songs with altered syntax and decreased complexity (Korsia and Bottjer 1991; Whaling, Nelson, and Marler 1995). Electrophysiological data demonstrate that early testosterone accelerates the maturation of neuronal firing patterns in song-related brain areas (White, Livingston, and Mooney 1999; Sizemore and Perkel 2011). Taken together, this work suggests pubertal hormones decrease plasticity, and early puberty may lead to premature closure of developmental critical periods.

In support of this idea, Piekarski and colleagues found that inhibition onto excitatory neurons in the cingulate cortex in female mice increases after puberty (Piekarski, Boivin, and Wilbrecht 2017). This increased inhibition is associated with a decrease in behavioral flexibility, as pre-pubertal animals show better performance on a reversal learning task compared to post-pubertal animals. Early puberty induction by treatment with ovarian hormones results in adult-like task performance. The parallel conclusions drawn from male songbirds and female mice point strongly to a conserved role for pubertal hormones in establishing adult-typical patterns of neural activity.

What are the gene expression programs that drive neuronal maturation during puberty? Do the genomic targets of gonadal hormones differ between birth and puberty? These questions remain open. Although there has been extensive investigation into both behavioral transitions and anatomical correlates of cortical maturation during puberty (reviewed in (Gee et al. 2018)), there are comparatively few studies on the dynamic changes in gene expression that must underlie these events. Walker et al. assessed gene expression in the POA and medial-basal hypothalamus from birth to adulthood and identified *Tac2* as a novel female-biased gene in the MBH, beginning pre-pubertally at P15. Shi et al. extensively analyzed the BrainSpan database, which includes up to 15 brain regions staged from 8pcw to 40 years of age (Shi, Zhang, and Su 2016). They found that although sex differences first arose during the second trimester, when the fetal testosterone surge occurs, most sex differences in gene expression were detected during puberty (8–19 years). However, the results from this study were highly variable due to low sample size, and only 3 sex-biased autosomal genes showed a consistent bias across developmental stages. Transcriptomic analyses of the maturing mesolimbic circuitry in rodents would reveal candidate factors that can alter neuronal firing or synaptic maturation, such as cell adhesion molecules, ion channels, and vesicle release machinery (Paul et al. 2017; Favuzzi et al. 2019). In the next section, we will discuss neural activity itself as a potential driver of sex differences in gene expression.

CROSSTALK BETWEEN HORMONE SIGNALING AND NEURAL ACTIVITY

Neuroscientists have traditionally separated the effects of estrogens into “genomic” and “non-genomic”. In this framework, genomic responses occur over hours to days via transcriptional regulation by estrogen receptors, while non-genomic effects are rapid signaling cascades initiated at the cell membrane, resulting in increased neuronal firing and

intrinsic excitability within minutes (Woolley 2007; Micevych and Dominguez 2009; Heimovics, Trainor, and Soma 2015; Stincic, Rønnekleiv, and Kelly 2018). However, as mentioned above, estradiol signalling can cause CREB phosphorylation, which itself can potentiate cascades of gene expression (Figure 3). In the brain, CREB phosphorylation typically occurs in response to neural activity. Neurotransmitters or depolarization cause calcium entry into the cell or release from intracellular stores, resulting in phosphorylation of CREB and other activity-regulated transcription factors. These factors rapidly induce the expression of immediate early genes (IEGs) such as *Fos*, *FosB*, *Egr1*, and *Npas4* within minutes (West, Griffith, and Greenberg 2002; Bloodgood et al. 2013). Conventionally used as histological markers for neural activity, these IEGs are themselves transcription factors and activate unique repertoires of late response genes (LRGs) in distinct cell types (Spiegel et al. 2014; Hrvatin et al. 2018; Tyssowski et al. 2018). Thus, it is likely that estrogens can induce IEG and LRG expression profiles that are entirely distinct from genes regulated by ER α /ER β .

Several lines of evidence suggest a link between steroid hormone levels and dynamic, activity-responsive gene programs. At the peak of the perinatal hormone surge, male rats have higher hypothalamic CREB phosphorylation than female rats (Auger, Hexter, and McCarthy 2001; Pérez, Chen, and Mufson 2003). Subsequently, on P1, males have elevated immunostaining for Fos, and CREB-binding protein (CBP), a cofactor that can bind CREB and hormone receptors, in VMH, MPOA, and the arcuate nucleus (Murphy and Segal 1997; Auger et al. 2002; Olesen and Auger 2005). Acute treatment with estradiol causes CREB phosphorylation in the brain within 30–60 min (G. Gu et al. 1996; Abrahám and Herbison 2005), and estradiol induces neurotensin (*Nts*) expression through CREB (Watters and Dorsa 1998). Accordingly, activity-dependent gene expression in females is likely to vary throughout the estrous cycle (Figure 5A). The rodent estrous cycle is short (4–5 days), with only 2 days separating the high-estrogen and low-estrogen time points. Duclot and Kabbaj compared the transcriptome of the rat mPFC in high-estrogen proestrus females, low-estrogen diestrus females, and males. Overall, the transcriptome of low-estrogen, diestrus females is more similar to that of males, and proestrus females show increased expression of the IEG *Egr1* and the LRG *Bdnf* (Duclot and Kabbaj 2015). *Bdnf* is also induced by estradiol in rat olfactory bulb and cortex (Sohrabji, Miranda, and Toran-Allerand 1995) and in the high vocal center (HVC) of juvenile male zebra finches (Dittrich et al. 1999).

These molecular findings are consistent with recent reports of steroid hormone modulation of neural activity throughout the estrous cycle. In female mice, progesterone acts through the membrane receptor PGRMC1 to silence a subset of neurons in the VNO, thereby blocking responses to male-specific major urinary proteins (MUPs) in diestrus (Dey et al. 2015). In the POA, estradiol increases activity of VTA-projecting *Nts*⁺ neurons to enhance the reward value of male odors (McHenry et al. 2017), and estradiol acts through ER β to stimulate parvalbumin neuron firing in the somatosensory cortex (Clemens et al. 2019). Estradiol also stimulates dopamine release (Becker and Chartoff 2019), which enhances VTA firing and cue-reward associations, suggesting that estrous primes the reward system to respond more effectively to salient stimuli (Calipari et al. 2017). Finally, increased female hormones during pregnancy are also thought to transcriptionally prime IEGs in the POA to facilitate the onset of maternal behavior in response to pup stimuli (Sheehan and Numan 2002; D. S.

Stolzenberg and Rissman 2011; Stolzenberg, Stevens, and Rissman 2014; Mayer et al. 2018).

Propagation of activity through a neural circuit could also cause changes in neural activity downstream of hormone-responsive brain areas (Figure 5B). This mechanism could account for sex differences in gene expression in regions that have minimal expression of steroid hormone receptors, such as the striatum and thalamus (Shughrue, Lane, and Merchenthaler 1997; Walker et al. 2018). Sex differences in activity-regulated gene programs could also arise as a consequence of distinct recruitment of neural ensembles by a sensory stimulus in females and males. In the posteroventral MeA, the pheromone ESP1 activates distinct populations of neurons in females and males. ESP1 is produced in the male lacrimal glands and facilitates both sexual receptivity in females and aggression in males. ESP1-responding neurons in the female MeApv project to the VMH, but ESP1-activated neurons in males project primarily to the POA (Ishii et al. 2017). Consequently, these two target regions would likely display sex differences in activity-dependent gene programs in response to a social stimulus (Figure 5C). In summary, sex differences in gene expression can be both a cause and a consequence of sex differences in neural activity.

EMERGING THEMES: INTEGRATING FINDINGS ACROSS THE FIELD

In this section, we will discuss a few recent publications that provide significant insight into the biology of sex differences in gene expression. Table 1 lists the brain regions studied and key genes identified in these papers, along with additional examples in which transcriptomic screens for sex differences were performed. We apologize for any omissions, as this table is not exhaustive but is meant to provide an overview of trends in the field, such as recent work in microglia. One recurring theme is that many papers primarily report enrichment of Gene Ontology (GO) terms rather than identifying individual statistically-significant candidate genes for future study. Furthermore, many of these GO terms are quite broad – “synapse”, “ion channel”, or even “cancer” – and do not provide much biological insight. The papers discussed below were selected because the authors’ design of their RNA-seq screen, or of follow-up experiments, revealed specific causal factors for future study. We recommend similar approaches to researchers wishing to investigate sex differences in their own model system.

Despite the number of papers using RNA-seq to identify sex-biased gene expression, the most fruitful screen thus far utilized cDNA microarrays. Xu, Coats, and colleagues employed both a well-designed experimental strategy and exhaustive histological validation of candidate genes to reveal 15 novel sex-biased genes (Table 1), most of which are more abundant in males. Although this number is well below that of differentially-expressed genes reported in other studies, to date, these 15 are the only genes identified in a screen for sex differences that were confirmed by *in situ* hybridization. 11 of these genes were recently replicated by a second group (Moffitt et al. 2018). Histological validation reveals regional specificity of gene expression that cannot be resolved by RT-qPCR interrogation of bulk dissections or tissue punches (Figure 6). In addition to careful analysis of the expression pattern of each sex-biased gene, Xu *et al.* obtained knockout lines for four of their genes to determine their necessity for sex-typical mating, territorial or parenting behavior. They

found that although these behaviors were largely normal, each gene contributed to a specific component of the behavior. Although the precise cellular roles of these genes in neuronal function have yet to be determined, this work stands out for its comprehensive approach that has rarely been emulated.

More recently, Labonté and colleagues carried out similar behavior validation of genes identified in a transcriptomic screen by viral overexpression or knockdown of candidates associated with sex-specific behavior phenotypes. They assessed gene expression in the brains of humans with major depressive disorder (MDD), a condition that affects a greater proportion of women than men and with more severity. They then compared their findings in humans to those from mice who had experienced chronic variable stress, a paradigm that produces depression-like behaviors. Labonté et al. assessed 6 brain areas from 48 human subjects as well as nucleus accumbens and ventromedial prefrontal cortex (vmPFC) from 40 mice. Comparison of depressed/stressed and control males and females revealed *Dusp6*, an ERK 1/2 phosphatase, as downregulated in the vmPFC of depressed women and stressed female mice but not in depressed/stressed males. Viral knockdown of *Dusp6* using RNAi increased ERK phosphorylation as well as excitability of a subset of vmPFC neurons. Although the female-specific requirement for *Dusp6* is not yet understood, this study stands out as an example of how specific sex-biased signaling pathways can be identified with a well-powered study design. Labonté et al. sequenced a relatively large number of individuals (9–12 biological replicates per experimental group) and used separate human tissue samples for candidate gene validation.

Another avenue to identifying robust sex differences is to look directly at hormone-induced genes. The acute transcriptional response to estrogens has been extensively characterized in non-neural tissues and is more robust than subtle baseline differences between the sexes (Carroll et al. 2006; Hewitt et al. 2012; Gertz et al. 2013; Palierne et al. 2016). An elegant 2017 study addressed the role of ERE-dependent and -independent effects of estradiol through the use of multiple genetic models (Yang et al. 2017). The ER α knock-in/knock-out (KIKO) mouse possesses two point mutations in the DNA-binding domain that dramatically reduce binding to EREs (Hewitt et al. 2014). However, KIKO ER α can still bind other hormone response elements and can activate additional transcriptional programs through tethering by other factors (Figure 3). Yang et al. assessed gene induction by estradiol in the arcuate nucleus of wild-type, KIKO and complete ER α KO animals. In addition to recapitulating known estradiol-induced genes such as *Cckar*, Yang et al. identified many additional targets and report mitochondrial and cellular respiration genes as uniquely induced by estradiol in the absence of ER α . Although this work was done exclusively in females, it is so far the most extensive analysis of estradiol regulation of gene expression in the brain. The genes identified are likely to be estrogen-regulated in other brain areas, potentially with distinct expression dynamics in the two sexes.

In addition to histological validation within a single study, reproducible expression across studies can also be used to discern *bona fide* sex-biased genes. We compiled multiple gene lists to determine if there were common sex-biased or estrogen-regulated genes identified in different laboratories (Figure 7). Intriguingly, *Moxd1*, a gene expressed more in male mouse BNST, POA and MeA (Tsuneoka et al. 2017; Moffitt et al. 2018), was also called as male-

biased in human anterior insula in the Labonté paper. *Tacr3* and *Cartpt* have been reported as both sex-biased and estrogen-regulated genes, while *Dusp6* shows a sex-bias and is also induced by neural activity. The human *Dusp6* gene is located within a locus that has recently been associated with attention deficit hyperactivity disorder (ADHD), a male-biased disorder (Biederman et al. 2002; Nussbaum 2012; Demontis et al. 2019).

THE BRAIN HETEROGENEITY CHALLENGE

Single cell RNA-seq (scRNA-seq) has revealed a high degree of transcriptional heterogeneity across cells in the mouse brain (Paul et al. 2017; Tasic et al. 2018; Moffitt et al. 2018). 117 and 65 transcriptomic neuron types have been described in the cortex and hypothalamic preoptic area, respectively (Tasic et al. 2018; Moffitt et al. 2018). Given this heterogeneity, it is now understood that RNA-seq of bulk brain tissue masks transcriptional changes within small populations of cells and fails to reveal differences in cell number. These issues are especially relevant to understanding sex differences, as males and females can differ in gene expression within sparse cell populations *and* in cell number. Without single-cell profiling, it is difficult to determine whether a gene is sex-biased due to increased expression in a specific class of neurons or a greater number of neurons expressing that gene (see Figure 4). Examples of this can be observed in the MPOA and dorsal raphe, where female mice contain more VIP neurons than male mice (Kim et al. 2017) and in the orbitofrontal cortex, where early postnatal stress reduces PV cell density in females but not males (Goodwill et al. 2018). Bulk RNA-seq of these regions would reveal a sex difference in *Vip* or *Pv* expression rather than a sex difference in cell number.

To detect sex differences in gene expression, it is important to enrich for cells that are expected to differ transcriptionally between males and females. Cells that express steroid hormone receptors or receive synaptic input from hormone receptor-expressing cells are most likely to differ between males and females and are therefore a logical starting point for studying sex differences in the brain (Figures 2,5). To isolate RNA specifically from steroid hormone receptor-expressing cells, the “translating ribosome affinity purification”, or TRAP, method may be applied (see Sidebar 1). In brief, TRAP uses a tagged ribosomal subunit allele to immuno-purify translating mRNA in a *Cre*-dependent manner. Using the TRAP method, two recent publications have identified major sex differences in gene expression in specific neuron populations. The first study used TRAP to profile gene expression in oxytocin receptor interneurons (OxtrINs) within the medial prefrontal cortex (mPFC) of male and female mice (K. Li, Nakajima, and Heintz 2016). Although males and females express similar levels of corticotropin-releasing hormone binding protein (*Crhbp*), which plays a critical role in the sex-specific behavioral response to oxytocin, 514 other autosomal genes are differentially expressed in OxtrINs between the sexes. Among these are several genes that have been implicated in sex-specific behaviors. For example, *Rxfp3*, a male-biased gene in mPFC OxtrINs, was previously shown to be male-biased in rats at the protein level in many hippocampal regions as well as in the basolateral, central, and medial amygdaloid nuclei (Meadows and Byrnes 2015). Male mice lacking the ligand for *Rxfp3* display elevated alcohol consumption and preference compared to wild-type animals, whereas female mice are unaffected by the deletion, implicating this receptor-ligand pair in sex-specific reward processing and/or stress response (Shirahase et al. 2016). Another

interesting candidate gene from this dataset, *Atp1a2*, was found to be female-biased in mPFC OxtRINs and has previously been linked to female-specific and estrous-cycle-dependent self-grooming and marble-burying behavior (Böttger et al. 2016). These examples highlight how cell-type-specific RNA-seq may lead to the identification of novel molecular mechanisms underlying sex-specific behaviors.

In the second study, the authors used TRAP to examine sex differences in gene expression within the locus coeruleus (LC) – a region involved in several functions, including learning, anxiety, and arousal (Mulvey et al. 2018). While few differences were observed in LC serotonergic neurons, 152 genes were differentially expressed between male and female noradrenergic neurons. One of the female-biased genes, *Ptger3*, which encodes for the inhibitory G protein-coupled receptor EP3, was further examined to determine whether sex differences in transcription predict sex differences in LC electrophysiology or associated behavior. Bath application of sulprostone, an EP3 agonist, caused a larger magnitude and longer duration of LC neuron hyperpolarization in female brain slices than male slices. At the behavioral level, EP3 agonism prevented stress-induced anxiety-like behavior, as measured by time and entries into the center zone of the open field test, in females but not males.

Sex differences revealed through single-cell RNA-seq—Single-cell RNA-seq provides the highest-resolution information and is the best approach to determine whether a sex difference arises from increased transcript numbers within a cell population or increased numbers of a specific cell type (Figure 4). This approach was recently taken to assess sex differences in MeA gene expression using DropSeq (P. B. Chen et al. 2019), which provides transcriptome information for high numbers of cells (>10,000) but with relatively low sequencing depth (Poulin et al. 2016). The authors did not identify sex differences in the proportion of cell types (ruling out Figure 1B) but did detect sex differences in gene expression in GABAergic neurons, although no novel sex-biased genes were validated by histology.

In contrast, the Correa lab found that three of the transcriptionally-defined cell types identified in their single-cell analysis of the VMH showed striking sex differences by in situ hybridization. They used a *Sfl-Cre* mouse line to selectively label the VMH for single-cell RNA-seq at p10, when sexual differentiation of the brain is complete but prior to puberty. The authors identified *Rprm* and *Tac1* as marking female-specific clusters in the VMHvl and *Pdyn* as a male-biased gene in the caudal VMHvl. Intriguingly, although *Rprm* and *Tac1* colocalize with ER α , neither adult gonadectomy nor genetic deletion of *Esr1* alters their expression. The authors employed the 4CG mouse model to demonstrate that the presence of *Sry* and a male reproductive tract, but not sex chromosome complement, results in a lower, male-typical expression pattern of these genes. They then went on to explore the function of *Rprm*, finding that its knockdown produced a decrease in thermogenesis, but not locomotion, complementing earlier work showing that stimulation of *Tac1*+ neurons in females leads to increased locomotion (Correa et al. 2015). This work is an excellent example of how scRNA-seq can be leveraged to identify functional populations of neurons that directly contribute to sex differences in physiology.

The studies discussed above show that RNA-seq of specific cell populations can reveal novel sex-biased genes involved in the molecular programming of innate behaviors. As scRNA-seq and TRAP become more common in the field, it is likely that additional sex-biased mechanisms relevant to physiological and disease states will be identified. Specificity can also be achieved by assessing the direct genomic binding sites of transcription factors (TFs), such as ER α . Previous attempts at studying these mechanisms by ChIP-seq have been stymied by the high number of cells required for the method, which prohibits analysis of specific brain areas. Newer approaches such as CUT&RUN (Sidebar 1) use low cell numbers, even single cells, to identify genomic sites of histone modifications or TF binding (Skene and Henikoff 2018; Hainer et al. 2019) and could potentially revolutionize the study of gene regulation in the brain.

Conclusion

Both molecular and circuit mechanisms can give rise to sex differences in the brain. Here, we have explored these mechanisms with a focus on the regions and neural pathways that directly contribute to sex differences in adult behavior. Most of these differences likely reflect internal state variations due to circulating hormones and physiology, rather than developmentally programmed “hard-wired” differences that evolve during juvenile or fetal brain. In the select brain regions where cell number is sex-biased, we predict that early-life hormone signaling specifies the survival and connectivity of specific functional cell types. This leads to constitutive sex differences in gene expression within these regions at the population level as well as activity-dependent gene programs in projection areas. In addition, sex differences in the proportion of cell types in these regions could underlie differences in neural coding and information processing in the circuitry that mediates social behavior.

As technological advances have revealed an increasing number of sex-biased genes, the next challenge will be to identify how these differences arise and evaluate how sex-biased genes contribute to sex differences in behavior and human disease. The ontogeny of sex-typical expression patterns may be identified by integrating data from multiple epigenomic assays, which should reveal sex-specific regulatory elements and their interacting transcription factors (Gray et al. 2015; Nord et al. 2015). Application of these approaches to the developing human brain is already underway (Ecker et al. 2017; M. Li et al. 2018; D. Wang et al. 2018) and likely to reveal conserved sex-biased or hormone-regulated gene programs (Hoffman et al. 2016; Ijaz and Hoffman 2016; Miller et al. 2016; Moe et al. 2016). Subsequent research in model organisms can then dissect the contribution of molecular sex differences to the development and function of specific neural pathways.

Many psychiatric and neurological disorders show a sex-bias in incidence, age-of-onset, or etiology. There is an increased incidence of neurodevelopmental disorders, including autism, ADHD, and developmental language disorders, in boys, suggesting the male brain is uniquely vulnerable during early life. In contrast, women are more likely to experience adolescent-onset mood disorders, such as depression and anxiety. Schizophrenia shows roughly equal incidence in the two sexes, but there are sex differences in the age-of-onset and symptoms. On average, men develop the disease earlier, but women show a second peak of incidence around menopause (Häfner 2003). It is thought that many psychiatric disorders

originate during neurodevelopment, highlighting a clear need for a better understanding of the unique developmental trajectories experienced by female and male brains (Manoli and Tollkuhn 2018). Defining the gene regulatory events that differ between the sexes throughout the lifespan is likely to reveal genes and pathways that may provide resilience or susceptibility to disease.

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Sidebar 1: Tools for studying gene regulatory mechanisms in specific cell types of the mouse brain

Several molecular genetic tools have been developed for studying gene regulation in the mammalian brain. Because steroid hormone receptors are expressed only in a subpopulation of cells within sexually dimorphic brain regions, it is critical to isolate specific cell types prior to downstream genomics assays. Cell-type targeting has been made possible with *Cre-loxP* and *Flp-FRT* recombination systems, which rely on cell-type-specific active gene promoters to facilitate knockin-knockout expression of fluorescent reporter or molecular-tagging alleles (Tsien et al. 1996; Daigle et al. 2018). When *Cre* and *Flp* animals are crossed, cells co-expressing two driver genes may be labeled, allowing for more refined targeting (Paul et al. 2017). Temporal control of cell labeling is also possible with tamoxifen-inducible *Cre* and *Flp* mouse lines (Danielian et al. 1998), which have been used extensively to track specific neuron populations throughout embryonic development.

Molecular-tagging alleles are used for isolation of RNA or chromatin for downstream genomics applications. The RiboTag mouse line carries an allele of the ribosomal protein gene *Rpl22* with a wild-type C-terminal exon flanked by loxP sites neighboring a copy of the C-terminal exon fused to three copies of the hemagglutinin (HA) epitope (Sanz et al. 2009). When crossed with a *Cre*-driver mouse line, the wild-type C-terminal exon is removed in cells expressing Cre recombinase, and the HA-tagged C-terminal exon is expressed. Using a method called translating ribosome affinity purification (TRAP), translating mRNAs may be immunopurified from microdissected brain tissue using an α -HA antibody and sequenced. Prior to this, several bacterial artificial chromosomes (BAC) containing a GFP-labeled *Rpl10a* allele driven by different cell-type-specific promoters were developed for transcriptional profiling of specific cell populations (Heiman et al. 2008, 2014).

A variation of the TRAP method, called Retro-TRAP, was later developed to determine the transcriptional profile of cell populations with a specific projection target (Nectow, Ekstrand, and Friedman 2015). This method uses a *Cre*-dependent adeno-associated virus containing the ribosomal protein gene *Rpl10a* tagged at its N-terminal end with an α -GFP camelid nanobody (AAV-FLEX-NBL10). Injection of this virus into a target brain region followed by injection of a GFP-expressing retrograde-tracing virus (CAV-GFP) produces GFP bound to the *Rpl10a*- α -GFP complex in a cell-specific and projection-specific manner. Translating mRNAs can then be immunopurified from the retrograde-labeled brain region with an α -GFP antibody.

Resolving the spatial distribution of gene transcripts is critical for linking gene expression to neuron morphology and connectivity. RNA imaging by fluorescence in situ hybridization (FISH) has traditionally been used to provide spatial information to RNA-seq data, as it uses highly specific fluorescently-labeled oligonucleotide probes to label transcripts in native tissue. The most advanced version of FISH - MERFISH - has recently been used to simultaneously image 155 RNA species at single-cell resolution in the POA and would be effective at assessing neuronal heterogeneity in other sexually dimorphic brain areas (K. H. Chen et al. 2015; Moffitt et al. 2016; Moffitt et al. 2018).

Gene regulatory programs involving interactions between cis-regulatory genomic elements and TFs underlie the immense transcriptional heterogeneity of the brain. Cis-regulatory elements, such as enhancers and promoters, are typically characterized by H3K27 acetylation, H3K4 dimethylation, and low DNA methylation, which yield an “open” chromatin state permissive of gene transcription. These properties have been studied with chromatin immunoprecipitation (ChIP-seq) of histone marks, bisulfite sequencing, and the assay for transposase-accessible chromatin (ATAC-seq). Implementing these techniques in brain has been accomplished using the isolation of nuclei tagged in specific cell types (INTACT) method (Deal and Henikoff 2011; Mo et al. 2015). Similar to TRAP and Retro-TRAP, INTACT uses a *Cre*-dependent MYC- and GFP-tagged nuclear membrane protein to enable immunopurification of nuclei from defined cell types. This method bypasses technical issues of fluorescence-activated sorting and removes the mitochondrial genome, which can occupy between 30–70% of ATAC-seq reads (Buenrostro et al. 2013). However, fl remains the best option for purification of nuclei from human post-mortem tissue and can be combined with immunostaining to purify specific populations for analysis of chromatin (Kundakovic et al. 2017).

Despite this improvement, identifying TF binding sites in a cell-type-specific manner within the brain remains a challenge. Traditional ChIP-seq of low-abundant TFs, such as hormone receptors, requires millions of cells to overcome epitope masking and degradation during crosslinking and chromatin shearing, respectively. A novel technique called cleavage under targets and release using nuclease (CUT&RUN) has been developed for studying TF binding in low cell numbers (Skene and Henikoff 2017; Skene and Henikoff 2018). CUT&RUN uses a protein A-micrococcal nuclease fusion protein to cleave nuclear DNA in the vicinity of antibody-TF complexes, eliminating the need for crosslinking or chromatin shearing. This approach has been used to determine TF occupancy in individual mouse embryonic stem cells (Hainer et al. 2019) and could potentially identify binding sites for TFs in discrete brain regions and cell types.

Figure 1. Intersection of developmental testosterone surges with neurodevelopmental events

Male mice (A) and humans (B) experience developmental T surges that delineate critical periods for sexual differentiation of the brain. Human testes begin to secrete T at approximately week 8 of gestation, peaking around week 16 and then declining until week 24. Fetal T levels are nearly as high as those during puberty. There is also elevated T during months 1–3 of infancy. Mouse testes become active in fetal development, and mice undergo a T surge on the day of birth. The testes are then inactive until puberty, which begins around postnatal week 4. At this time, the brain is still undergoing significant neuronal migration, particularly of inhibitory interneurons, while gliogenesis and synaptogenesis are still increasing. Human brain development occurs on a much longer timescale with the bulk of neuronal proliferation occurring during the fetal T surge. Events that occur during T surges are likely to give rise to sex differences in brain function, as female ovaries do not undergo significant hormone release until puberty.

Figure 2. Sex differences in steroid hormone receptor expression in the mouse brain

Sex differences in steroid hormone receptor expression have been reported in several brain areas involved in social behaviors. These areas include the anteroventral periventricular nucleus (AVPV), medial amygdala (MeA) posterior dorsal (MePD) and posterior ventral (MePV) regions, medial preoptic area of the hypothalamus (MPOA), posterior bed nucleus of the stria terminalis (BNSTp), and ventrolateral region of the ventromedial hypothalamus (VMHvl). AR androgen receptor; ER β estrogen receptor β ER β estrogen receptor β ; PR progesterone receptor.

Figure 3. Mechanisms of ER α -mediated gene regulation

Estrogen receptor α (ER α) acts through various binding partners and intracellular signaling pathways to regulate gene expression in a tissue- and cell-type-specific manner. (A) Following ligand binding, ER α can directly influence transcription by binding canonical estrogen response elements (EREs), activator protein 1 (AP-1) transcription factor complex, or pioneer factors (PFs), which infiltrate heterochromatin and impart cell-type-specificity to ER α genomic binding (Droog, Mensink, and Zwart 2016; Gertz et al. 2013). ER α also receives post-translational modifications (PTM) from diverse signaling pathways independent of ligand (Bennesch and Picard 2015). Many extracellular molecules, such as growth factors, cytokines, and peptide hormones, can initiate ligand-independent activation of ER α . (B) ER α can also indirectly regulate gene expression by controlling the phosphorylation of cAMP response element (CRE) binding protein (CREB). CREB specifically recruits CREB-binding protein (CBP) - a transcriptional coactivator that serves a dual function in gene regulation as a histone acetyltransferase and scaffold for transcriptional machinery (Dyson and Wright 2016).

Figure 4. Developmental programming of sex differences in gene expression

Two simplified scenarios by which perinatal estradiol signaling could drive persistent sex differences in gene expression. Depicted is a schematized postnatal BNST, which expresses more gene X in males than females at the bulk tissue level (bar graph). Circles, squares, and triangles correspond to different cell types in the BNST. In (A) transient exposure to estradiol results in permanent remodeling or reorganization at the promoter of gene X in a subset of BNST cells (circles) expressing ER α . This opened chromatin facilitates increased transcription of gene X, similar to estradiol upregulation of Ar expression in mouse BNST (Juntti et al. 2010). In (B) estradiol promotes cell survival in males relative to females. Estradiol increases expression of pro-survival Bcl family genes that inhibit the BAX/BAK apoptosis pathway (Zup et al. 2003; Nancy G. Forger et al. 2004). In this model, although the expression of gene X in a distinct cell type is similar between males and females, the male BNST contains more X-expressing cells. The gene X expression difference would appear similar in models A and B at the bulk tissue level. Thus cellular resolution is necessary to distinguish between models A and B.

Figure 5. Sex differences in activity-dependent gene expression

Estradiol and other steroid hormones can induce sex differences in gene expression via activity-dependent transcription. A) During proestrus, high levels of estrogens can activate neurons (orange bordered cells) in brain areas such as the MPOA (McHenry et al. 2017) or VTA (Calipari et al. 2017). Neural activity increases calcium release, CREB phosphorylation and upregulation of immediate early genes such as *Fos* (West, Griffith, and Greenberg 2002). IEGs activate or repress cell-type-specific late response genes (LRGs). Shown here is the growth factor *Bdnf*. This mechanism could lead to distinct gene expression profiles between high-estrogen proestrus females and low-estrogen diestrus females or males as in (Duclot and Kabbaj 2015). B) Neural activity in select hormone-responsive neurons could be propagated to downstream brain areas, which would then report their own unique signature of activity-regulated genes. This process could induce sex

differences in gene expression in areas that do not express steroid hormone receptors. C) A stimulus may activate distinct populations of neurons in the same brain area in males and females (Ishii et al. 2017), causing sex differences in both local activity-responsive genes and in the two downstream target brain areas.

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Figure 6. Histological validation of sex differences in gene expression
In situ hybridization for two genes identified in a screen for sex differences in gene expression. Sytl4 is expressed more highly in male BNST, and its expression is decreased in the absence of circulating gonadal hormones (A-D). Brs3 is more abundant in female BNST (E-H) and MeA (I-L) and is upregulated in castrate males but unchanged in castrate females. Modified from (X. Xu et al. 2012).

Figure 7. Sex-biased or estrogen-regulated genes identified in multiple studies

Shown in bold type are genes identified in at least two papers as showing sex-biased or hormone regulated expression in the brain. Superscript letters indicate the publications. Many estrogen-regulated genes have also been found in screens for activity-dependent gene expression (grey gene names). Underlined genes have been confirmed by histology. Not shown are steroid hormone receptors and aromatase, which have a well-established sex bias.

Table 1:
Screens for sex differences in gene expression in the mammalian brain

The studies listed performed screens to identify genes with differential expression between female and male brains. Genes validated in the original study by histology or qPCR in additional tissue samples are noted. Y chromosome and X chromosome escape genes are excluded.

Publication	Brain region	Key validated genes
(X. Xu et al. 2012)	Adult mouse hypothalamus microarray	<i>Brs3</i> , <i>Esr1</i> : female. <i>Cartpt</i> , <i>Chodl</i> , <i>Dgkk</i> , <i>Ecel1</i> , <i>Gabrg1</i> , <i>Gtra3</i> , <i>Gpr165</i> , <i>Greb1</i> , <i>Pak3</i> , <i>Rps6ka6</i> , <i>Sytl4</i> : male <i>Cckar</i> , <i>Irs4</i> , <i>Nnat</i> : both, region-specific
(Walker et al. 2012)	Postnatal rat POA/MBH timecourse, qPCR	<i>Esr1</i> , <i>Kiss1</i> , <i>Tacr3</i>
(Trabzuni et al. 2013)	Adult human, multiple areas, microarray	GO terms only
(Armoskus et al. 2014)	Neonatal mouse cortex and hypothalamus	<i>Mid1</i> , <i>Erdr1</i> , female
(Duclos et al. 2015)	Adult rat mPFC, males, diestrus or proestrus females, RNA-seq	<i>Igf2</i> , <i>Igf2</i> , <i>BDNF</i> , <i>Fos</i> : male. Rgs9: female
(Hodes et al. 2015)	Adult mouse nucleus accumbens RNA-seq	<i>Dnmt3a</i> : female
(Nugent et al. 2015)	Neonatal rat MPOA, RNA-seq	<i>Ppp1r9b</i> : male
(K. Li, Nakajima, and Heintz 2016)	Adult mouse mPFC, Oxt neurons, BAC-TRAP RNA-seq	none
(Werling, Parikshak, and Geschwind 2016)	Human cortex. Adolescent and adult RNA-seq, prenatal microarray	none
(Shi, Zhang, and Su 2016)	Reanalysis of BrainSpan data (www.brainspan.org)	none
(Marrocco et al. 2017)	Adult mouse CA3 Gpr3 neurons, BAC-TRAP, RNA-seq	GO terms only
(Mulvey et al. 2018)	Adult mouse locus coeruleus, Slc6a2 neurons, BAC-TRAP microarray	<i>Ptger3</i> : female
(Villa et al. 2018)	Adult mouse microglia, RNA-seq	<i>Shank3</i> , <i>Fxyd1</i> , <i>Aqp1</i> , <i>Timp3</i> : female <i>Akt1s1</i> , <i>Trem1</i> , <i>S100a9</i> , <i>Cxcl2</i> : male
(P. B. Chen et al. 2019)	Adult mouse MeA, DropSeq	<i>Brs3</i> : female, <i>Greb1</i> : male (from (X. Xu et al. 2012)). <i>Fam84a</i> , <i>Ankrd55</i> : male
(van Veen et al. 2019)	P10 mouse VMH, sorted from Sf1-Cre reporter, scRNA-seq	<i>Tac1</i> , <i>Rprm</i> : female, <i>Pdyn</i> , <i>Ndr</i> : male