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Lost in translational biology: Understanding sex differences to inform studies of diseases of the nervous system

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Abstract

Female and male humans are different. As simple and obvious as that statement is, in biomedical research there has been an historical tendency to either not consider sex at all or to only use males in clinical and in preclinical model system studies. The result is a large volume of research that reflects the average biology and pathology of males even though we know that disease risk, presentation, and response to therapies can be different between females and males. This is true, albeit to differing degrees, for virtually all neurological and psychiatric diseases. However, the days of ignoring sex as a biological variable are over – both because of the realization that genetic sex impacts brain function, and because of the 2014 mandate by the U.S. National Institutes of Health that requires that "sex as a biological variable" be addressed in each grant application. This review is written for neuroscientists who may not have considered sex as a biological variable previously but who now are navigating the best way to adapt their research programs to consider this important biology. We first provide a brief overview of the evidence that male versus female differences in the brain are biologically and clinically meaningful. We then present some fundamental principles that have been forged by a dedicated but small group of ground-breaking researchers along with a description of tools and model systems for incorporating a sex differences component into a research project. Finally, we will highlight some key technologies that, in the coming years, are likely to provide critical information about sex differences in the human brain.

1. Introduction: basic biological processes differentially impacting the male and female brain

Understanding the mechanism of how a population responds to a therapy or is more susceptible to an environmental or genetic insult is at the core of translational medicine. A relatively recent push towards personalized medicine offers the hope of understanding and developing custom treatments for maladies at the level of the individual human subject. Thus, rather than relying on the general trends of populations to diagnose and treat

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individuals, the field seeks to identify the underlying genetic and external influences that will predict precisely who will develop a disease or respond to a particular clinical intervention. We know that genomic variation between individuals can influence the susceptibility to disease, response to treatment, and the presentation of biomarkers. However, we generally discuss genomic variation within a given human population in the context of the inherited genetic signatures that have been passed down through a specific familial and ethnic lineage. But while ethnic and geographical genomic variation clearly plays a role in differential human biology, the greatest biological variability is between males and females. Without external hormonal intervention or rare sex chromosomal anomalies, sister/brother fraternal twins are more biologically and genetically different from each other than either one is from a same sexed individual of an entirely different ethnic background (Genomes Project et al., 2015; Zerbino et al., 2018).

So, what are the factors that drive differential biology between human males and females? At the highest level there are three primary effectors: hormonal environment, genetic composition, and social influences. While it can be tempting to speculate on the effects of society's different treatment and expectations of men and women on health outcome, it is an extraordinarily complicated subject that biologists and medical researchers are woefully ill-equipped to address in a rigorous scientific manner. Because of this, we will predominantly limit this particular review to the hormonal and genetic drivers of sex differences in the brain.

We are currently seeing a surge of interest in studying sex differences in human biology. However, historically the effect of sex on biology and disease has been minimized. On the genetic level we have effectively rounded an XX genotype to an XY genotype citing X-inactivation to dismiss the effect of the second X chromosome in women and dismissing the Y chromosome as small and primarily involved in sex determination during embryogenesis. However, we now know that well over 100 X-linked genes escape inactivation showing full to partial bi-allelic expression in adult female tissues (Disteche, 2012; Tukiainen et al., 2017). In addition, there are at least 26 protein-coding genes as well as many long-non-coding RNAs and miRNAs on the Y chromosome (Skaletsky et al., 2003). Quite telling is the fact that 98% of human embryos with Turner Syndrome (defined by a 45, XO genotype) do not survive gestation (Cockwell et al., 1991; Saenger, 1996). That extraordinary embryonic mortality rate is a direct result of the lack of either an X or a Y chromosome in the second sex chromosome position and underscores the importance of X/Y chromosome complement beyond sex determination.

It is worthwhile mentioning that we will use the term sex to mean genetic sex, and it will be assumed that the hormonal environment follows the genetics in the studies referenced. While this is the case with the vast majority of humans, there are people for whom those assumptions do not apply. In cases of sex reversal (XX with male external genitalia and XY with female external genitalia), genital development is unlinked to genetic sex. While sex reversal is thought to be rare, occurring an estimated 1 in 10,000 people, it is a condition that can likely go undiagnosed (and therefore underestimated) as people can live relatively normal lives unaware that their genetics do not match their external genitalia (Martins et al., 2017). More commonly, it is estimated that between 0.1 and 0.7% of the population are

transgender or gender non-conforming and up to 25% of these individuals are on cross-hormone therapy (Unger, 2016; Williams Institute, 2017). These numbers would imply that more than 100,000 people in the US alone have a sex hormone milieu that fits their gender but is opposite to their genetic sex. Gender, though often erroneously used as a synonym of sex, is a term that combines both the internal and external perception of an individual's sex (as defined by the World Health Organization). While the influence of gender roles might ultimately impact neural biology, to date there are few major studies that differentiate sex and gender in analyses of diseases of the human brain. Thus, in this review, references to males assumes an XY genotype and a circulating hormonal environment dominated by testosterone while a reference to females assumes an XX genotype and a circulating hormonal environment dominated by estrogen. Finally, we will use the term "sexually dimorphic" to refer to traits that are systematically different between males and females and "sex difference" to refer to measurements that have statistically significant differences in male-female population averages. To be clear, most of the sex differences observed are differences in the population average of a particular measurement with considerable overlap between men and women. Thus, one way to approach studying sex as a biological variable is to consider sex as a differentiation axis that can be used to evaluate natural variations in human biology. In the case of sex differences, the particular variations would tend to be ones influenced by sex hormones, X and Y chromosomes, differential cultural environments or a combination of those.

2. Sex differences in neurological diseases, psychiatric disorders, and beyond

The influence of sex on risk and presentation of neurologic disease and response to clinical interventions is becoming increasingly clear. Virtually every neurodegenerative and neuropsychiatric disease shows some variation, often striking, between males and females. Males have a higher incidence of autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), while females are at a higher lifetime risk for Alzheimer's disease (AD), major depressive disorder (MDD), post-traumatic stress disorder (PTSD), and multiple sclerosis (MS) (reviewed in (Zagni et al., 2016)). However, sex differences in these diseases are much more nuanced and interesting than a simple differential susceptibility would imply. Below we highlight a few examples of sex differences in specific disorders and diseases of the nervous system.

Autism Spectrum Disorder

The prevalence of autism shows a striking sex difference. A 2014 report from the Center for Disease Control and Prevention reported that Autism Spectrum Disorder (ASD) occurs in 1.6% of all 8yr old children and is 4 times more common in boys than in girls (Baio et al., 2018). While the overall prevalence of ASD diagnoses has risen in the past 10 years, reports of a sex bias toward males have been consistent (Fombonne, 2005). ASD is diagnosed in children as young as 2 years old indicating that the initiating problem would have likely had to occur during fetal development or in the very early post-natal period, prior to overt gender-specific socialization and about a decade before pubescent hormones differentiate

male and female biology more dramatically. Because of the strong difference in prevalence, there are numerous studies of sex differences in ASD in humans and in animal models, and multiple thorough reviews have been written on the topic (McCarthy and Wright, 2017; Rubenstein et al., 2015; Werling and Geschwind, 2013). Evidence from numerous studies reveal that there are well validated sex differences in the clinical phenotypic presentation of ASD and suggest that both genetic and hormonal influences contribute to these differences.

Schizophrenia

The lifetime prevalence rate for schizophrenia is similar for males and females but the average age of onset, morphological disruptions in the brain, and clinical presentations have been shown to be quantifiably different (reviewed in (Mendrek and Mancini-Marie, 2016)). Males have a higher risk for developing the disease early in life while females have a greater risk later in life. The exact window of risk varies from study to study but the general conclusion that males tend to develop the disease early and females late is consistent across many studies (Abel et al., 2010; Leung and Chue, 2000; McGrath et al., 2008; Salem and Kring, 1998; van der Werf et al., 2014). Additionally, men tend to present with more severe structural abnormalities of the brain, having reduced frontal and temporal volumes than women, and men with schizophrenia tend to show a reversal in the normal left-right asymmetry in the grey matter in the inferior parietal lobe while women do not (Mendrek and Mancini-Marie, 2016). Several lines of evidence have indicated a role of sex hormones in the presentation and progression of schizophrenia, though a direct role of the sex chromosomes themselves also has been implied by observations that Klinefelter patients (47, XXY) have a higher incidence of schizophrenia than do individuals with a traditional sex chromosome complement (DeLisi et al., 2005; van Rijn et al., 2006).

Alzheimer's disease

It is often stated that females have a higher risk of Alzheimer's disease (AD) than males of the same age, and it is clearly true that females bear a considerably greater burden from AD than males. There are approximately 3.3 million women compared with 2.0 million men living with AD in the United States (Alzheimer's, 2016) and the estimated lifetime risk for AD is about double for women compared to men (20% compared to 10%). However, much (though not all) of this sex disparity is a result of the increased lifespan of females. Age is the strongest risk factor for AD, and age-related risk spikes dramatically as one approaches their late 80's and 90s. While some European studies and one US study reported a higher prevalence of AD in women than in men, a recent meta-analysis of many studies from both Europe and the US showed that when combined, the difference was no longer significant when controlling for age (Fiest et al., 2016; Nebel et al., 2018). Nonetheless, there is consensus that there are clinical differences in progression between men and women. Females are subject to a more rapid decline in cognition after diagnosis than males (Henderson and Buckwalter, 1994; Holland et al., 2013; Lin et al., 2015; Proust-Lima et al., 2008; Read et al., 2006; Tifratene et al., 2015). Furthermore, studies of the pathological hallmarks of AD (Aβ plaques and tau-containing tangles) also show differences between the sexes. In the analysis of postmortem data from 1,453 individuals, it was found that women have a significantly higher tangle density than men, but only borderline differences in plaque burden (Oveisgharan et al., 2018). In a neuroimaging study of cognitively normal

individuals, it was shown that while there are no differences between men and women with regards to level of plaque burden, females with a high plaque burden show more rapid cognitive decline than men with the equivalent plaque burden (Buckley et al., 2018).

There is evidence that the sex differences in AD are at least partly the result of post-menopausal estrogen decline in women (Doraiswamy et al., 1997; Henderson et al., 1994; Mulnard et al., 2000; Paganini-Hill and Henderson, 1994; Tang et al., 1996). For example, pre-menopausal oophorectomies and surgical menopause at an early age increase the risk for cognitive impairment (Bove et al., 2014; Rocca et al., 2007). In addition, there is evidence that estrogen replacement therapy begun close in time to menopause onset can lessen the risk and symptoms of AD in women (Doraiswamy et al., 1997; Henderson et al., 1994; Mulnard et al., 2000; Paganini-Hill and Henderson, 1994; Shao et al., 2012; Tang et al., 1996). However, the precise molecular mechanism of how estrogen may affect AD pathology and cognition is not clear.

Parkinson's disease

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta that results in motor as well as non-motor symptoms. Both the incidence and prevalence of PD are significantly higher in men than in women, with men having approximately a 60% greater prevalence and an earlier average age of onset (Elbaz et al., 2002; Hirsch et al., 2016; Twelves et al., 2003; Wooten et al., 2004; Zagni et al., 2016). In addition, there is evidence that females have less severe motor symptoms than men (Haaxma et al., 2007). At the morphological level, researchers have observed sex differences in the basal ganglia in both animal models and humans. Female mice have been reported to have higher numbers of dopaminergic neurons in the substantia nigra (Beyer et al., 1991; Cantuti-Castelvetri et al., 2007), while in humans, PET imaging studies have shown male-female differences in dopamine transporter binding (Haaxma et al., 2007; Kaasinen et al., 2015; Wong et al., 2012). At a molecular level, gene expression differences in male vs female dopaminergic neurons in the human substantia nigra have been reported, as well as differences in lipid profiles in the substantia nigra in men vs women (Cantuti-Castelvetri et al., 2007). Additional studies using the available tools and technologies described below will be necessary to tease apart the molecular mechanisms underlying elevated male risk for PD.

Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating autoimmune disease. It is highly heterogeneous in its course, with patients showing very different rates of progression of the disease, and some having a benign form that does not progress beyond the initial presentation. There is a well-established sex bias in MS risk and course, with women having a higher risk for MS, and men more likely to have a rapidly progressing form of the disease (Compston et al., 2006; Koch et al., 2010; Tomassini et al., 2005). In the past decades, the female-to-male ratio of MS has increased (now approximately 3:1) due to a puzzling increase in female cases of MS in many different parts of the world (Kingwell et al., 2015; Maghzi et al., 2010; Orton et al., 2006; Trojano et al., 2012). Several studies have implicated sex hormones in the differential risk and presentation of MS between males and females.

Notably in females there is a correlation of disease onset with the onset of puberty (Ramagopalan et al., 2009), a decline in MS relapse rate during pregnancy (Alroughani et al., 2018; Confavreux et al., 1998), and a worsening of symptoms with menopause (Bove et al., 2016; Smith and Studd, 1992). Sex chromosome complement also is thought to contribute to the male-female differences in MS rates and progression, and has been studied using models of MS coupled to the “four-core genotype” mice described in the next section (Du et al., 2014; Smith-Bouvier et al., 2008). Further investigation of the molecular mechanisms underlying sex differences in MS will both increase our understanding of disease mechanisms and aid in the identification of improved therapies.

Pain

In addition to neurodevelopmental and neurodegenerative diseases, sex differences have been observed in basic neurological functions of individuals who have no chronic disease, notably in pain perception and response to opioid treatment (Loyd and Murphy, 2014; Rosen et al., 2017; Sorge and Totsch, 2017). In these studies, it has been consistently observed that females have a much higher prevalence of chronic pain than males. for Additionally, in response to discrete pain events, women exhibit a higher sensitivity, rate the pain higher, and have a lower overall tolerance pain (reviewed in (Mogil, 2012)). Many experiential and sociological explanations for these differences have been proposed including differing gender role expectations of pain tolerance, and different life experiences (such as child birth) affecting an individual’s application of the pain scale. But there also are relevant measurements that hint at the biological basis of some of the differences observed. A positron emission tomography (PET) study that imaged brains of males and females during pain tolerance tasks demonstrated that males had a higher activation of the mu opioid receptor than women in discrete brain regions, specifically the amygdala, anterior thalamus, and ventral basal ganglia (Zubieta et al., 2002). Consistent with this observation, male rodents show a consistently greater molecular response to morphine treatment than females (Loyd and Murphy, 2006). While this doesn’t address chronic pain, the observation indicates that on average the endogenous analgesic response is quantifiably different between males and females. These are important observations in a field where effect sizes in humans are, by necessity, self-reported and thus challenging to quantify across different subjects.

Sex differences in pharmacologic response

Clinical studies in humans have demonstrated sex differences in the response to many classes of psychotropic and sedative/hypnotic drugs. One example is morphine, which was described above. Another, famous example of this is differential response to the sleep-aid zolpidem (Ambien), which has a significantly more persistent effect in women than in men. This can lead to dangerously high levels of the sedative in the blood streams of women during the waking hours the day after taking the medication (a primary concern being driving or operating machinery while still under the influence of the drug). The longer-term effects of the drug in women seems to be an indirect effect of the medication being metabolized more slowly in women compared to men (Greenblatt et al., 2000; Olubodun et al., 2003; Tripodianakis et al., 2003). In response to the data, the FDA announced in 2013 a reduced recommended dosage of zolpidem for women relative to men. Sex differences in pharmacologic response are not limited to Ambien and morphine; women also have a

tendency to respond better to selective serotonin reuptake inhibitors (SSRIs), respond more strongly (and adversely) to anti-anxiety medication, and have a more persistent response to benzodiazepines than men (reviewed in (Lange et al., 2017; Sramek et al., 2016)).

Above is a selection of the many examples of sex differences across disease risk, presentation and response to neuromodulators. There are numerous other examples in the primary literature that underscore the undeniable truth that there are measurable differences in the basic neurobiology between human males and females. These differential clinical measures imply the existence of variant molecular activity between human males and females that can result in a long-term impact on disease. In addition to the processes that directly affect brain function, some differences certainly result from indirect effects due to differences in cardiovascular biology, liver metabolism, microbiome composition, and a number of other biological functions that are different between men and women. So, in answer to the implied underlying question of this section “why is studying sex as a biological variable in the brain important?”- it is important because the biology, disease risk, pharmacology, and pathology are *measurably* different between the sexes. It is worth stating that while men and women are clearly more biologically alike than they are different, the culmination of numerous small variances over a lifetime can result in significant changes to overall health as we age. A more intimate knowledge of exactly what those differences are at the molecular level will ultimately have a positive impact on clinical outcomes.

3. Existing paradigms in sex differences

While the new NIH mandate and research funding initiatives have sparked a strong current interest in studying sex differences, there is an “old guard” who have been steadily advancing knowledge about sex differences in neurobiology and pathology since well before it was popular or easy to do so. This handful of researchers dedicated to the topic have had to overcome decades of skepticism and overt pushback regarding the recognition of sex differences in the brain. Here, we summarize a subset of the established paradigms that sculpt the current understanding of the differential basic biology between the male and female brain.

A historic theory of mammalian sexual differentiation that had been widely accepted since the 1940’s was that it consisted of a linear two-step process: 1) sexual *determination* is directed by the chromosome complement (traditionally XX or XY) that instructs the embryonic genital anlage to differentiate into fetal ovaries or testes respectively, and 2) hormonal secretions from the developed genitalia (predominantly estrogen from the ovaries and testosterone from the testes) then determine *all* aspects of the differential biology (and pathology) between males and females. In this model, exposure to androgens during development actively “masculinized” the brain and the female brain was considered to be the “default” result of lack of androgen exposure. Importantly, this historic model broke hormone action on the brain into early (neonatal and perinatal) effects that are classified as being “organizational” and permanent, and late, (post- pubertal) effects that are classified as “activational” and transient. The rigidly linear historic model completely discounted any direct effect of genes on the second X or the Y chromosome on postnatal biology. The theory was driven by the extraordinarily strong effect of exogenous hormones on human

biology and by a technological inability to clearly test the effects of the sex chromosome genes in adult cells. Though it is occasionally still repeated as current, it has been shown to be a highly oversimplified model with major caveats (reviewed in (Arnold, 2017; Fitch and Denenberg, 1998; Koebele and Bimonte-Nelson, 2015; McCarthy et al., 2017b)). A large, and growing, body of work have demonstrated that a) sex chromosomes influence feminization and masculinization of the brain outside of hormonal influences; b) full feminization of the mammalian brain requires active signaling by estrogens rather than simply being a passive "default" pathway; and c) male and female hormones exert organizational effects in the mammalian brain well beyond early development.

The "General Theory of Sexual Differentiation"

Work from the Arnold lab and others has allowed researchers to clearly separate the effects of sex hormones from the effects of sex chromosomes in a mouse model, and has demonstrated that sex chromosome complement has a direct effect on many processes in adult cells irrespective of hormonal environment (Arnold and Chen, 2009; De Vries et al., 2002; Koopman et al., 1991). Outside of the critical role of the Y chromosome in directing differentiation of male gonads and a lifetime of androgen exposure vs. estrogen exposure, there are at least 5 potential mechanisms by which the sex chromosomes can differentially affect biology between males and females: 1) Expression of Y chromosome genes in non-gonadal tissues; 2) X-inactivation escapers having higher gene transcript dosage in XX vs. XY cells; 3) Differential imprinting between maternal and paternal X chromosomes (males only ever inherit maternal X chromosomes); 4) High expression of the Xist gene in every XX cell with potential downstream effects on autosomal genes; and 5) Presence of the large inactive X chromosome in XX cells acting as a heterochromatic "sink" that then impacts autosomal gene expression. For a more detailed description of each of these possibilities, see (Arnold, 2017).

This fundamental shift in thinking regarding the sex chromosomes was made possible by the development of a valuable set of animal models that uncoupled gonadal sex from genetic sex. The "four core" genotypes mouse model leverages the power of the Y-linked *Sry* gene to singularly determine male gonadal development in the embryo regardless of the presence or absence of the remaining Y chromosome. By starting with a mouse containing a spontaneous 11Kb loss of ChrY (XY^-) which included the endogenous *Sry* gene (Lovell-Badge and Robertson, 1990), and breeding to a mouse carrying a genomic *Sry* transgene inserted into an autosome (XY*sry)* (Koopman et al., 1991), the gonadal phenotypes of offspring mice were unlinked to the complement of sex chromosomes (De Vries et al., 2002). Thus, by simply breeding the resulting XY^-*sry* males (where the *sry* gene has effectively been moved to an autosome) to XX females, "four core" genotypes are generated which are: XY^- females, XX females, XY^-*sry* males, XX*sry* males. This elegant system has served as a tool to uncover clear actions of genetic chromosome complement -irrespective of hormonal milieu - in many biological processes including but not limited to: embryonic numbers of TH+ dopaminergic neurons (higher number in XY relative to XX (Carruth et al., 2002)), the sensitivity of response to morphine in pain tests (XY more sensitive than XX, (Gioiosa et al., 2008a; Gioiosa et al., 2008b)), and vulnerability in multiple sclerosis and

systemic lupus erythematosus models (greater effects on XX than XY (Smith-Bouvier et al., 2008)).

For those not familiar with the gene complement on the X and Y chromosomes, it should be noted here that the tips of the X and Y chromosomes in humans share homologous sequences called the pseudoautosomal regions (PARs). Because of their homology, crossing over between the X and Y occurs within the human PARs during meiotic recombination, and thus the genes in these regions are inherited in the same manner as autosomal genes. The section of the Y chromosome between the PARs is referred to as the male specific region (MSR) and consists of some genes that are entirely unique to the Y and some genes that have structurally similar paired genes on the X chromosome having arisen from a common ancestral gene. It has been demonstrated that some of the unique Y chromosome genes (including the testes determining factor *Sry*) are both expressed and function in the adult mammalian brain (Czech et al., 2012; Czech et al., 2014; Dewing et al., 2006; Mayer et al., 1998; Milsted et al., 2004). Additionally, the paired genes existing on both the X and the MSR of the Y chromosomes are generally escapers of X-inactivation which would seem to effectively balance their activity between XX and XY cells. However, while these paired genes are similar, they are not identical in either sequence or in transcriptional regulation and thus may confer different biology between XX and XY cells. One example of this in humans is a pair of genes, *TSPY* and *TSPX*, arising from a common ancient ancestor but located on the MSR(Y) and the X chromosomes respectively. Females have two transcribed copies of *TSPX* (*TSPX* is an X-inactivation escaper) while males have a maternal *TSPX* and a paternal *TSPY*. While the naming scheme may imply a common gene function that is simply located on different sex chromosomes, the two genes have been shown to have *opposite* functional effects on the androgen receptor. This difference is mediated by an inhibitory domain in TSPX that is absent in TSPY (Li et al., 2017).

More broadly, any gene that escapes X chromosome inactivation will be more highly expressed in XX cells than in XY cells. In a recent, extensive survey of 29 different tissues from 449 humans, researchers demonstrated that at least 23% of X chromosome genes are incompletely inactivated, and that the specific genes that escape inactivation vary across tissues and individuals (Tukiainen et al., 2017). The same study demonstrated that, surprisingly, most PAR genes are more highly expressed in males than in females. Finally, there are the genetic sex differences resulting from imprinting inequality. While males have only a maternal X chromosome, females have both a maternal and a paternal X chromosome. X-linked genes with differential expression due to maternal vs. paternal imprinting will have strongly different male/female expression patterns in adult tissues. For a review of the functional implications of genomic imprinting on brain development and function, please see (Huang et al., 2018).

Masculinization vs feminization of the fetal rodent brain

Traditionally, basic research on the impact of sex on embryonic development and maturation of the mammalian brain was necessarily limited to animal models. While there is much still undiscovered that connects the observed mechanisms in rodents to equivalent processes in humans, the current knowledge in animal models offers a fascinating insight in to sex

specific regulation of fundamental biological processes such as neurogenesis, glial proliferation, and synaptic function (reviewed in (McCarthy et al., 2017a)). Elegant work has illustrated how the rodent brain can be either “masculinized” or “feminized” during embryonic development depending on the hormonal environment. Embryonic rodent brains are protected from the effects of circulating maternal estrogen by alpha-fetoprotein which binds and sequesters circulating estrogen (Bakker et al., 2006). After gonadal differentiation, male and female embryos secrete fetal testosterone and estrogen, respectively, but while the fetal estrogen in female embryos is sequestered by alpha-fetoprotein (like the maternal estrogen), embryonic testosterone in male embryos is not and passes into the developing brain. In a fascinating twist, the aromatase enzyme, synthesized in the male embryonic brain, converts fetal testosterone to estrogen which then activates a transcriptional pathway resulting in a masculinization of the developing brain (Bakker et al., 1993; Bakker et al., 2006; Baum, 1979; MacLusky and Naftolin, 1981; VanRyzin et al., 2018). Thus, the female sex hormone estrogen (synthesized in the brain) is responsible for masculinization of the male rodent brain. In females, the sequestration of gonadal estrogen coupled to the lack of testosterone results in “feminization” of the brain.

Researchers have used this knowledge of rodent development to artificially masculinize the female brain or feminize the male brain. Treatment of newborn female pups with either testosterone, or else eliminating embryonic alpha-fetoprotein, results in brain masculinization of females while blocking the aromatase that converts testosterone to estrogen feminizes male brains. But what does it mean to be masculinized or feminized? In the case of the developing rat brain, several reproducible differences have been described with masculinization of the female brain or feminization of the male brain. One very intriguing finding is that during a particular developmental time window (postnatal day 4), there are a consistently greater number of microglia in specific regions of the masculinized rodent brain including the amygdala, parietal cortex, hippocampus, paraventricular nucleus of the hypothalamus, and the medial preoptic area (mPOA) (Schwarz et al., 2012). Additionally, mPOA of the masculinized brain has two-fold greater dendritic spine density than the feminized brain, which is a direct result of estrogen receptor activating the cyclooxygenase 2 gene that in turn stimulates prostaglandin synthesis in the developing masculinized mPOA (Amateau and McCarthy, 2002; Amateau and McCarthy, 2004; Wright et al., 2008; Wright and McCarthy, 2009). Beyond these observations are numerous detailed descriptions of sex differences in the neurophysiological, morphological and neurochemical makeup of the feminized vs masculinized rodent brain that are thoroughly reviewed elsewhere (McCarthy et al., 2017a). In some cases, the differences observed during development are transient, and the functional consequences of the particular trait on the adult brain are not yet known.

Male vs Female Endocannabinoid-based Synaptic Regulation

The endocannabinoid system modulates many biological processes that have been shown to be variable between the sexes including stress response, appetite regulation, thermoregulation, social behavior and anxiety, metabolism, analgesia, sleep, and reproduction. The discovery that there is a sexually dimorphic mechanism of action in this particular system hints at a potential molecular explanation for the described differences.

The continuing story of how the endocannabinoid response differs between males and females starts more than 35 years ago with the observation that physiological concentrations of 17-β-estradiol (E2) increased excitability of hippocampal pyramidal cells in electrophysiological analyses in brain slices (Smejkalova and Woolley, 2010; Teyler et al., 1980; Wong and Moss, 1992). Numerous follow-up analyses refined the understanding of this initial observation uncovering the specific molecular players that mediate the observed E2 response (Smejkalova and Woolley, 2010; Teyler et al., 1980; Wong and Moss, 1992). About a decade ago it was further shown that rather than relying on gonadal hormones for neuronal signaling, E2 is actually produced in both male and female hippocampal neurons through the local action of P450 aromatase (Hojo et al., 2004; Woolley, 2007). Then in a landmark study, while following up on a curious observation that E2 signaling is reduced in ERα knockout mice (it had previously been thought that E2 effect in the hippocampus was mediated by ERβ), it was demonstrated that E2 strongly suppressed inhibitory postsynaptic potentials in CA1 pyramidal neurons of the hippocampus in females but not at all in males (Fugger et al., 2001; Huang and Woolley, 2012). Unlike the previously observed excitatory effect, this E2 effect on inhibitory currents was shown to be mediated through ERα and required mobilization of the endocannabinoids and the activity of the Cannabinoid Receptor 1 (CB1R) (Huang and Woolley, 2012). This effect was further shown to require IP_3 signaling which is differentially regulated (through direct interaction of the IP_3R with mGluR1) in male and female hippocampal neurons (Tabatadze et al., 2015). Interestingly, this same study demonstrated a tonic endocannabinoid activity in females that is in Whether absent males. this different tonic activity contributes to the differential E2 response, and whether this is modulated directly by the sex chromosomes or some other mechanism is currently unknown. While these studies are necessarily done in a rodent model, they demonstrate clear molecular differences that affect the functioning of the endocannabinoid system between the brains of mammalian males and females.

Challenges relating rodent models to humans

The ability to freely manipulate the hormonal milieu and genetic composition of developing rodents has allowed researchers to perform near perfectly controlled comparisons between males and females as highlighted above. However, not all mechanisms will be conserved between rodents and humans and so it is important to understand how these different observations from model systems relate to human biology. It is still unclear if and to what extent the differential endocannabinoid mechanisms observed in rodents will apply to humans, however a recent tracer-based imaging study reported a 40% greater availability of CB1R in men relative to women (Laurikainen et al., 2019). With respect to brain masculinization in humans, insight can be gained by analyzing the brains of individuals with specific genetic conditions such as sex reversal due to complete androgen insensitivity syndrome (CAIS) resulting in XY females. A recent imaging study demonstrated that CAIS women displayed feminized brain morphology in some brain regions including thicker parietal and occipital cortices and stronger connection in the default mode network, but had masculinized morphologies in other regions including in the thickness of the motor cortex and the volume of the caudate nucleus (Savic et al., 2017). While the mechanism of the role of specific hormones in the masculinization or feminization of the embryonic human brain is still debated (Luoto and Rantala, 2018; Puts and Motta-Mena, 2018), the CAIS imaging

study, along with many others, point to a role for both hormonal environment and genetic complement as drivers in the masculinization and feminization of different parts of the human brain.

4. Emerging insights in sex differences: human studies

Relatively new technologies available in the last few decades are providing an unprecedented depth of information about the human brain. Here, we highlight two such technologies: 1) new methods of *in vivo* neuroimaging and 2) omics-level analyses such as bulk and single cell RNA sequencing, proteomics, epigenomic profiling and genome sequencing. These technologies are become more widely available due to both a reduction in cost and the development of user-friendly analytic tools. With more widespread application across multiple large cohorts, the power to detect and therefore study sex differences in the human brain will be realized.

Large Scale Neuroimaging Studies of Males and Females

It has long been known that there are morphological differences between the male and female brains of mammals and non-mammalian vertebrates, notably with the early observation that a region in the songbird brain that controls vocalization is visibly larger in male birds than in female birds (Nottebohm and Arnold, 1976). From that observation stemmed a series of studies interrogating differential morphology in rodent brains that led to the of the much referenced “sexually dimorphic nucleus discovery of the preoptic area” (SDN-POA) (Gorski et al., 1980) which is larger in male than in female rats due the previously mentioned effects of embryonic estrogen on the male brain (Davis et al., 1996). The identification of the SDN-POA was very important for the field of sex differences, and it became a model system for studying the mechanisms by which hormones impact brain structure. It’s discovery also affirmed the value of comparative approaches to studying sexual differentiation.

After description of the SDN-POA in rodents there were a series of descriptions of male-female differences in human brain structure and morphology using post mortem tissue, which compared differences in average size between specific nuclei or fiber tracts (reviewed in (Hines, 2002)). These studies were limited by the high degree of inter-individual variability owing to differences in age, health, environment and cause of death that, when coupled to low numbers of individuals, made it difficult to fully rule out other potentially confounding causes of the observed differences. The tremendous advancements made in imaging technology have facilitated some well-controlled large studies that have greatly increased our understanding of the sex differences in the structure and morphology of the living human brain (Jahanshad and Thompson, 2017). A recent highly-powered study using 5,216 living human subjects (2,750 females and 2,466 males ranging in age from 44–77) from the UK biobank analyzed brain imaging data, all generated from a single MRI scanner to minimize technical variability (Ritchie et al., 2018). The study found that, while on average males had a higher total brain volume and raw surface area, females had a higher cortical thickness and white matter complexity. They also saw a significantly greater variability of raw structural measurements among males than among females. A resting state

fMRI connectivity analysis in the same study demonstrated several sex differences including a stronger connectivity of the default mode network in females and a stronger connectivity in the sensorimotor and visual networks in males. Importantly, in addition to identifying new sex differences in brain structure, this study also confirmed a multitude of sex differences described in previously published studies that had lower numbers of individuals (reviewed in (Jahanshad and Thompson, 2017)).

It is worth noting here that the identification of sex differences in the human brain through imaging studies has been an area of controversy. Multiple early studies reporting sex differences in the brain between men and women were overhyped in the popular press and misused by some as biological evidence supporting female inferiority. Several of these studies were underpowered, and their findings not replicated in larger cohorts. These unfortunate events coupling weak study design to overinterpretation of results was quite damaging to the larger field of sex differences research, leading some to make conclusions such as “The history of sex-difference research is rife with innumeracy, misinterpretation, publication bias, weak statistical power, inadequate controls and worse” (Eliot, 2019). For the myriad of reasons discussed throughout this review, these past events should not deter researchers from considering sex as an important biological variable. Rather, they should remind us to be rigorous in our experimental design, and precise and clear in relaying the implications of our findings.

One of the primary reasons for investigating sex differences in the brain is to better understand and treat diseases of the nervous system. One powerful approach for investigating disease is to use advanced neuroimaging to follow the trajectory of pathological changes in the brain that relate to disease course. For example, diffusion tensor imaging and T1- and T2-weighted MRI have been used to describe sex differences in structural presentation and progression of MS and acquired demyelinating syndrome (Longoni et al., 2017; Schoonheim et al., 2014). A second example comes from the field of Parkinson’s disease research. PET and single photon emission computed tomography (SPECT) imaging studies have suggested that women have higher dopamine transporter densities than men in certain regions of the basal ganglia, supporting the hypothesis that the higher incidence of PD in men is in part determined by a higher “dopaminergic reserve” in women (Kaasinen et al., 2015; Laakso et al., 2002; Lavalaye et al., 2000; Mozley et al., 2001; Varrone et al., 2013; Yamamoto et al., 2017).

Longitudinal neuroimaging studies, wherein the same human subjects receive multiple imaging sessions over time, reduce the issues related to inter-individual variation. For studies of aging and AD, new longitudinal imaging studies for the pathological hallmarks of AD (Aβ-rich plaques and tau containing tangles) are providing key information about the early events and trajectory of the disease. A recent study using neuroimaging of plaques and tangles longitudinally in aging individuals was able to define tau and Aβ propagation patterns, and showed an association of tangles with cognitive levels (Sepulcre et al., 2018). In a related longitudinal study, it was shown that females with a high Aβ plaque burden show faster cognitive decline compared to males with equivalent plaque burdens (Buckley et al., 2018). The steadily increasing numbers of participants in studies such as these will be

instrumental in defining disease trajectory in males versus females, providing clues into the initiating events leading to differential risk.

Large scale omics level analyses of human brain: differential genetic regulation

Clearly there are quantifiable sex differences in the context of neurologic disease and the healthy human brain. In order to help translate these findings into actionable steps for personalized medicine, researchers need to understand the underlying neural chemistry that drives the observed differences. However, once molecular differences between men and women are observed, the identification of the *drivers* of those sex differences can be quite challenging. Transient differences in gene regulation, whether in response to hormones or differential expression of a sex chromosome gene, could affect a temporary change in a basic cellular process that results, over time, in potentially important differences. Thus, when molecular differences are identified, they could be nascent causal events (which may no longer be observable at later time points), or downstream indirect effects not related to causation but potentially relevant to clinical intervention. While there is a goal of understanding the mechanistic role of individual genes in neurobiology, our current era of genome-wide analyses has shifted much of the focus toward describing the entirety of the chemical make-up of a particular brain region or cell-type. Coupling these descriptions with a comparison of molecular signatures between different populations then can be used to highlight pathways that drive biological differences. A recent explosion of large cohort omics-level studies of the human brain includes tissue and single-cell RNA sequencing (RNAseq), unbiased and targeted proteomics, lipidomics, epigenetic profiling, and metabolomics. These are highly valuable techniques for identifying pathways relevant to sex differences in the brain but can be cost intensive and unapproachable for some researchers. Fortunately, advances in data-sharing technology along with new sharing guidelines enacted by funding institutions and a general positive trend toward open-science initiatives have collectively resulted in a number of collaborative data platforms and unprecedented access to large data sets.

Examining molecular sex differences in a comprehensive manner requires large numbers of samples. Fortunately, there are now several accessible databases representing large cohorts and tissue collections each with unique populations, clinical measurements and goals. One example is the Genotype-Tissue Expression (GTEx) project (Carithers et al., 2015). Data collected includes RNAseq and whole genome or exome sequencing. All of the data is open access, and the current release (v7) includes data from 11,688 samples, 53 tissues (including 13 different regions of the brain), and 714 healthy donors. The donors all range in age from 20–79, with 34.2% female and 65.8% male. The GTEx data portal (http://gtexportal.org) allows one to search for any gene, and returns expression levels across tissues, with a radio button that breaks down the data by sex. One of the first studies using GTEx data was the previously discussed analysis of X-chromosome inactivation across tissues and individuals (Tukiainen et al., 2017).

There are handful of other open access omics datasets of human brain that are targeted to more specific populations and allow for examinations of molecular sex differences in disease states. One such initiative was established through the NIA-led Accelerating Medicines

Partnership-Alzheimer's Disease (AMP-AD) Target Discovery and Preclinical Validation Project (https://www.nia.nih.gov/research/dn/amp-ad-target-discovery-and-preclinical-validation-project). Data from this project can be found through the AMP-AD Knowledge Portal (https://www.synapse.org/ampad), which houses data obtained from both human and model systems. Within this database are three major sources of human brain data: the Mount Sinai Brain Bank (Allen et al., 2016; Wang et al., 2018), the Mayo Clinic (Allen et al., 2016; Wang et al., 2018), and the Religious Order Study (ROS) and Memory and Aging Project (MAP) of Rush University (Bennett et al., 2012a; Bennett et al., 2012b; De Jager et al., 2018). A variety of different omics level molecular data are acquired from the brain in these studies. For example, for the ROS and MAP cohorts, the following data sets are available (number of human donors included shown in parentheses): SNP (2,090); WGS (1,196); RNAseq (639); miRNA (744); H3K9Ac-ChIP-Seq (728); and DNA methylation (740). All of these studies in AMP-AD have comparable proportions of males and females, providing rich data sets to interrogate sex differences at the molecular level. A large meta-analysis of expression across different brain regions using the AMP-AD database uncovered two separate gene expression clusters with consistent and strong heterogeneity between males and females in AD-related expression (Logsdon et al., 2019).

The CommonMind Consortium (CMC) and PsychENCODE Consortium (PEC), which are focused on psychiatric disorders such as schizophrenia and bipolar disorder, are other examples of efforts aiming to collect large-scale omics level data from human brain. Data generated by these consortia is distributed through the PEC and CMC knowledge portals hosted on the Sage Bionetworks Synapse system: https://www.synapse.org/pec and https://www.synapse.org/cmc. While the primary driving force behind these projects is not the study of sex differences, these databases may be sufficiently powered to interrogate certain sex differences. A recent study using transcriptome and epigenome data from PsychENCODE sought to comprehensively compile human brain data across developmental time, brain region, and cell type (Li et al., 2018). Included in the study was an analysis of sex differences in the transcriptome over developmental time from 8 weeks post conception to adult (40 yrs). This study identified modules of co-expressed genes that were enriched for male-biased or female-biased expression during particular developmental windows. Interestingly, many of these modules showed sex-bias only during a specific developmental window, and some even switched from being male-biased to female-biased (or vice-versa) between different developmental windows. These findings were based on data from 18 males and 23 females spread over a ~41-year age range. Follow up studies interrogating larger numbers of males and females at targeted developmental windows will provide greater power to define the molecular differences between male and female brain development.

Gene expression studies in specific diseases have the potential to uncover differential mechanisms of disease in men and women. For example, transcriptomic studies of corticolimbic brain areas revealed that different sets of transcripts are significantly upregulated or downregulated in males versus females with MDD (Labonte et al., 2017; Seney et al., 2018). Importantly, some of the male-female differences observed in the human brain also were observed in the mouse brain in a chronic variable stress model (Labonte et al., 2017). The linking of studies of sex differences in the human brain to studies in animal

models is a powerful approach for the identification of convergent mechanisms of brain function and disease.

While these expression analyses can highlight molecular differences in the brain, genetic studies such as genome wide association studies (GWAS) and family-based studies provide a method for identifying genetic influences on disease and prioritization of pathways likely to play causative roles. However, few genetic studies separate data by sex, mainly because this dramatically lowers the power of the study. Thus, aside from X-linked diseases, for most genetic risk factors of neurological disease it is not known whether there are differential impacts on men versus women. One exception to this is the ε4 allele of the apolipoprotein E (APOE) gene, which is the greatest genetic risk factor for late-onset Alzheimer's disease (Farrer et al., 1997). For many decades, the ε4 allele of APOE was described as being more detrimental for women than for men (Bretsky et al., 1999; Farrer et al., 1997; Payami et al., 1996; Poirier et al., 1993). However, a recent meta-analysis of 27 independent studies including ~58,000 participants revealed that the elevated risk for AD in female ε4 carriers over males was restricted to earlier age ranges while the lifetime risk of AD conferred by the ε4 allele is not different between men and women (Neu et al., 2017). In addition to studies of relative risk, neuroimaging studies have shown differences between male and female ε4 carriers. These include differences in: hippocampal atrophy (Holland et al., 2013); cortical thinning (Sampedro et al., 2015); and connectivity in the default network (Damoiseaux et al., 2012). Future studies such as these that couple neuroimaging with examinations of genetic risk factors will provide a powerful resource for interrogating sex differences in human disease.

5. Conclusion

There is insufficient space in any review article to cover all of the work (both historical and emergent) that has provided mechanistic insights into observations of sex differences in neurobiology and disease. We have presented here a relatively small sampling of observations that demonstrate the extraordinary reach of the descriptions of differential neurobiology between females and males. These differences and dimorphisms are wide-spread, persistent and have been shown to result in impactful long-term differences in clinical measures. Understanding the mechanism of exactly how these differential clinical outcomes develop over time holds a very real promise of significantly advancing personalized medicine for everyone.

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Highlights

- Virtually every neurodegenerative and neuropsychiatric disease shows differences between males and females with respect to risk, presentation, trajectory and/or pathology.
- While there is genomic variation across ethnicity, the greatest genetic variability between individuals is between males and females.
- Sex differences in the brain may be a result of differential gonadal hormones, X and Y chromosome complement, differential societal influences or a combination of these.
- Most of the sex differences observed are differences in the population average of a particular measurement with considerable overlap between men and women.
- New studies using neuroimaging and large-scale omics approaches are providing an everexpanding understanding of sex differences in human neurobiology and disease