Sex-Dependent Mechanisms of Synaptic Modulation

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Introduction

The recent emphasis on including sex as a variable in preclinical neuroscience is motivated by a goal of making the results of basic research relevant to both sexes. Although sex inclusion is often interpreted as meaning that researchers should prioritize the investigation of sex differences above other scientific interests, this is not the case. Sex differences do, however, provide the rationale for balancing sex in the subjects of preclinical studies. Because of the growing number of identified differences between males and females, including in brain areas and processes for which there is little reason, *a priori*, to expect the sexes would differ, it is clear that experimental results from one sex cannot be assumed to apply to both (Shansky and Woolley, 2016). Thus, to the extent that preclinical studies provide crucial new ideas and information to stimulate and guide clinical research, conducting vertebrate animal experiments in both sexes broadens their potential impact. Conversely, limiting animal experiments to one sex (or failing to note the sex of the animals used) runs the risk of missed opportunities for understanding fundamental mechanisms as well as potentially costly mistakes if and when results from one sex are applied to both without validation.

Here, I will discuss three types of sex differences in the brain; illustrate two of these with examples from our work on neurosteroid estrogen modulation of synaptic transmission in the hippocampus; and explain some of the choices we have made in experiments that have revealed "intrinsic" sex differences. These topics address two questions that come up often in discussions with colleagues and trainees: (1) Why should I include both sexes in my experiments? and (2) How should I include both sexes in my experiments?

Types of Sex Differences

Quantitative differences

Broadly speaking, sex differences can be divided into two categories: quantitative and qualitative. In a quantitative sex difference, each sex has or does something, but one sex has or does more of that thing than the other sex. This would most commonly be revealed in an experiment by a difference between the sexes in the measured distributions of a particular variable (Fig. *1A*). Examples of quantitative sex differences include those of height, responses to stress, and, at the population level, the incidence of numerous diseases and disorders.

The majority of sex differences in the brain that have been identified are quantitative differences, which may contribute to many skeptics' views that sex inclusion in animal research is more trouble than it's worth. That is, if sex differences manifest simply as shifts (often small ones) (Maney, 2016)

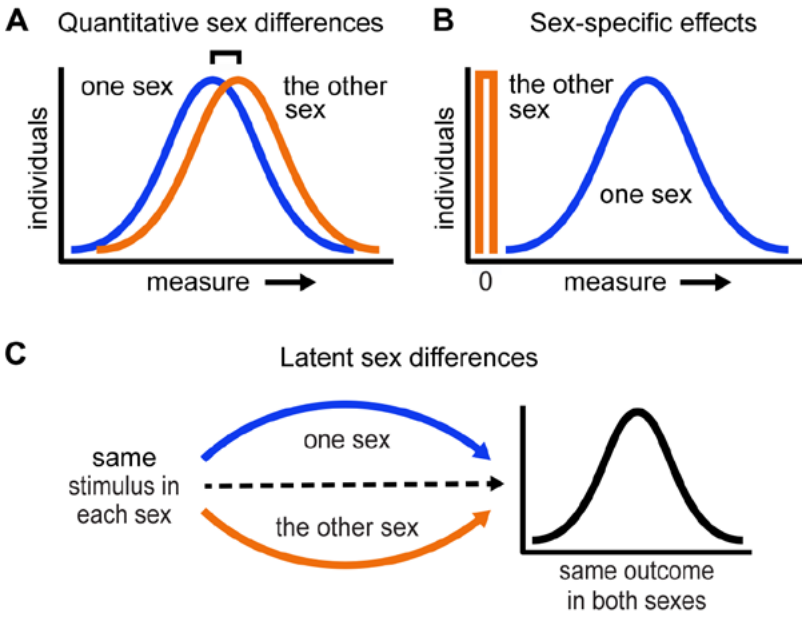

Figure 1. Types of sex differences. ***A***, Quantitative sex differences are evident as differences between the sexes in the distributions of an experimental measure. ***B***, Sex-specific effects are evident when an experimental variable can be measured in one sex but the other sex scores zero for measures of that variable. ***C***, Latent sex differences are indicated when a particular stimulus produces the same outcome in both sexes but acts through distinct underlying mechanisms in each sex.

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in the distributions of experimental measures, then including both sexes in a study could increase variance without much payoff in terms of fundamental new information gained. Moreover, accommodating additional variance could require increasing the number of animals needed either to achieve comparable statistical power in an experiment that combines sexes or to conduct separate analyses within each sex. These concerns have prompted a "limited resources" argument for focusing experiments on one sex or the other.

In many cases, however, quantitative sex differences are an indication of distinct underlying mechanisms in each sex. For example, it is well known that men and women differ in the incidence of major depressive disorder (MDD), reflecting a quantitative sex difference. Yet analyses of gene expression in corticolimbic brain areas of male and female MDD patients versus healthy controls reveal that profoundly different sets of transcripts are significantly upregulated or downregulated in men versus women with MDD (Labonté et al., 2017; Seney et al., 2018). Moreover, in the Labonté et al. study (2017), sex-dependent gene-expression differences in human patients were mainly recapitulated in a chronic variable stress model in mice. These types of observations strongly suggest that quantitative sex differences in disease incidence, of which there are many, signal mechanistic differences between the sexes and that animal studies will be useful for understanding the basis of those differences.

Qualitative differences

In contrast to quantitative differences, qualitative sex differences show directly that males and females differ in fundamental mechanisms. Qualitative sex differences come in (at least) two varieties: sex-specific effects and latent sex differences. In a sex-specific effect, one sex has or does something that the other sex does not have or do. Thus, one might map the distribution of a measured variable in one sex and find that all members of the other sex score zero for that measure (Fig. *1B*). In addition, in some cases, males and females show opposite responses. For example, in the Seney et al. study (2018), while only 73 of 1027 MDD-regulated genes were common to both sexes, 52 of those genes in common were regulated in opposite directions in men compared with women.

Although some sex-specific effects are predictable (being related to reproductive physiology or behavior), others are not. Several years ago, we discovered sex-specific molecular mechanisms in studies of inhibitory synaptic modulation in the hippocampus, discussed in more detail below (Case 1). These effects could not have been anticipated based on known sex differences in behavior or mechanistic differences apparent in the published literature. Additional sex-specific effects are surely on the horizon as more neuroscientists begin to use both sexes in their work. One potential impact of sex-specific molecular signaling is the possibility that therapeutics derived from mechanistic studies that focus on only one sex could be ineffective or have unanticipated consequences in the other sex.

A second type of qualitative sex difference revealed in our studies of synaptic modulation is what we have termed "latent sex differences." In a latent sex difference, a particular stimulus produces the same outcome in both sexes, but this outcome is achieved through distinct underlying mechanisms in each sex (Fig. *1C*). Latent sex differences, by definition, would not be discoverable by comparing simple stimulus-response relationships in each sex; rather, such differences can be identified only through mechanistic studies done in each sex.

Latent sex differences are reminiscent of De Vries's description of compensatory sex differences (De Vries, 2004), which posits that the significance of some sex differences may be to compensate for other sex differences, making males and females more similar at the behavioral level rather than more different. The extension of this concept to molecular mechanisms of synaptic modulation, explained in more detail below (Case 2), is also meaningful when translating basic studies into the development of therapeutics. Latent sex differences indicate that molecular mechanisms targeted for drug development may differ between males and females even in the absence of an overt sex difference in disease.

Case 1: Sex-Specific Mechanisms of Inhibitory Synaptic Modulation

We discovered sex-specific mechanisms of inhibitory synaptic modulation quite by accident, during studies aimed at understanding neurosteroid estrogen actions in the hippocampus. Although estrogens are commonly thought of as reproductive hormones important mainly in females, they are also synthesized as neurosteroids in the hippocampus of both sexes. There, they activate downstream signaling initiated by extranuclear estrogen receptors (ERs) to influence seizure susceptibility (Sato and Woolley, 2016), synaptic plasticity (Vierk et al., 2012), and memory (Tuscher et al., 2016). We found that the steroid 17β-estradiol (E2) acutely suppresses perisomatic

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inhibitory synapses in the hippocampus of ovariectomized female rats but not of castrated (or gonadally intact) male rats (Fig. 2A) (Huang and Woolley, 2012). This sex specificity was surprising because E2 was already known to acutely regulate excitatory synapses in the hippocampus of both sexes (Case 2, below).

Further experiments using electrophysiological, biochemical, anatomical, and molecular techniques showed that E2 suppresses inhibitory synapses in females through membrane-associated estrogen receptor-alpha (ERα), which interacts with metabotropic glutamate receptor-1 (mGluR1). When E2 stimulates this interaction (in females), it results in activation of phospholipase C (PLC) and the production of inositol triphosphate (IP3); in turn, IP3 activation of the IP3 receptor increases intracellular calcium and leads to postsynaptic mobilization of the endocannabinoid anandamide (AEA), which is transported across the cell membrane to inhibit presynaptic GABA release (Fig. 2B) (Tabatadze et al., 2015). Interestingly, although the hippocampus of males has all the molecular components of this pathway, E2 does not stimulate ERα–mGluR1 or mGluR1–IP3R interactions in males. Thus, the molecular signaling activated by neurosteroid estrogens differs profoundly between the sexes.

These experiments led to the discovery of a second sex-specific effect with immediate translational implications. We found that an inhibitor of fatty acid amide hydrolase (FAAH, the enzyme that hydrolyzes AEA) suppresses inhibitory synapses in the hippocampus of females, but not males (Fig. 2C) (Tabatadze et al., 2015). This indicates tonic release of FAAH-sensitive endocannabinoids in the hippocampus of females that is absent in males. Endocannabinoids are known to influence many diverse aspects of physiology and behavior, including learning and memory, motivational state, appetite, responses to stress, and pain; they are also involved in neurological disorders such as epilepsy. As such, the enzymes that regulate endocannabinoid levels are targets for therapeutic development. Indeed, when our study was published, the same FAAH

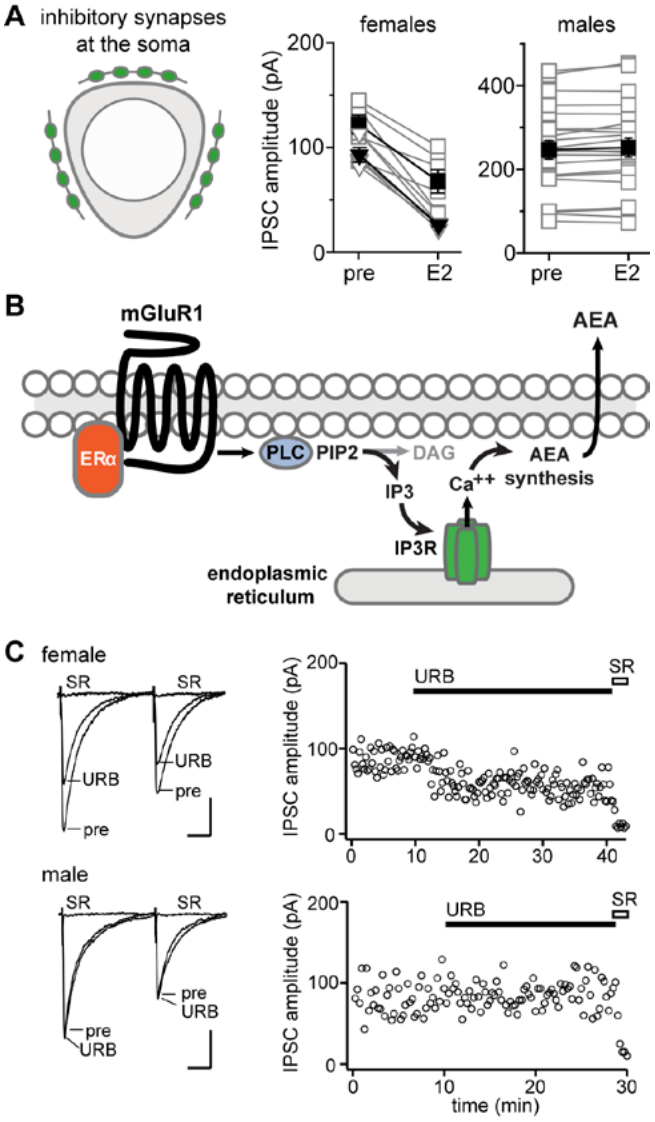

Figure 2. Sex-specific mechanisms of inhibitory synaptic modulation in the hippocampus. ***A***, Inhibitory postsynaptic currents (IPSCs) evoked by stimulation of perisomatic synapses are suppressed by E2 in females, with no effect in males. Squares represent compound IPSCs; triangles represent unitary IPSCs. Individual recordings are in gray; means ± SEM are in black. ***B***, Schematic of the sex-specific mechanism by which E2 mobilizes the endocannabinoid AEA to suppress GABA release only in females. DAG, diacylglycerol; PIP2, phosphatidylinositol 4,5-bisphosphate. ***C***, The FAAH inhibitor URB 597 (URB) suppresses inhibitory synapses in females but not in males. SR, a $GABA_A$ receptor blocker. Modified with permission from Huang and Woolley (2012), Figs. 1, 4; copyright 2012, Elsevier; and Tabatadze et al. (2015), Figs. 8, 9; copyright 2015, The Authors.

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inhibitor that we used (URB 597) had already been tested in a human clinical trial, presumably without the knowledge that it could affect the brains of males and females differently. Two previous animal studies (Hajos et al., 2004; Kim and Alger, 2004) had reported no effect of URB 597 on inhibitory synapses in the hippocampus. However, these previous studies were done only in males, which is also true of the majority of animal studies suggesting endocannabinoid metabolic enzymes as therapeutic targets (Fowler, 2015). This latter point underscores the importance of balancing sex in preclinical studies so that researchers can determine whether molecular mechanisms that suggest drug targets operate similarly or differently between the sexes.

Case 2: Latent Sex Differences in Excitatory Synaptic Modulation

A second line of research on neurosteroid estrogens focuses on excitatory synaptic modulation. It has been known for decades that applying E2 to rat hippocampal slices can potentiate excitatory synapses in both sexes (Teyler et al., 1980; Wong and Moss, 1992). However, initial studies aimed at understanding the mechanism(s) of this effect were done in different sexes and came to different conclusions about the mechanisms involved. Kramar et al. (2009) studied male rats and found that E2-induced synaptic potentiation is caused by a postsynaptic increase in glutamate sensitivity, whereas our group studied female rats and found that potentiation occurs through a presynaptic increase in glutamate release probability (Smejkalova and Woolley, 2010). Both groups reported that estrogen receptor-beta (ERβ) is critical to E2-induced synaptic potentiation.

To resolve this apparent discrepancy, we tested how E2 or agonists of each of three ERs (ERα, ERβ, and G-protein coupled estrogen receptor-1 [GPER1]) affect miniature EPSCs (mEPSCs), which can distinguish presynaptic versus postsynaptic modulation. These experiments showed that E2 itself increases both mEPSC frequency (presynaptic) and mEPSC amplitude (postsynaptic) in both sexes (Fig. 3A), although mainly in separate subsets of cells in each sex (Oberlander and Woolley, 2016). Then we found that, in females, an ERβ agonist increased

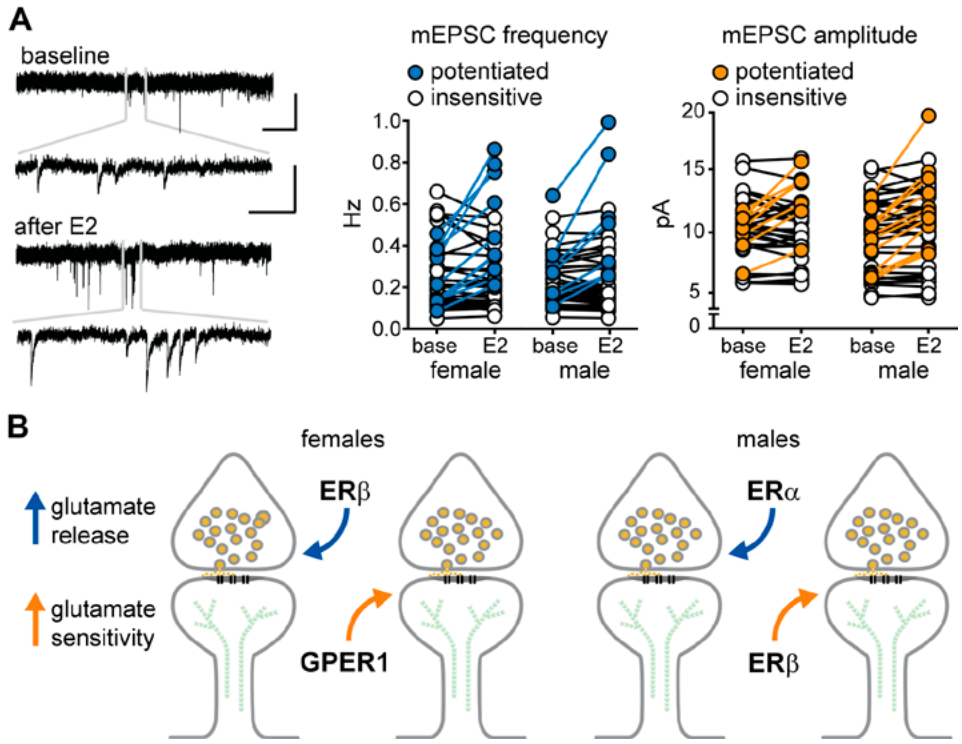

Figure 3. Latent sex differences in excitatory synaptic modulation in the hippocampus. ***A***, E2 increases both mEPSC frequency (blue) and mEPSC amplitude (orange) in a subset of hippocampal neurons in both females and males with no apparent sex differences. Colored symbols show cells with an effect of E2; open symbols show cells with no effect. ***B***, Schematic showing how presynaptic and postsynaptic effects of E2 on hippocampal excitatory synapses are mediated by a distinct combination of estrogens receptors in each sex. Modified with permission from Oberlander and Woolley (2016, 2017), Figs. 1, 8; copyright 2016 and 2017, The Authors.

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mEPSC frequency (not amplitude), whereas in males, it increased mEPSC amplitude (not frequency). Thus, the conflict in the literature resulted from a sex difference! We then found that the postsynaptic component of potentiation in females is mediated by GPER1 and the presynaptic component in males is mediated by ERα, completing the puzzle of E2 potentiation of excitatory synapses. Together, these results demonstrated a latent sex difference in which E2 produces the same outcome in males and females—increased synapse strength through both presynaptic and postsynaptic modulation—but this outcome is mediated by a distinct combination of ERs in each sex (Fig. *3B*).

As was the case for sex-specific effects, this latent sex difference in neurosteroid estrogen action is important for the translation of basic mechanisms to clinical studies. For example, ERβ agonists have been suggested as therapeutics for Alzheimer's disease (Zhao et al., 2015) and are currently in a clinical trial for negative and cognitive symptoms in schizophrenia (ClinicalTrials.gov, 2013). Given the distinct effects of ERβ activation on presynaptic versus postsynaptic components of synaptic transmission in the hippocampus of females versus males, it is reasonable to speculate that ERβ agonists may have different physiological/behavioral consequences in women versus men treated with these drugs.

Studying Intrinsic Sex Differences versus Hormone Effects

Some sex differences in the brain are intrinsic differences that do not depend on circulating gonadal hormones. Intrinsic sex differences are driven by many related factors, including the direct effects of sex chromosome genes, the organizational effects of hormones during early development, and epigenetic chromatin modifications (Arnold, 2017). The majority of experiments described above were performed in animals that were gonadectomized as adults, eliminating circulating hormones as drivers of the sex differences we observed. This reflects a conscious choice that has both advantages and limitations. The principal advantages are to simplify experiments by reducing the number of variables that differ between males and females and to establish baselines on which circulating hormones act in each sex. Gonadal hormones have been shown to affect a wide variety of endpoints, however, including synaptic plasticity in the hippocampus (Warren et al., 1995; Good et al., 1999; Harte-Hargrove et al., 2015). As such, when translational implications of particular research findings arise, we also test our findings in gonadally intact males and females (e.g., Sato and Woolley, 2016). This is important because gonadal hormones are an essential component of physiology and, of course, most patients who would be treated with drugs are gonadally intact.

The most straightforward way to design a sex-inclusive experiment is to use both sexes in a 50:50 ratio and plot data from individual subjects by sex. This is how we begin all our experiments. Indeed, this approach led one of my colleagues to discover a completely unanticipated sex difference in the neurophysiology of cerebellar nuclear neurons in prepubertal mice, and in the responses of those neurons to mutation of the autism-linked *Gabrb3* gene (Mercer et al., 2016). If and when the possibility of a sex difference is indicated, variance in an initial dataset can be used to estimate the sample sizes necessary to evaluate sex differences statistically, if this of interest. Irrespective of whether sex differences are a focus of the research being conducted—or are even apparent in a dataset—reporting the number of males and females in each experiment and plotting data by sex in figures increases the value of reported research results. This practice is the best way to establish for the broader scientific community, now and in the future, whether specific research findings apply to one sex, the other sex, or both.

Acknowledgments

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