SHORT COURSE 1:

Sex Differences in the Brain: Balancing Sex in Preclinical Research

Organized by Jill Becker, PhD, and Jessica Tollkuhn, PhD

SOCIETY *for* NEUROSCIENCE

NEUROSCIENCE 2018

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NEUROSCIENCE 2018

SHORT COURSE 1

Sex Differences in the Brain: Balancing Sex in Preclinical Research

Organized by Jill Becker, PhD and Jessica Tollkuhn, PhD
Friday, November 2, 2018
8 a.m.–6 p.m.
Location: San Diego Convention Center • Room: 6B

TIME	TOPIC	SPEAKER
7:30–8 a.m.	CHECK-IN	
8–8:10 a.m.	Opening Remarks	Jill Becker, PhD • University of Michigan Jessica Tollkuhn, PhD • Cold Spring Harbor Laboratory
8:10–9 a.m.	Developmental Origins of Sex Differences in the Brain	Margaret M. McCarthy, PhD • University of Maryland
9–9:30 a.m.	The Stress Response: Sex-Specific Neural Mechanisms	Debra Bangasser, PhD • Temple University
9:30–10 a.m.	Behavior Paradigms I: Sex-Specific Responses	Rebecca Shansky, PhD • Northeastern University
10–10:30 a.m.	BREAK	
10:30–11:20 a.m.	Sex-Dependent Mechanisms of Synaptic Modulation	Catherine Woolley, PhD • Northwestern University
11:20–11:50 a.m.	Behavior Paradigms II: Reward and Affective Behaviors	Laura O'Dell, PhD • University of Texas, El Paso
11:50 a.m.–12:20 p.m.	Dissecting Sex-Typical Neural Circuitry	Jessica Tollkuhn, PhD • Cold Spring Harbor Laboratory
12:20–1:20 p.m.	LUNCH — ROOM 33	
1:20–2:10 p.m.	Adolescence and Puberty	Deena Walker, PhD • Icahn School of Medicine at Mount Sinai
2:10–3 p.m.	The Importance of Estradiol as a Neuroprotectant in Both Sexes	Colin Saldanha, PhD • American University
3–3:50 p.m.	How to Study Female and Male Rodents — Takeaway Lessons	Jill Becker, PhD • University of Michigan
3:50–4 p.m.	Summary and Introduction of Breakout Sessions	Jill Becker, PhD and Jessica Tollkuhn, PhD
4–4:15 p.m.	BREAK	

AFTERNOON BREAKOUT SESSIONS • PARTICIPANTS SELECT FIRST DISCUSSION GROUPS AT 4:15 P.M.

TIME	BREAKOUT SESSIONS	SPEAKERS	ROOM
4:15–5 p.m.	Group 1: Stress, Learning, and Vulnerability	Debra Bangasser, PhD and Rebecca Shansky, PhD	29A
	Group 2: Reward Circuitry and Addiction: Differential Effects of Drugs of Abuse in Females and Males	Jill Becker, PhD and Laura O'Dell, PhD	29B
	Group 3: Neuroimmune Interactions: The Role of Microglia	Margaret M. McCarthy, PhD and Colin Saldanha, PhD	29C
	Group 4: Sex Differences in Gene Expression: Contributions of Steroid Hormones and Sex Chromosomes	Deena Walker, PhD and Jessica Tollkuhn, PhD	29D
5–5:15 p.m.	BREAK		

AFTERNOON BREAKOUT SESSIONS • PARTICIPANTS SELECT SECOND DISCUSSION GROUPS AT 5:15 P.M.

5:15–6 p.m.	Same as sessions above

Table of Contents

Introduction

Many, if not most, neurological and psychiatric disorders show a sex bias in incidence, etiology, or age of onset. Yet most neuroscientists still perform their experiments in a single sex, usually males. To address this discrepancy, the National Institutes of Health now mandates that researchers consider "sex as a biological variable" in their study design. It is therefore crucial for scientists to possess a sound understanding of how sex differences in the brain manifest and how these differences can influence development, brain function, and disease susceptibility.

In addition to contributions from sex chromosomes, sex differences in the vertebrate brain are regulated largely by steroid hormones that originate both in the gonads and locally in the brain. These hormones act in early life to specify sex-typical neural circuitry and, following puberty, to modulate neural activity and signaling pathways within the sexually differentiated brain. Recent work has made significant progress toward illuminating sex differences in sophisticated behavior paradigms, mapping the circuitry that mediates sex-typical behavioral responses, and defining molecular programs that both instruct and reflect these differences. The study of sex differences is now rapidly expanding beyond the realm of a specialized field to emerge as a principle consideration in the design of reproducible, clinically relevant research.

This course is designed to enable neuroscientists to incorporate both sexes into their preclinical research. Participants will become familiar with molecular, neural circuit, and behavioral differences between the sexes, with a focus on rodents. Leading experts will review fundamental concepts and the latest discoveries, including the developmental origins of sex differences, gonadal steroid hormones, stress and vulnerability, reward and affective behaviors, and adolescence and puberty. Experimental design considerations and statistical analyses will also be discussed.

How to Study Female and Male Rodents

Jill B. Becker, PhD

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Ann Arbor, Michigan

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Introduction

This chapter discusses how to think about and determine the appropriate manipulations and procedures for investigating sex differences in, and the effects of gonadal hormones on, experimental outcomes in adult rats and mice. I will also discuss estrous cycles, surgical procedures, and hormone treatments. I will conclude with a discussion of variability and statistical methods that can be used to minimize animal numbers when adding sex as a biological variable to your research.

What Is a Sex Difference?

The first question researchers usually ask is whether there is a sex difference in a trait. The answer to this question is not a simple "yes" or "no"; it turns out to be more complicated. As illustrated in Figure 1*A*, males and females can exhibit different traits, as is true for reproduction. For many traits, however, both females and males exhibit the trait, but there are differences in how it is expressed (Figs. 1*B*,*C*) or the mechanisms that mediate it (Fig. 1*D*) (Becker et al., 2016; Becker and Koob, 2016; Sanchis-Segura and Becker, 2016). When a sex difference is found, some investigators will want to determine more about the neurobiological processes that are responsible for the differences.

Effect of Gonadal Hormones on a Trait

One of the next questions that will arise is whether gonadal hormones have an effect on the trait. Two approaches can help determine whether this is the case. One can examine whether the female's behavior varies with the estrous cycle. Alternatively, one can remove the gonads by ovariectomy (OVX) or castration (CAST) and then selectively replace hormones. We will address the estrous cycle first.

Determining Estrous Cycle Stages

The estrous cycle is the product of the hypothalamic-pituitary-gonadal (HPG) axis that results in a cyclic release of ovarian hormones, ovulation, and sexual receptivity. It is analagous to the human menstrual cycle, except that in rats and mice, the cycle is much

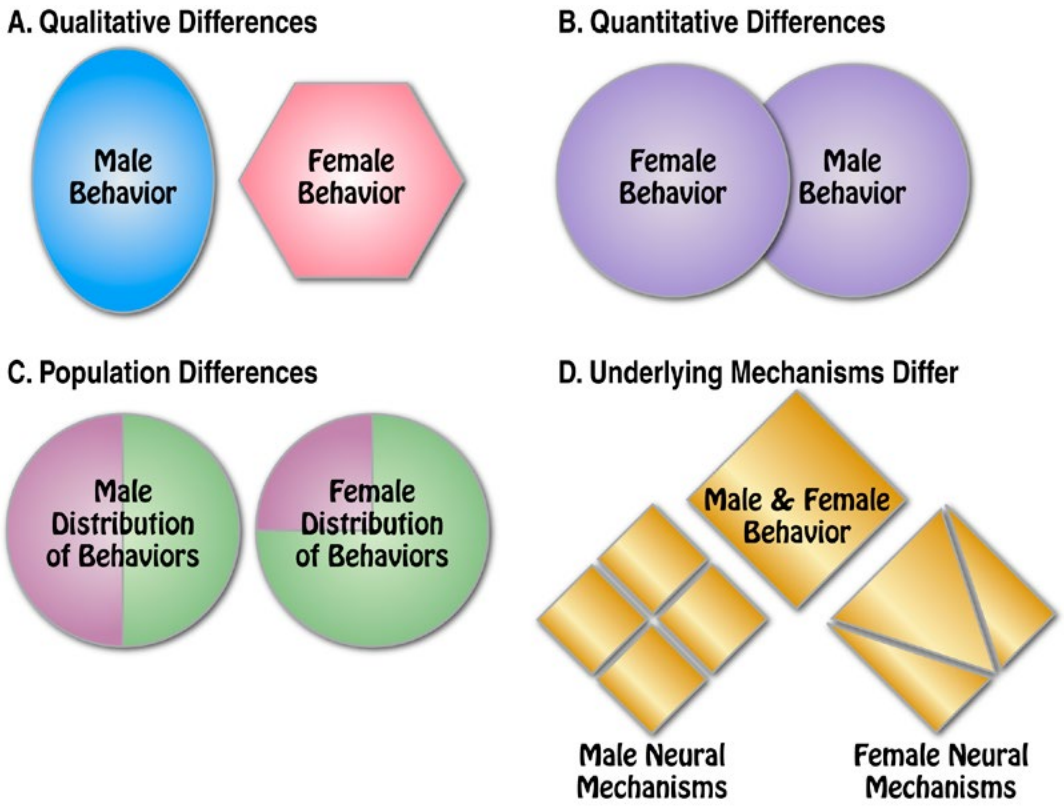

Figure 1. Four types of sex differences that can be observed: qualitative, quantitative, population, and mechanistic, also referred to as compensatory, divergent, or latent sex differences. Reprinted with permission from Becker and Koob (2016), Fig. 1. Copyright 2016, American Society for Pharmacology and Experimental Therapeutics.

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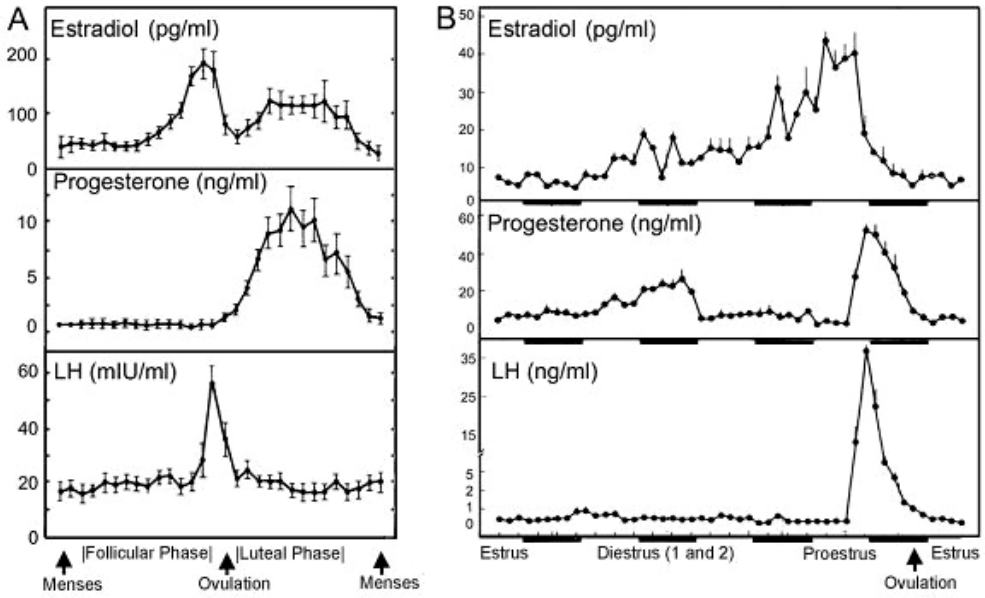

Figure 2. Patterns of estradiol, progesterone, and luteinizing hormone (LH) in the female human (***A***) and rat (***B***) during the reproductive cycle. Time unit of the *x*-axis in ***A*** is days; in ***B***, *x*-axis is hours. ***B***, Dark bars, Dark period of the day/night cycle. Note that during the follicular phase in humans and its analog in rats (diestrus), 17β-estradiol rises but progesterone secretion remains low. After the LH surge, progesterone is elevated in both rats and women. In women, the corpus luteum also secretes some 17β-estradiol, whereas in rats, during the brief luteal phase, 17β-estradiol concentrations decline. Reprinted with permission from Becker et al. (2005), Fig. 2. Copyright 2005, The Endocrine Society and Oxford University Press.

shorter (4 or 5 days), the luteal phase is truncated, and there is no menstruation (Fig. 2). This means that when studying rodents, the greatest intracycle variation in circulating hormones occurs when comparing late proestrus/early estrus (the period immediately before and after ovulation) with diestrus (the period of lowest circulating hormones). Thus, initial investigations into whether the estrous cycle has an effect on a trait could begin by comparing estrus with diestrus. This is usually sufficient to determine whether endogenous ovarian hormones are exerting an effect, as long as the animals were correctly evaluated for stage of the estrous cycle (see below). For methods used to collect and assess vaginal cytology data, please see Becker et al. (2005).

Because of the rapidly changing serum concentration of hormones that characterizes the estrous cycle in rats and mice, time of day is critical for interpreting cycle stage and even vaginal smears. Stages of the estrous cycle, relative to the day–night cycle, are illustrated in Figure 3. It is a generally accepted practice that one needs at least two complete estrous cycles to correctly determine from the vaginal smears where a female is in the cycle. This is because the vaginal cytology needs to be interpreted in context. For example, the image depicted for proestrus in Figure 3*B* has mostly round cells with dimples in the middle that are charcteristic of proestrus (nucleated epithelial cells), but it also has a few irregualrly shaped cells that are characteristic of estrus (cornified cells).

The images in Figure 3*B* were obtained during the dark phase of the cycle, but it is possible to obtain smears that resemble proestrus during the morning (light phase) of diestrus 1 (also referred to as metestrus). Without the information about the preceding and following days' vaginal cytology, even an experienced neuroendocrinologist could not tell proestrus and metestrus smears apart and successfully determine which stage of the cycle the rat is in. Even estrus can be misleading, as stress and experimental manipulations can result in a prolonged period of estrus that may or may not reflect a true estrus. In my experience, an animal that is not cycling regularly (exhibiting 1 day of estrus every 4 or 5 days) does not show the same behavioral, neuroendocrine, or neurochemical patterns as animals that are cycling regularly, and so the animal is excluded before data collection. Thus, it is important to accurately stage your female animals if looking for effects of the estrous cycle.

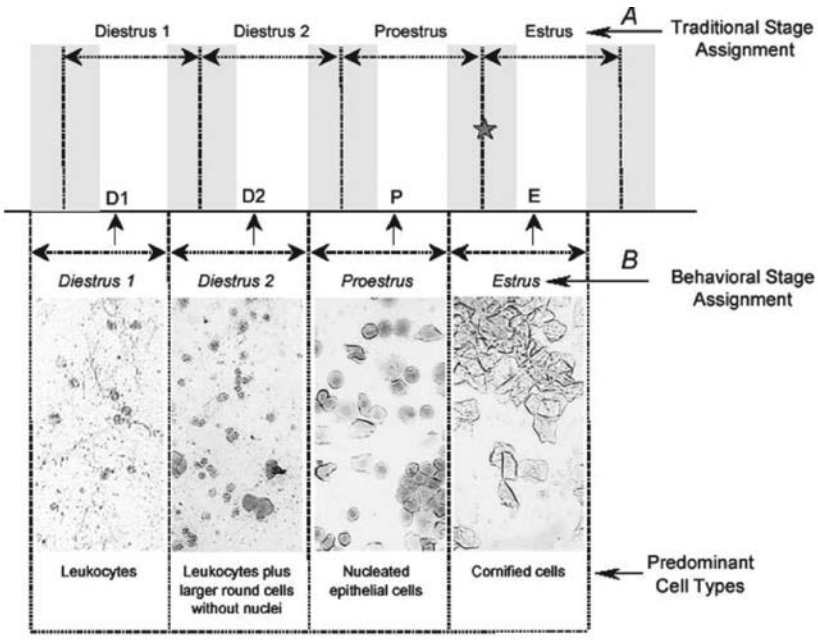

Figure 3. Stage assignment across a rat's 4 d estrous cycle in relation to a 12 h light/12 h dark cycle and samples of vaginal cytology. Top, Shaded bars denote successive 12 h dark periods. Vertical arrows, Time (early-to-middle light phase) when vaginal cytology is typically sampled. Gray star, Time of ovulation (4–6 h into the dark phase). Bottom, Representative photomicrographs and a brief summary of the cell types that predominate during each cycle stage. ***A***, Traditional stage assignment. ***B***, Behavioral stage assignment during the four successive dark periods. Reprinted with permission from Becker et al. (2005), Fig. 3. Copyright 2005, The Endocrine Society and Oxford University Press.

As Figure 4 shows, by testing males and females on proestrus/estrus and diestrus, one can see both sex differences and effects of the estrous cycle. Although it may be easier to present and discuss data as depicted in the top diagram, the bottom diagrams more clearly conceptualize results as rapidly changing in response to changes in ovarian hormones. Thinking about and modeling the dynamic nature of systems that are changing can help one to understand the systems, how they are related to each other, and how they related to hormonal changes during the estrous cycle.

This brings up another point: the time course of steroid hormone effects on brain and behavior can range from milliseconds to days. Thus, one cannot assume that changes in traits associated with a phase of the estrous cycle are caused by specific hormones in the blood at the time of an event without removing the endogenous source of the hormones (the gonads) and then selectively replacing the hormone or hormones. For example, estradiol rapidly enhances striatal dopamine (DA) release in females within seconds to minutes, but changes in sexual receptivity do not occur until at least 48 hours after estradiol. Additionally, the dose of hormone to use does not always have a linear dose-response profile. This means that hormone replacement needs to be carefully considered, for which physiological doses of hormones usually are the most efficacious.

Surgical approaches

OVX is usually done from a dorsal approach (Stout Steele and Bennett, 2011; Idris, 2012). In the Becker Lab, we usually take vaginal smears for 10 days post-OVX to ensure that residual hormones have cleared the system and that the OVX was successful. If the ovary is not handled gently, some ovarian cells may be left in the system and produce sufficient estradiol to interfere with subsequent experimental manipulations. CAST is usually done from a ventral approach (Idris, 2012). The testes are easier to externalize than the ovaries, so it is easier to be sure that the entire testis has been removed. This is convenient because there is no simple bioassay for testosterone levels analogous to vaginal smears for estradiol. Finally, following OVX or CAST in order to control for hormone exposure in the diet and bedding, it is recommended that (1) a phytoestrogen and soy-free diet be used (there are commercially available diets that meet this requirement); and (2) alternative bedding should be used because bedding made from corn cob has endocrine disruptors that have been found to decrease fertility (Markaverich et al., 2007).

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Steroid Hormone Administration

How to replace hormones after OVX or CAST depends on the goals of the experiment (discussed in more detail in Becker et al., 2005). The method and hormones used will depend on whether one wants to determine if a sex difference is caused by gonadal hormones, for example, by exploring how and when the hormones of the estrous cycle are influencing a trait. The administration of gonadal hormones is influenced by their chemical characteristics: 17β-estradiol, progesterone, and testosterone are steroidal, which means they are not soluble in aqueous solutions and require oil or another solvent for dissolving. Their chemical structure also means that they rapidly cross the blood-brain barrier.

Esterified versus free hormone

Because circulating estradiol does not remain elevated for very long after systemic injection of 17β-estradiol, slower-release, esterified forms of estradiol are often used in physiological research. A variety of esters and other modified versions have been used, such as ethinylestradiol, estradiol valerate, or estradiol dipropionate. However, the most commonly used form is estradiol-3-benzoate, which is estradiol with a benzoic acid esterified in the third carbon position. This form is hydrolyzed in vivo to the physiologically active estradiol. Progesterone, in contrast, is injected only in an unmodified form. Testosterone is usually administered as testosterone or testosterone propionate.

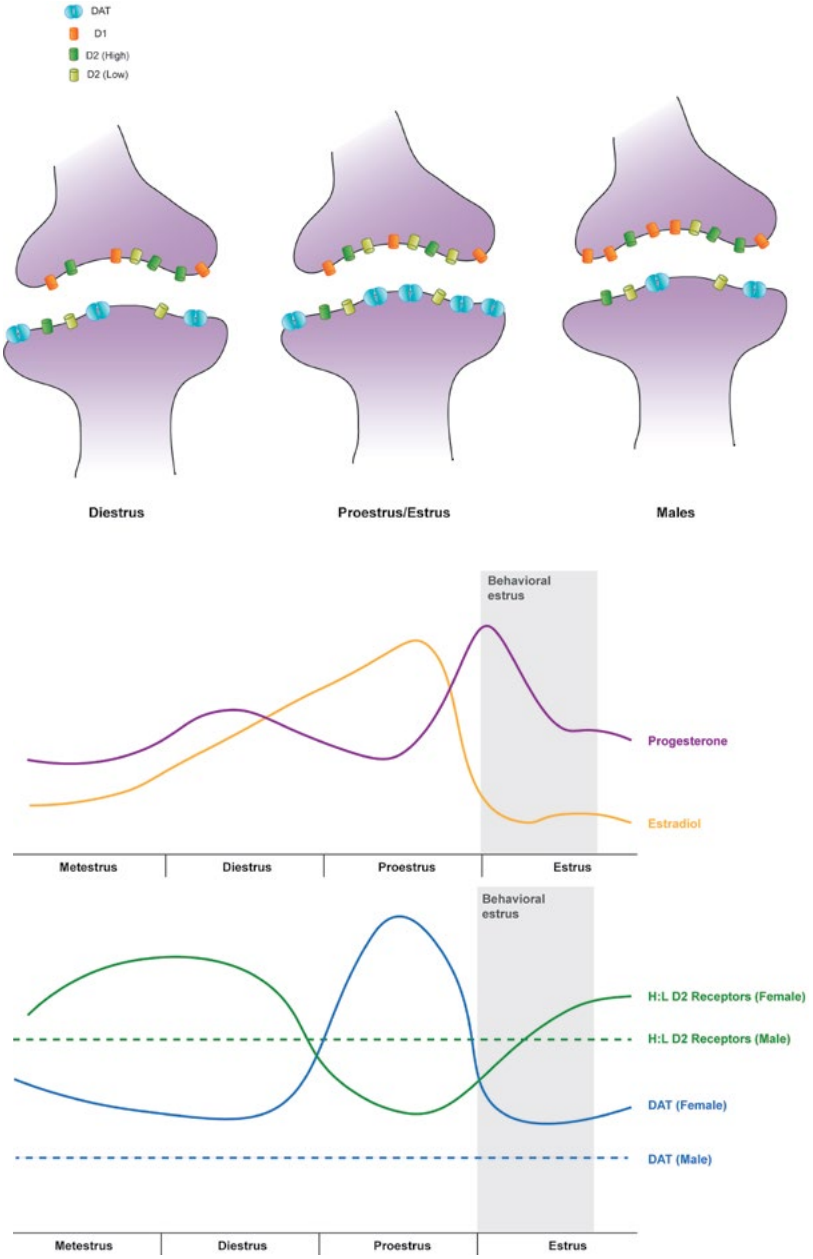

Figure 4. Sex differences and dynamic changes in dopamine (DA) receptors and dopamine transporter (DAT) across the estrous cycle. Expression of DAT (top, blue ovals) is higher in females (solid blue line) than males (bottom, dashed blue line) overall and increases the morning of proestrus, when levels of estradiol (E2) are peaking and levels of progesterone (P) begin to rise. Males have greater expression of D1 DA receptors (top, orange rectangles) than females, and D1 DA receptor expression does not change across the estrous cycle in females. Expression of D2 DA receptors (top, green rectangles) is constant across the estrous cycle, but the ratio of high to low D2 DA receptors is dependent on both sex and reproductive state. Diestrus females have a higher level of high vs low D2 DA receptors than males (bottom, dashed green line), but as E2 and P rise during proestrus (center graph), the ratio of high to low D2 DA receptors in females (bottom, solid green line) decreases to lower than the level observed in intact males. Modified with permission from Yoest et al. (2018), Fig. 2. Copyright 2018, Elsevier.

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Estradiol, progesterone, and testosterone can be administered through subcutaneous, intravenous, intramuscular, intraperitoneal, and intracranial routes. For long-term treatments, steroid hormones (either in crystalline form or dissolved in peanut oil) have been enclosed in a small length of silicone tubing. They can also be administered either by a mini-pump that delivers a consistent dose of hormone for days, or by commercially available pellets that deliver a particular dose of steroid hormone daily when implanted subcutaneously. Each mode of hormone replacement has its appropriate place, but different routes and regimens may provide discrepant results. In some cases, such differences in response to different treatments have been exploited to better understand hormone–behavior relationships.

The most typical mode of administration when attempting to induce lordosis behavior is one, two, or three daily injections of estradiol benzoate followed by progesterone. These treatments reliably induce the expression of feminine sexual behavior. In some cases, chronic daily injections of estradiol benzoate have been given without progesterone, although progesterone is essential for the facilitation of sexual receptivity during the estrous cycle and for the full complement of sexual behaviors in rats. When the treatment regimen requires or can accommodate prolonged exposure to the hormones, estradiol, progesterone, and testosterone have often been administered in the form of crystalline hormone implants in silicone tubing implanted subcutaneously. Lipophilic steroid hormones dissolve through the wall of the silicone tubing and are released at a constant rate that depends on the surface area (length × diameter) of the capsule and the thickness of the capsule wall. To implant and remove silicone tubing capsules, the animals have to be anesthetized. However, they do not need to be handled daily as would be the case with injections. A similar mode of administration has been used in mice. When estradiol is administered chronically, progesterone treatment once per week is quite effective at inducing sexual receptivity. It must be noted, however, that although this might be useful in certain types of studies when prolonged elevation of estradiol concentrations is desired, the treatments bear no similarity to the patterns seen during the estrous cycle.

Intracranial and intravenous administration

Sex steroid hormones are extensively metabolized by the liver. To bypass this metabolism and prevent the delay in hormone delivery to the neural site of action, hormones can be infused by cannula directly into the cerebral ventricles. If the hormones' neuroanatomical site of action is being investigated, the hormones can be implanted directly into specific neuroanatomical areas. Similarly, steroid hormones have been administered intravenously either to increase the amount of unmetabolized hormone reaching the brain or to deliver the hormone to the brain as rapidly as possible.

Absence of hormone in blood does not mean absence of hormone

The decrease in blood concentrations of a steroid hormone does not indicate it is no longer active. Rather, steroids are retained by cell nuclear receptors for a considerable time after the decline in blood concentrations. For example, 18–24 hours after an intravenous injection, estradiol is still bound by cell nuclear estradiol receptors, functioning as transcription factors, long after circulating blood concentrations of estradiol have declined. Thus, sexual receptivity in the female rat or mouse results from the effects of estradiol and progesterone at intracellular receptors in the brain when circulating hormone levels are low.

Rapid effects of steroid hormones

It is well established that steroid hormones have rapid, membrane receptor–mediated effects in addition to their classic slow-acting effects (Rønnekleiv and Kelly, 2005; Thomas, 2008; Yoest et al., 2018). In many systems, it is thought that the membrane receptors and nuclear receptors collaborate to amplify the response to circulating hormones (Razandi et al., 2002; Levin and Hammes, 2016). This means that the speed at which a hormone treatment response is observed reveals information about the mechanism through which the hormone is acting.

Summary

This section discussed the most common modes of administration and regimens used in ovarian hormone replacement treatment of rats and mice. Each has its advantages and disadvantages, and each can be applied to the study of sex differences. A great deal of thought must go into choosing the particular hormones administered, their form, and the mode and timing of administration, and a good deal of thought must go into providing equitable treatments in males and females. Fortunately, much is already known about the effects of varying particular parameters on physiological responses, so well-informed choices are possible.

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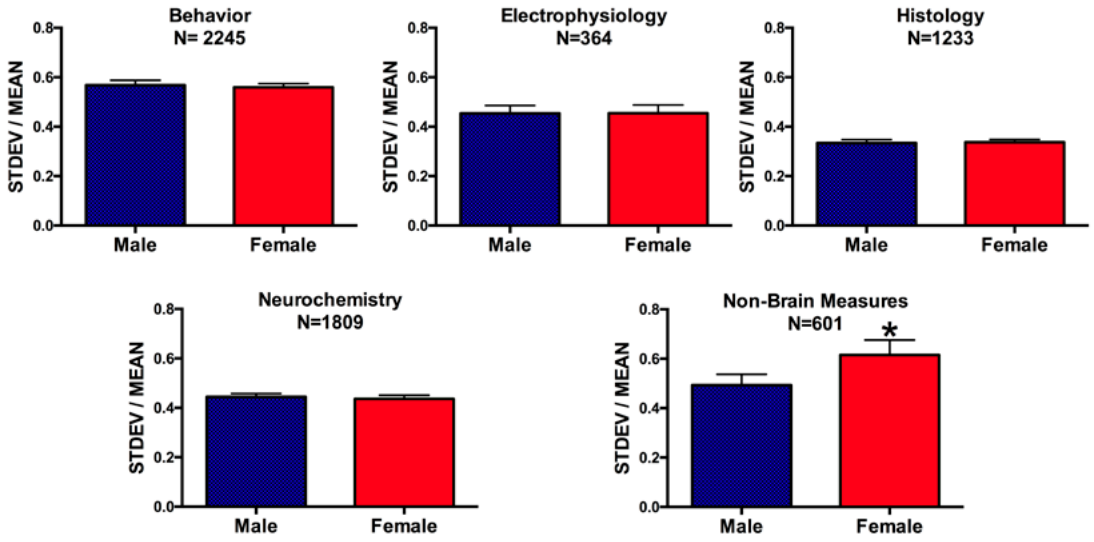

Figure 5. Trait variance as indicated by the SD (STDEV) divided by the mean for behavioral measures, electrophysiological measures, histological measures, neurochemistry, and nonbrain measures. *N*, number of data points each for males and females. For nonbrain measures, greater variability was seen for females. *Females > males ($p = 0.03$; Mann–Whitney *U* test). Lines above bars indicate SEM. Reprinted with permission from Becker et al. (2016), Fig. 1. Copyright 2016, The Authors.

Variability in Male and Female Rats and Mice

In a recent meta-analysis, we investigated whether female and male rats differed in their variability in studies that focused on neuroscience outcomes (Becker et al., 2016); other investigators have analyzed variability in male and female mice (Prendergast et al., 2014). Both reports found that female rats and mice are not more variable than males when females are used without regard to the estrous cycle, or when female rats are studied at specific days of the estrous cycle (Fig. 5) (Becker et al., 2016). The coefficient of trait variability (CV) was defined as the standard deviation (SD) divided by the mean. In this study, we found that even for data points on which males and female differed significantly, the CV did not differ between the sexes. This finding contradicts the idea that using females in neuroscience research will result in greater variability.

Both studies went on to look at the distribution of CV ratios (female CV/[female CV + male CV]) to determine whether, at tails of distribution, females or males would be represented more. As can be seen in Figure 6, the distribution of CV ratios is relatively symmetrical. Some sex differences appear in the tailing for only three of the measures, and for two of those, the males showed greater variability than the females.

If females do not exhibit greater variability, what are the other drawbacks of including them in preclinical research? One big concern has been that it would be necessary to double the number of animals studied, thereby increasing the cost and time to carry out the research. In a recent article, Annaliese Beery explained that with the appropriate use of statistics, this is not necessarily the case (Beery, 2018). The use of a factorial approach (Fig. 7) allows an investigator to analyze the results for both males and females without losing power relative to analyses of only one sex. Three scenarios are described: Scenario 1: no sex differences; Scenario 2: large sex difference with no interaction; and Scenario 3: sex difference with large interaction (males and females show the opposite response).

Dr. Beery concludes, "Although the factorial approach is powerful, it is not without potential weaknesses. ANOVA on sex *x* treatment generates 3 *F* values at $p = 0.05$. This leads to a higher collective type I error rate than one *t*-test (explaining why the 'treatment' factor performs as well in ANOVA as the *t*-test in Scenario 1). This is important to keep in mind if additional factors are added. Also, whereas Scenario 3 is extreme, when interaction effects are more intermediate, they will be less easily detected. If assessing sex differences is a primary rather than secondary goal, increased sample size will improve detection of interactions" (Beery, 2018).

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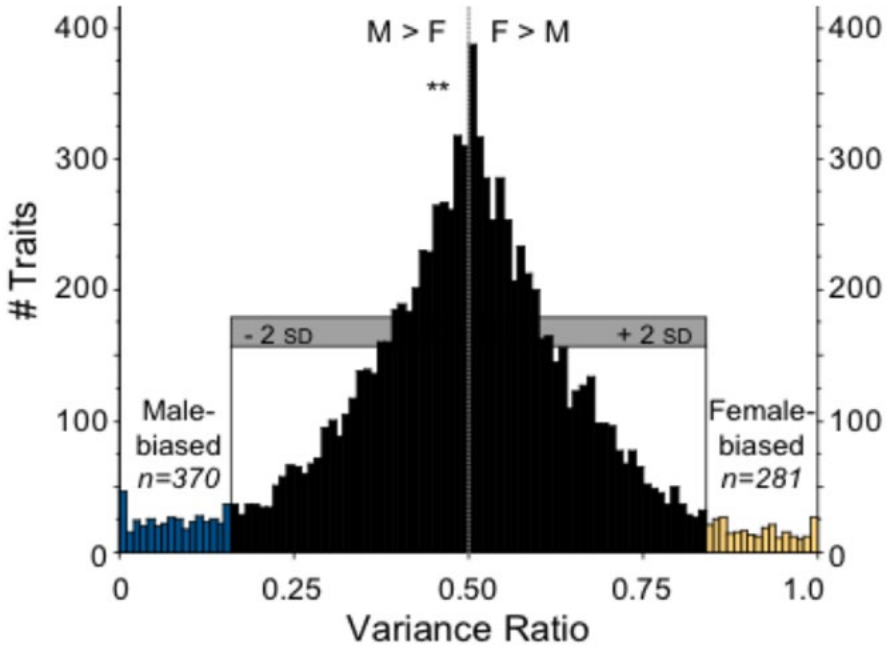

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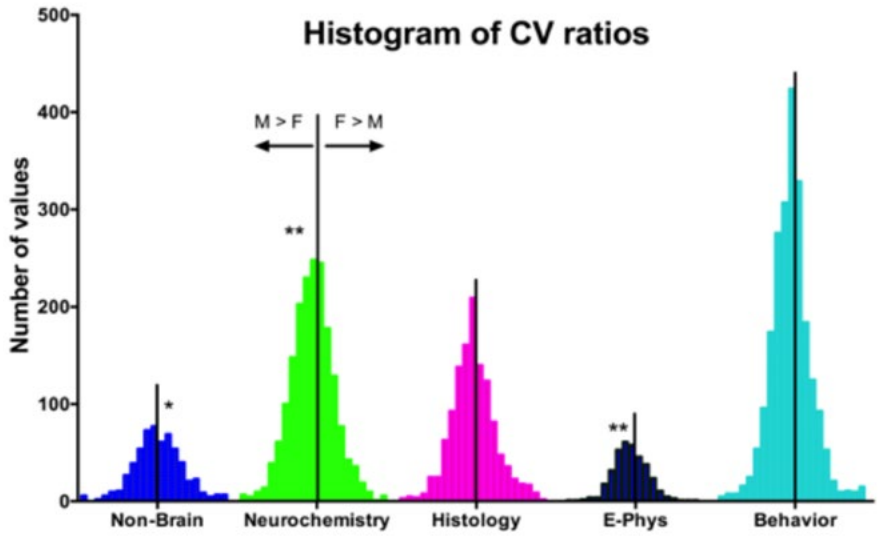

Figure 6. Distribution of CV ratios in mice (left) and rats (right). ***A***, CV ratios were assessed in male and female mice across >9900 measurements of traits. Variability was similar in males and females, with more male-biased than female-biased traits, and a mean variance ratio significantly lower than 0.5. Modified with permission from Beery (2018), Fig. 1*b*. Copyright 2018, Elsevier. ***B***, CV ratios depict variability among the values obtained. A value of 0.5 (vertical black line) indicates that males and females are the same. Values to the right of the vertical black line for each trait show where females are more variable than males; values to the left of the black line show where males are more variable than females. *Females were more variable than males on the Nonbrain measures ($p < 0.0001$). **Males were more variable on the E-Phys trait ($p = 0.037$) and the Neurochemistry trait ($p = 0.0196$). Reprinted with permission from Becker et al. (2016), Fig. 3. Copyright 2016, The Authors.

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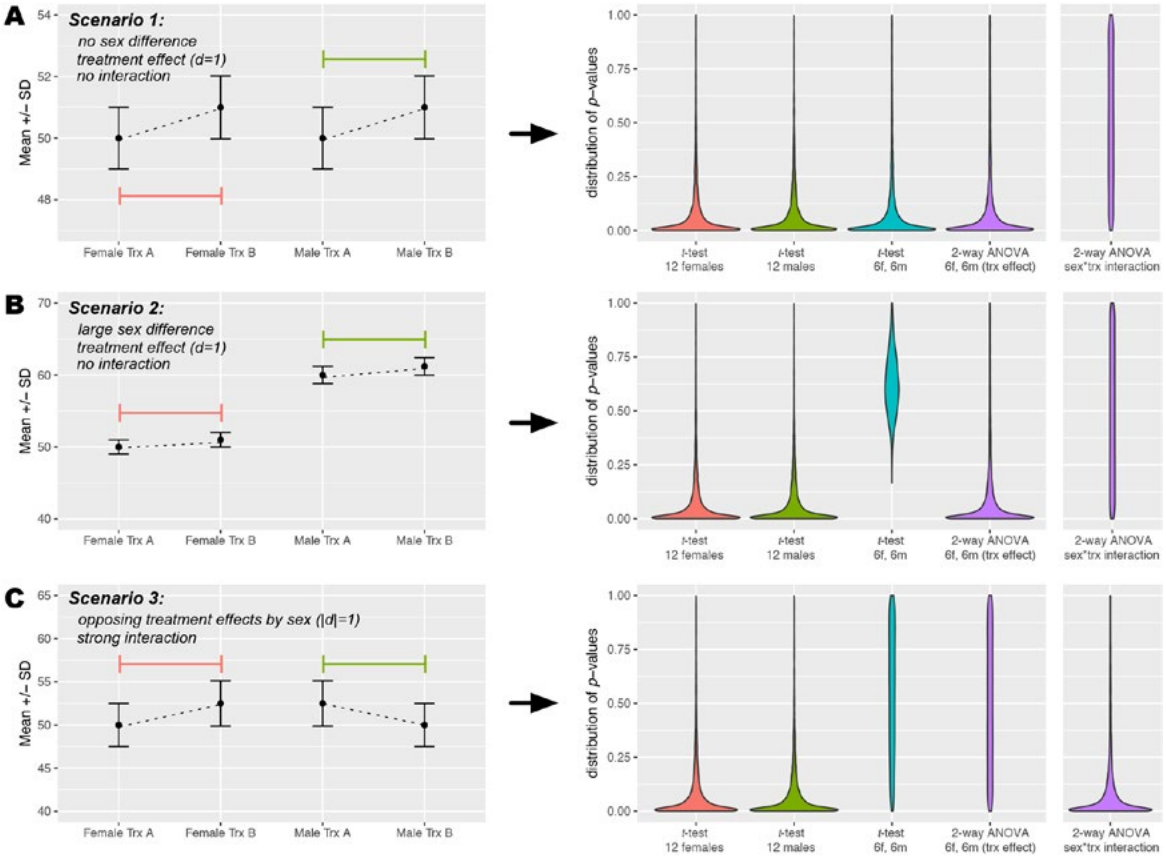

Figure 7. Simulated *p*-value distributions for different group compositions and treatment effects. The statistical outcomes of two-group and factorial tests used simulated data. Consider an experiment with two treatments ("A" and "B," e.g., hot vs cold room temperature) and an outcome measure (e.g., distance traveled). In each scenario, there is a ± 5% change in mean deviation and SD in females and males. ***A–C***, Left panels, Mean and SD of each group used to generate 10,000 possible samples of subjects (12f and 12m, or 6f and 6m) receiving each treatment, according to a Gaussian distribution. CVs (SD/mean) were constant for each sex/treatment combination so that the spread of groups with different means would be equivalent. Effect sizes for treatment A versus B comparisons in all male or female groups were matched (Cohen's $d = 1$ using SD of lower group, or 0.97–0.99 using pooled SD). ***A–C***, Right panels, Violin plots of *p*-values generated for *t*-tests between different kinds of groups in treatments A versus B (first 3 datasets) or from the treatment factor from two-way ANOVA on mixed-sex groups (purple plot). ANOVAs were run with sex and treatment as factors, and an interaction term. The fifth plot (far right, purple) represents the distribution of *p*-values of the ANOVA's interaction term across runs. Even with a large sex difference, no loss results from using half males and half females in the experiment when a factorial analysis is used, as long as there is no interaction. When an interaction is present, factorial analysis cannot detect a unified treatment effect. However, the strong interaction effect indicates that subgroup analysis by sex and possible follow-up experiments are merited. ***A***, In Scenario 1, no sex difference is seen between males and females. This is a common result in which there is no cost to mixing sexes. All analysis methods yield equivalent effects of treatment, and two-way ANOVA on sex and treatment indicates no interaction effect. ***B***, In Scenario 2, a large sex difference and a moderate treatment difference appear. This is the oft-feared scenario in which simply pooling males and females reduces statistical power. Whereas loss of power occurs when a *t*-test is used to compare across treatments, two-way ANOVA results in no loss of power for detecting treatment effects, as the test quantifies treatment differences relative to the mean of each subgroup. ***C***, Scenario 3 represents a possible "worst-case scenario" in which a large treatment effect is seen in females and an equally sizable but opposite effect is seen in males. Here, pooling males and females results in the eradication of a treatment difference. However, the ANOVA interaction effect will very likely be significant, signaling that sex-specific follow-up study is strongly indicated. Reprinted with permission from Beery (2018), Fig. 2. Copyright 2018, Elsevier.

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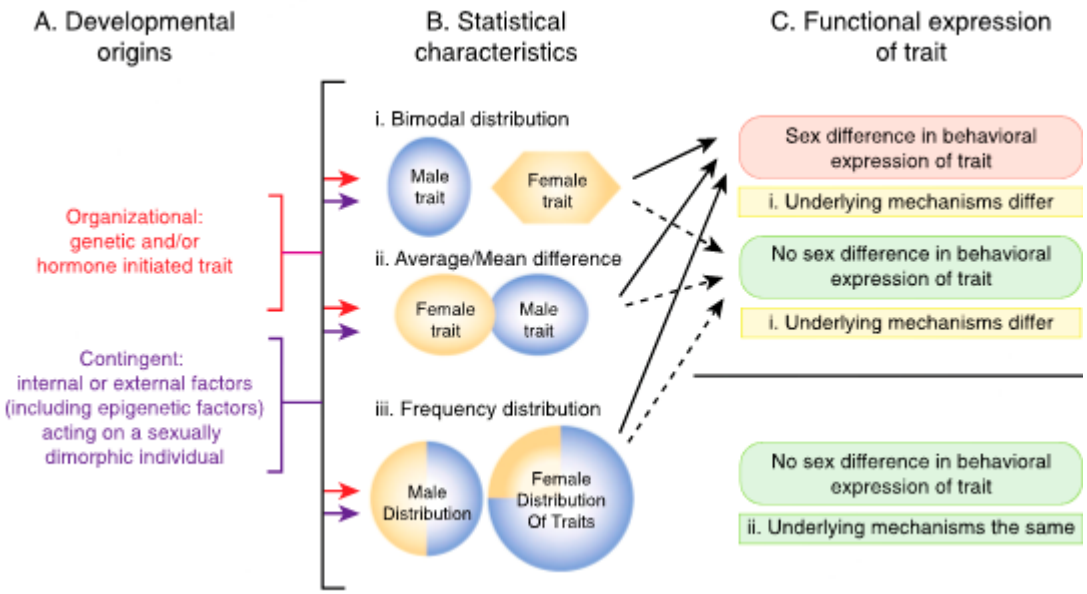

Figure 8. Developmental origins, statistical characteristics, and functional expression of sex differences in the brain. ***A***, Developmental origins of sex differences may arise from organizational influences or be contingent on interaction with internal or external factors. Organizational origins are defined as genetic (XY/XX chromosomes), gonadal hormone influences during critical/sensitive periods of development, and placental influences. Contingent origins include internal or external factors, e.g., epigenetic traits induced by environmental exposure, effects of stress *in utero* or postnatal, and nutritional factors ***B***, Statistical characteristics describe different types of sex differences that occur due to multiple developmental processes. Sex differences may exist in four forms, three of which involve differences in behavioral output: *i*, Bimodal distribution; *ii*, Average or mean differences; and *iii*, Frequency distribution or population differences in trait occurrence. The fourth form of sex difference occurs when behavioral expression of a trait is statistically similar between males and females but the underlying mechanisms differ significantly. ***C***, Traits may be functionally expressed differently in females and males. The sexes may show similar expression of the trait (by the measures used) but get to the trait (***i***) by different underlying mechanisms or (***ii***) via the same mechanism. Reprinted with permission from Becker and Chartoff (2018), Fig. 1. Copyright 2018, Springer Nature.

Although an interaction as illustrated in Scenario 3 may not be what an investigator is hoping to find, it could be the most interesting outcome. A statistical technique known as bootstrapping provides realistic confidence intervals and can help an investigator decide, in cases where the outcome is unclear or there is an interaction (as in Scenario 3), whether more animals should be tested. Bootstrap resampling is the use of simulated datasets, generated by computer from existing measurements, to estimate confidence intervals (Efron, 1979; Iwi et al., 1999; Dixon, 2006). It can also be used to estimate bias and variance or to simulate a population from available sample data. It will take into account Poissonian errors in the measurement process and variations among individuals. Bootstrap resampling is not a substitute for eventually running more experimental animals, but it can provide the statistical evidence for whether doing so is likely to result in a significant outcome or whether the result in question is more likely spurious.

Conclusion

Statistical sophistication and modern methods of data treatment and analysis can help make the study of sex as a biological variable easier to incorporate into experiments in the neuroscience laboratory. However, given what we know about the multiple types of sex differences (Fig. 1) and how they originate, they cannot take the place of looking at one's data and making knowledgeable decisions about what types of analyses are appropriate for the dataset collected. As Figure 8 illustrates, the data treatment in Figure 7 is most relevant if an average or mean difference has been found, but it is less relevant if a bimodal distribution or frequency distribution (population) difference in the expression of a trait appears. When studying the latter, nonparametric statistics become more appropriate for performing analyses.

Moving forward, the study of sex as a biological variable and the future of sex-differences research are eagerly anticipated. This chapter has dealt with thinking about experimentation that is relevant

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primarily to adult animals, but of course, the adult is the product of its developmental origins, which include organizational factors as well as internal and external factors throughout development. The functional expression of a trait can differ in males and females, as can the mechanisms mediating a trait. Even the neural circuitry is likely to differ between the sexes. These sex differences are fundamental to our understanding of the brain and essential for the effective development of translational therapies for mental health and neurological disorders, so future studies that include sex as a variable are crucial.

Acknowledgments

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Genetic and Neural Circuit Approaches to Studying Sex Differences

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Introduction

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

Sexual Differentiation of the Brain Is Regulated by Gonadal Hormones

The neural circuitry that controls innate social behaviors develops under the control of gonadal hormones (Phoenix et al., 1959; Simerly, 2002; Arnold, 2009; McCarthy et al., 2009; Wu and Shah, 2011). Male mice undergo a surge of testosterone at birth that subsides within hours (Motelica-Heino et al., 1988; Corbier et al., 1992). This circulating testosterone is converted to estradiol directly in the brain by aromatase (MacLusky and Naftolin, 1981; Amateau et al., 2004). Estradiol is the primary endogenous estrogen, although estrone and estriol also bind estrogen receptors (ERs); here, we primarily use the general term "estrogen" for simplicity. Pharmacological and genetic experiments have demonstrated that this brain-derived perinatal estrogen is the primary driver of sexual differentiation of the rodent brain and permanently establishes sex-typical differences in the structure and function of neural circuitry that mediates sex-specific behaviors in the adult (Honda et al., 1998; Rissman et al., 1999; McCarthy, 2008; Wu and Shah, 2011). Females given estradiol at birth display male-typical fighting behavior as adults, with no additional hormone supplementation (Wu et al., 2009). This sensitivity to estradiol is lost by the second postnatal week (Gerall et al., 1967; Motelica-Heino et al., 1993; Toda et al., 2001). Although sex differences in neural circuitry are specified during this postnatal critical period, sex-typical behaviors are not displayed until puberty, when the male testes produce testosterone and female ovaries make estrogens and progesterone. These hormones are acutely required in adult life: gonadectomy abolishes mating and aggression, but circuit structure remains intact and behaviors can be restored by giving exogenous hormones. Although testosterone is the primary driver of adult male-typical behaviors, estradiol alone can restore some mating and territorial behaviors (Södersten, 1975; Kimura and Hagiwara, 1985; Cross and Roselli, 1999; Bakker et al., 2004). Therefore, estrogen acts both to modulate postnatal male-typical circuit development and to "activate" circuits for sex-typical behaviors in adulthood. Although estrogen is the primary driver of sexual differentiation in rodents, both estrogen and testosterone signaling are required for full masculinization of adult rodent behaviors.

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

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

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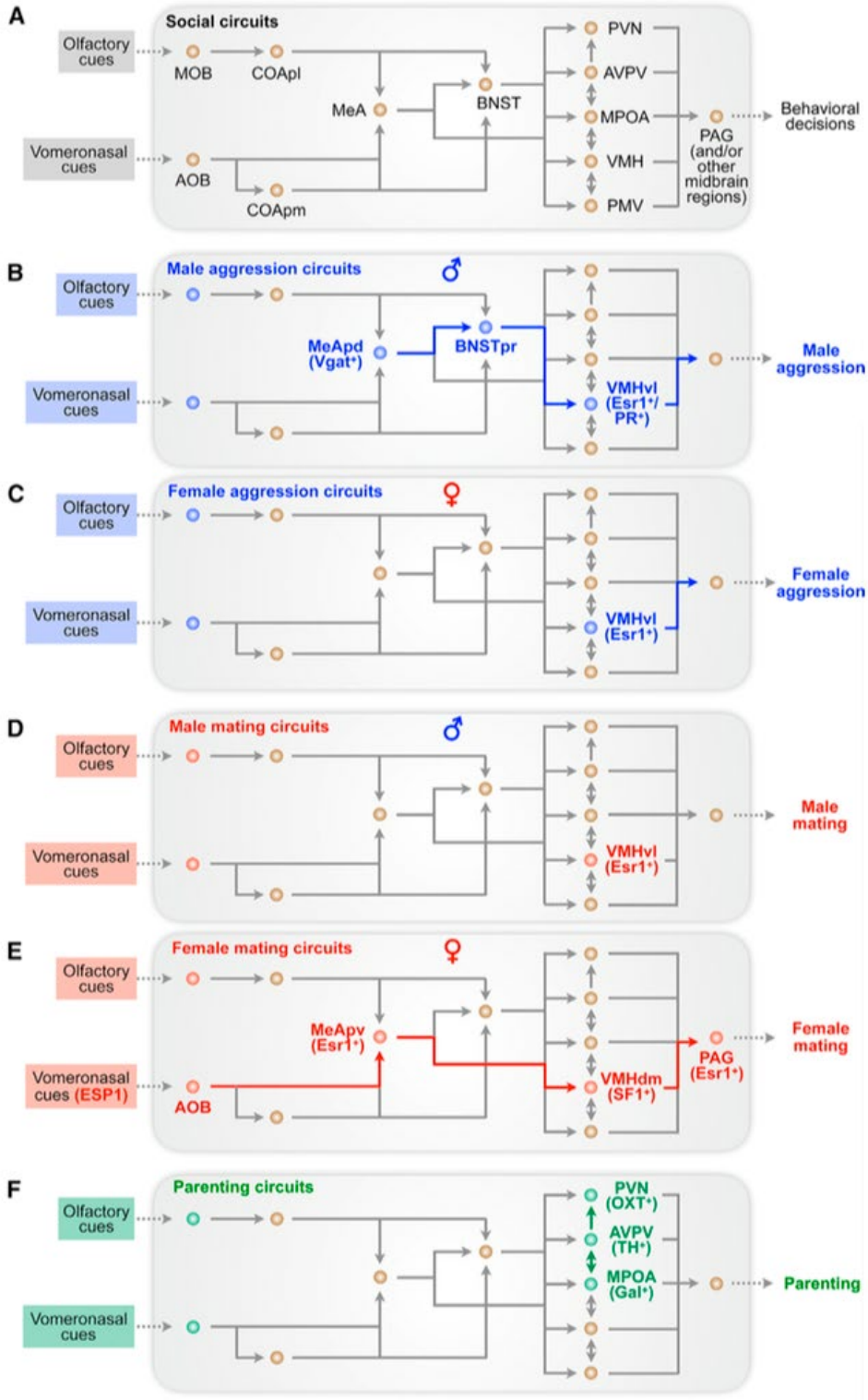

Figure 1. Current understanding of circuits and brain regions in males and females implicated in different social behaviors. ***A***, Overview of key social behavioral circuits and regions. ***B, C,*** Circuits involved in male (***B***) and female (***C***) aggression. ***D, E,*** Circuits involved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

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

Sex Chromosome Influences on the Brain

Sex chromosomes also contribute to sexual differentiation of the brain, both directly through their own genetic content, and indirectly through regulation of gonadal development (Arnold, 2004; Cox et al., 2014; Arnold et al., 2016; Bramble et al., 2017). Sex chromosome aneuploidies are some of the most common genetic disorders in humans, affecting nearly 1 in 400 live births (Lenroot et al., 2009). These disorders are associated with cognitive and behavioral symptoms, particularly in social skills and motor abilities (Hong and Reiss, 2014). Notably, language and spatial abilities appear to correlate with sex chromosome dosage. Females with X monosomy show normal or increased verbal and lexical abilities and visuospatial deficits, whereas individuals with sex chromosome polysomy have language impairments that increase with the number of chromosomes, while their spatial skills are often enhanced (Crespi, 2008; Lenroot et al., 2009; Hong and Reiss, 2014). Brain imaging studies have identified a relationship between sex chromosome dosage and brain volume (Lenroot et al., 2009) and highlight specific chromosomal effects in cortical (Lin et al., 2015) and subcortical (Raznahan et al., 2015; Reardon et al., 2016) brain areas. Mouse models of sex chromosome aneuploidies have been used to discern the effects of sex chromosomes on specific behaviors, including social behaviors, anxiety, feeding, and nociception (Cox et al., 2014). The most widely used model is that of the "four core genotypes." This system employs two modified alleles of the testis-determining *Sry* gene: one in which *Sry* has been deleted from the Y chromosome, resulting in genetic males that resemble females, and another in which *Sry* has been inserted on an autosome to generate XX animals that develop testes (De Vries et al., 2002). Comparison of these mutants with wild-type XX and XY animals thereby permits the dissociation of sex chromosome complement from gonadal development.

Location of Hormone-Receptor-Expressing Neurons in Rodents

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

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

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

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

Genetic Tools for Steroid Hormone Receptors

Mutant mouse alleles exist for all four SRs, making it possible to test the contribution of an individual receptor to sex differences in behavior phenotypes. Consequently, the requirements of ERα and AR for male-typical behaviors have been extensively characterized (Rissman et al., 1999; Juntti et al., 2008, 2010; Zuloaga et al., 2008). Male mice mutant for AR also show decreased spatial memory and increased anxiety (Zuloaga et al., 2008; Juntti et al., 2010). In contrast to ERα, ERβ does not appear to be necessary for the display of mating and aggression. Rather, ERβ null males show increased levels of aggression and altered social investigation (Handa et al., 2012a). Estrogen is anxiolytic, and studies support a role for both ERs in modulating anxiety and the HPA axis (Handa et al., 2012b; Handa and Weiser, 2014). Conditional alleles for gonadal hormone receptors have been generated, thereby permitting the deletion of these receptors with a variety of cell-type-specific Cre lines. Recent studies from the Herbison and Tollkuhn labs have deleted *Esr1*, the gene for ERα, from either excitatory or inhibitory neurons using vGlut2-Cre and vGAT-Cre driver lines, respectively. Females lacking ERα in excitatory neurons show altered hypothalamic-pituitary-gonadal axis function, early puberty onset, and infertility (Cheong et al., 2015). Surprisingly, loss of *Esr1* in vGlut2-positive neurons does not affect male-typical behaviors, but deletion of *Esr1* in GABAergic neurons dysmasculinizes male sexual and territorial behaviors. The expression of *Ar* and *Esr2* (ER-β) is also feminized: there is less *Ar* in the BNST of mutant males and more *Esr2* (Fig. 2) (Wu and Tollkuhn, 2017).

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

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

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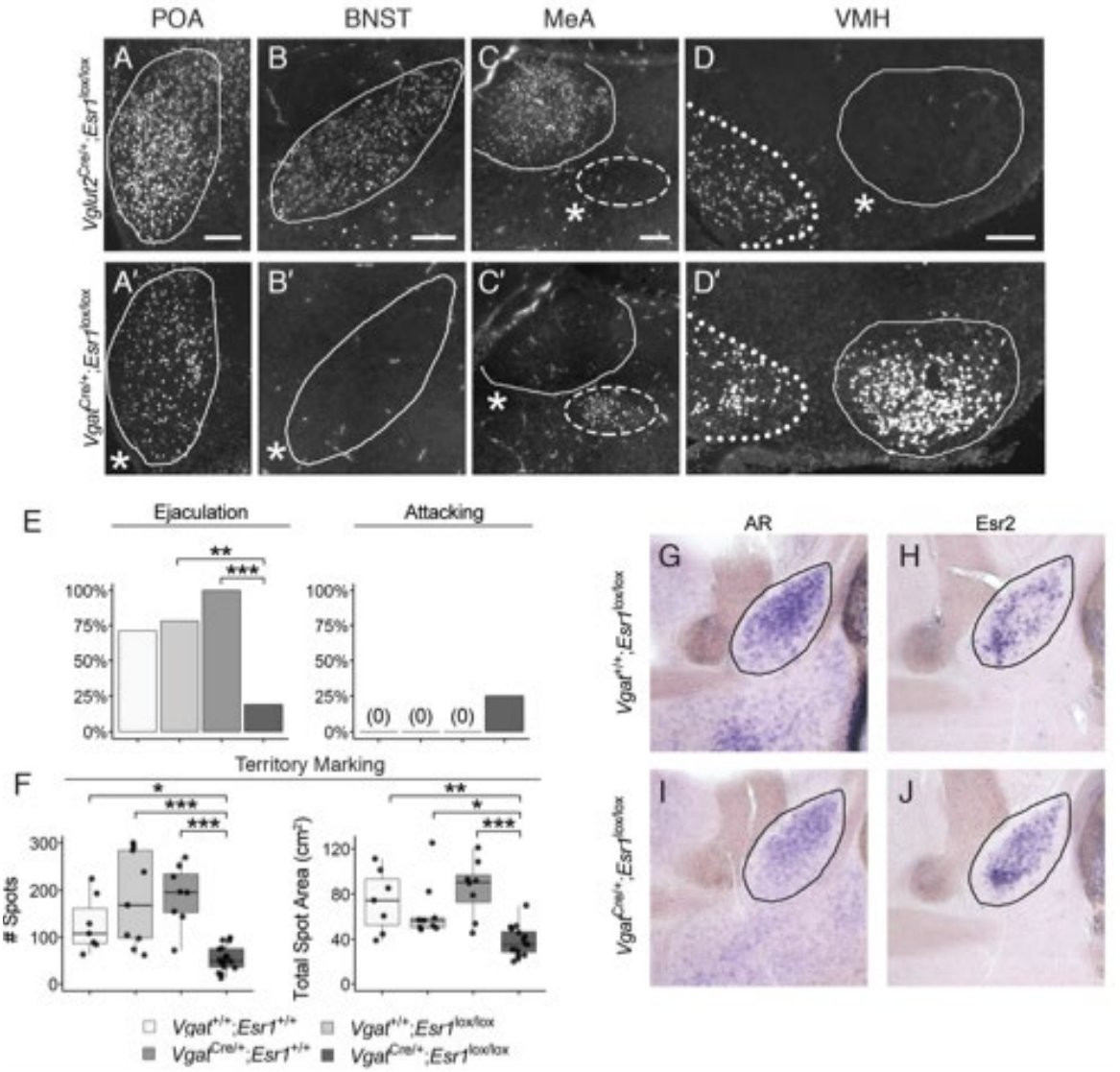

Figure 2. Deletion of ER-α (*Esr1*) in inhibitory neurons dysmasculinizes behavior and gene expression. Representative images of ER-α immunostaining in postnatal day (P) 0 pups lacking *Esr1* in excitatory (***A–D***) and inhibitory (***A'–D'***) neurons. Solid lines outline the MPOA, BNST, MeApd, and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

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

Sex Differences in Neural Activity

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

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are used in female sexual behavior. The first strategy is seen in the PR/ERα neurons in the VMHvl, which receive inputs from the MeApd and send much stronger projections to the AVPV of females compared with males (Yang et al., 2013). Intriguingly, a parallel adjacent pathway from the posteroventral MeA (MeApv) to the dorsal VMH appears to employ the second strategy. The sex pheromone exocrine-secreted peptide 1 (ESP1) is secreted from the lacrimal glands of males and acts to facilitate sexual receptivity in females through the vomeronasal receptor V2Rp5 (Haga et al., 2010). As with other pheromones, neurons in the vomeronasal organ send projections to the accessory olfactory bulb (AOB) and then to the MeA, BNST, and cortical amygdala (Stowers and Logan, 2010). ESP1 activates distinct ensembles of neurons in the MeApv of females and males, resulting in sex-specific outputs from a common circuit: female-active neurons project to the VMHvl, and male-active neurons project to the POA. The MeApv-to-VMHvl projection promotes the display of a sexually receptive lordosis posture in females via activation of ERα neurons in the PAG. Importantly, the projections themselves are the same in the two sexes; the sex difference lies in the populations of cells that respond to ESP1 (Ishii et al., 2017).

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

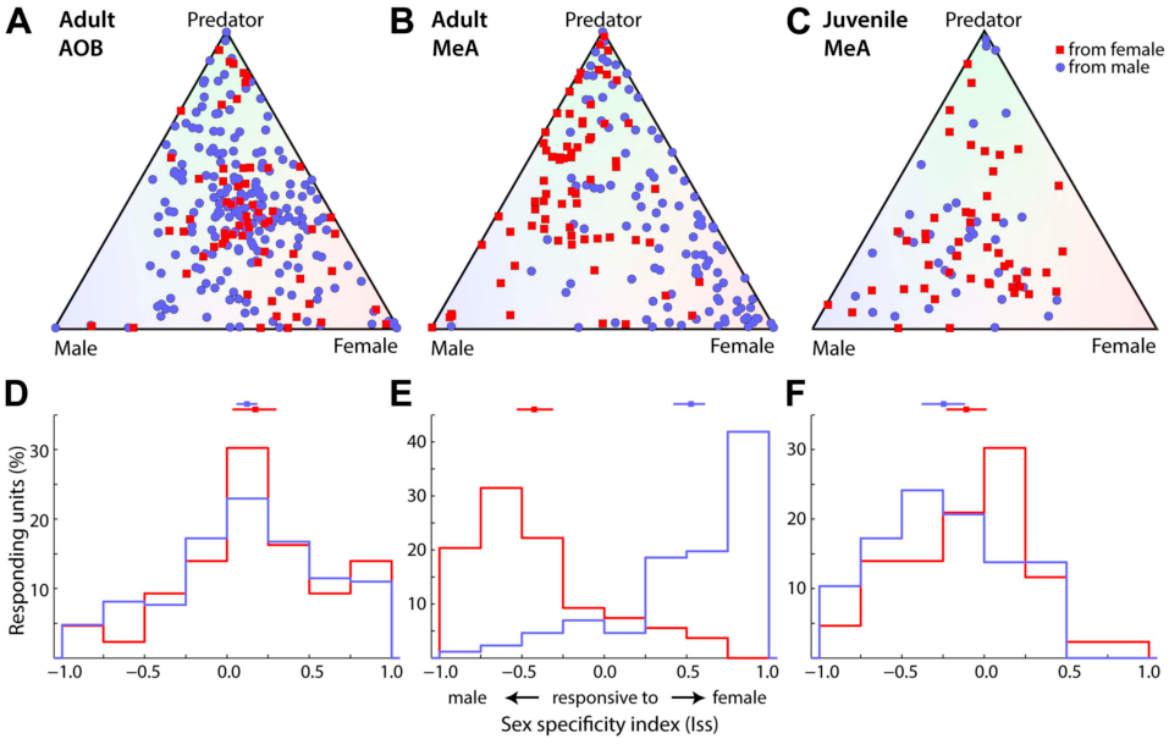

Figure 3. Sexual dimorphism of adult MeA responses. ***A***, Responses of AOB neurons to vomeronasal stimuli in adult male (210 units) and female (64 units) mice. ***B***, Responses of MeA neurons to vomeronasal stimuli in adult male (106 units) and female (91 units) mice. ***C***, Responses of MeA neurons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

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Summary

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

Acknowledgments

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How to Study the Origins of Sex Differences in Brain and Behavior

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Introduction

This year marks the 48th Annual Meeting of the Society for Neuroscience. It is also almost 60 years since the discipline of neuroendocrinology was born, after seminal papers established that gonadal hormones during fetal development exert enduring changes on the brain, which then determine adult reproductive behavioral and physiological phenotypes (Phoenix et al., 1959). During this time span, the fields of neuroscience and neuroendocrinology have lived largely parallel lives, having insufficient influence on each other. This has contributed to the situation we have today, in which neuroscience has advanced almost exclusively by studying the brains of male animals, and neuroendocrinology has largely emphasized endocrinology at the expense of neuroscience. But today, all that is changing. Part of the change involves making more accessible and appealing the study of sex differences in the nervous system by (1) demystifying females (they are not just fluctuating hormones) and (2) conveying the heuristic power of contrasting fundamental neuroscience processes in males and females. Toward that end, I will present some fundamentals.

Sex Determination, Sexual Differentiation, and Sex Differences

Sex determination begins with genetics and the sex chromosomes. All mammals and birds, some insects, most fish, and a smattering of amphibians and reptiles are sex-determined as a function of specialized chromosomes that differ between males and females. In mammals, XX and XY chromosomes determine male and female, and the same goes for *Drosophila*. But in birds, females are WZ while males are WW. In mammals, it is a single gene on the Y chromosome, *Sry*, that directs the bipotential gonadal anlage to differentiate into a testis (Goodfellow and Lovell-Badge, 1993). If that gene is missing or mutated, or if there are two X chromosomes, the gonadal precursor will develop into an ovary. Formation of a testis occurs extremely early in development, when the brain is still a gelatinous mass, and becomes an active endocrine organ shortly thereafter, synthesizing hormones that repress the survival of the female reproductive tract (e.g., uterus, cervix, vagina) and promote the survival of the male reproductive tract (vas deferens, seminal vesicles, etc.). By the second trimester in humans, and during the third week of pregnancy in rodents, the testis of a male fetus is synthesizing close to adult levels of androgens (Fig. 1). It is this phase of

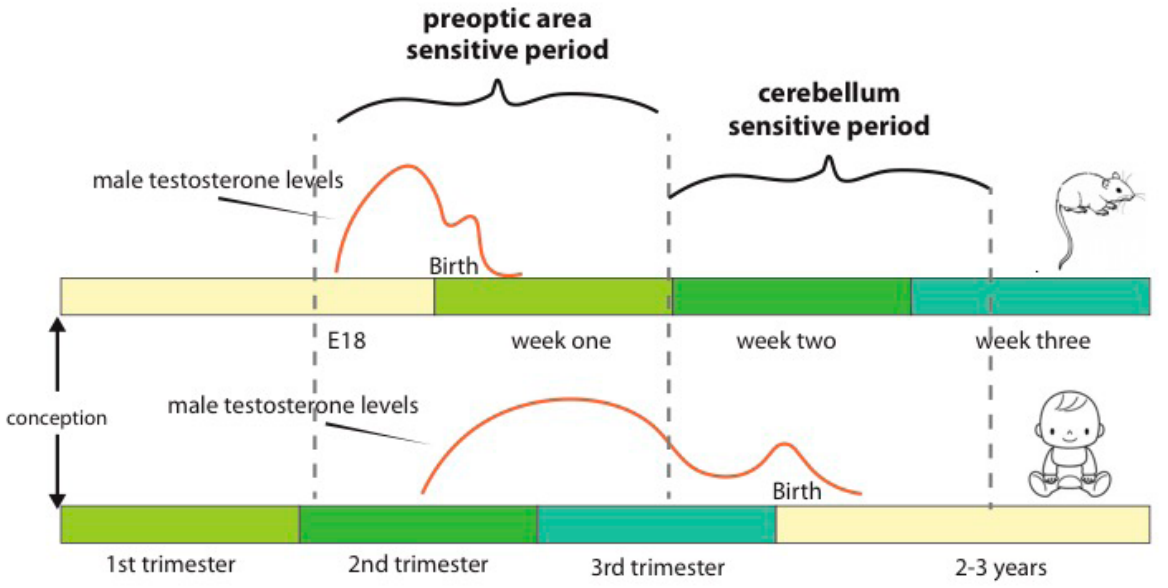

Figure 1. Sensitive periods at different life stages of rats and humans. The developmental profile of the rat is shifted from that of humans, in that a newborn pup is roughly equivalent to a mid- to late-gestation human fetus. The sensitive period for sexual differentiation of the preoptic area in the rat is operationally defined by the onset of testicular androgen production in male fetuses on E18 and the loss of sensitivity of females to exogenous hormone treatment by the end of PN week 1. In humans, the sensitive period for the preoptic area begins during the second trimester with fetal androgen production and probably ends before birth (although this conclusion is constrained by a lack of experimental data). The sensitive period we have identified in cerebellar development occurs during PN week 2 in the rat, which corresponds to the peripartum period in the human. Factors constraining the sensitive period in the rat are the onset and offset of gene-expression profiles. Whether a similar profile exists in humans is currently unknown. Reprinted with permission from McCarthy and Wright (2017), Fig. 4. Copyright 2017, Elsevier.

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steroid production that drives sexual differentiation of the brain by simultaneously initiating multiple distinct cellular and molecular process that can be collectively referred to as "masculinization." The purpose of masculinization is to impart anatomical and physiological changes that will ensure that, later on, the brain residing in the male-determined body will support spermatogenesis, motivation to mate with females, territorial defense and/or competition or aggression against other males, and so on. The result of sexual differentiation of the brain is sex differences. However, brain sex differences can be achieved in other ways or even reversed, as will be discussed below.

Critical versus sensitive periods

The formation of the brain proceeds in epochs. Some of these are intrinsic programs, and others are periods of sensitivity to stimuli, either internal or external, such as the need for light for the proper formation of the visual system. These are called "critical periods" because the exposure must occur during a specific developmental epoch or the window of opportunity is forever lost. Sensitive periods are distinct from critical periods; they are times when a perturbance will have an enduring effect that would not occur if the exposure happened at another time. *In utero* inflammation and the risk of autism spectrum disorders or schizophrenia in the offspring are emerging examples of sensitive periods and their consequences. The sexual differentiation of the brain is unique in that it consists of a critical period for masculinization: exposure to androgen must occur during that developmental window and therefore exists in only one sex (Fig. 2). But there is also a sensitive period for females when exposure to exogenous androgens can induce masculinization, if only during a specified period (Fig. 3). In our rodent animal models, this period extends into postnatal (PN) week 1; thus, treating

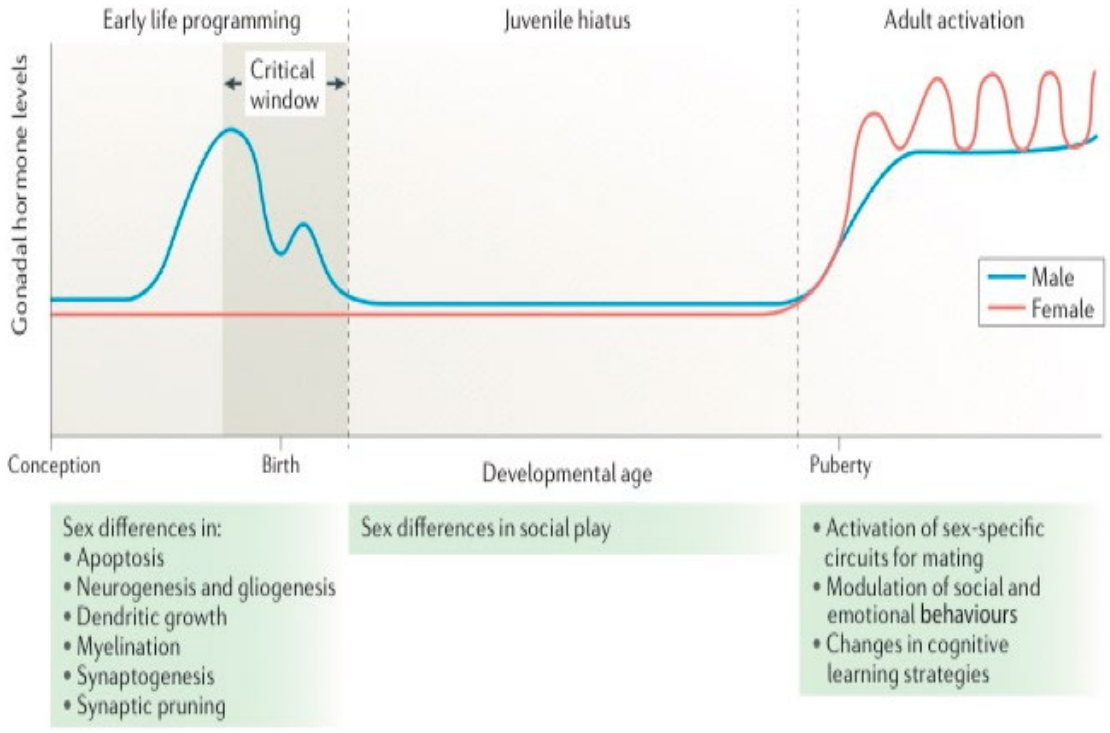

Figure 2. Early life programming of adult sex differences. Steroidogenesis by the perinatal male testes results in elevated circulating testosterone, which is aromatized to estrogens in the brain. Combined androgen and estrogen action modifies multiple developmental process throughout the brain in a regionally specific way during a narrow, sensitive time window. These processes include cell genesis, neuronal migration, dendritic growth, synaptogenesis, and synaptic pruning and cell death, among others. Within a few days of birth, the elevated steroids in males decline to undetectable and are equivalent to the female's. Both remain there during the juvenile hiatus, a time of heightened rough-and-tumble play behavior by males. After puberty, both sexes reestablish gonadal steroidogenesis that is dimorphic in amount and patterning. This hormonal milieu acts upon the neural substrate that was organized early in life to promote the expression of sex-typic physiology and behavior, the most obvious of which is copulatory. Reprinted with permission from McCarthy et al. (2017), Fig. 1. Copyright 2017, Nature Publishing Group.

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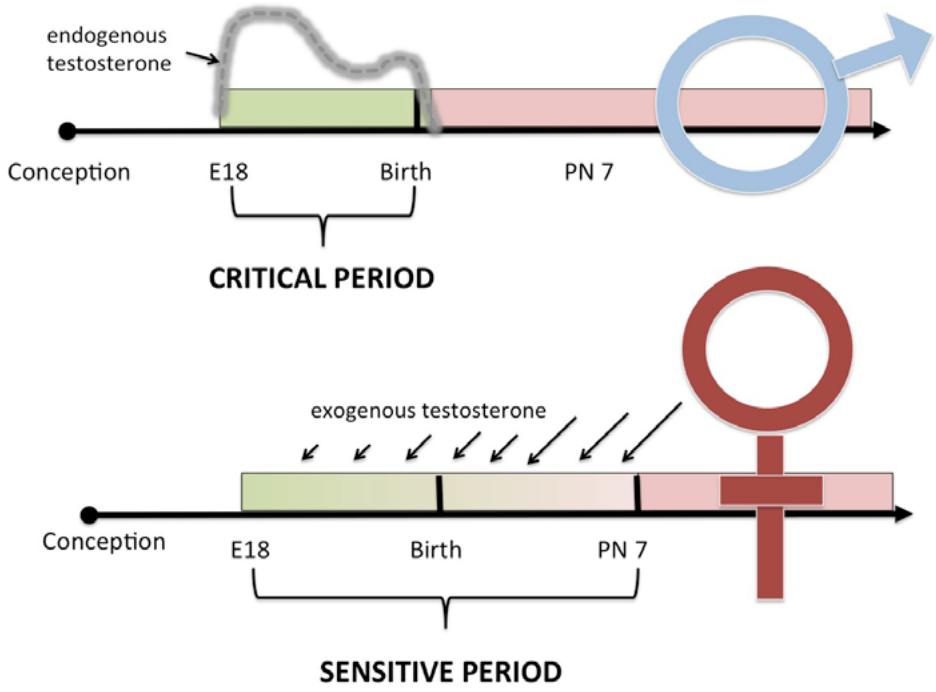

Figure 3. The critical and sensitive periods for sexual differentiation. Masculinization of the brain occurs during a critical period that begins with the onset of endogenous testosterone production from the fetal testis on E16 (mouse) or E18 (rat). Circulating testosterone levels fall within hours of birth, and the critical period ends shortly thereafter as the process of masculinization irrevocably proceeds. Females are not exposed to endogenous testosterone as the ovaries are quiescent; therefore, gonadally derived hormone exposure is limited to testosterone exposure from their littermates. Females also remain sensitive to exogenous testosterone treatment for up to 1 week after birth, with increasingly larger doses (indicated by larger arrows) required as sensitivity wanes. After 7–10 days, the process of feminization irrevocably proceeds. Because of the unique synthesis of testosterone in males but the shared sensitivity of both sexes to this steroid hormone, males have a short critical period whereas females have a longer sensitive period. The ability to sex-reverse females postnatally with exogenous testosterone provides a highly useful but imperfect tool for the study of sexual differentiation. Reprinted with permission from McCarthy et al. (2018), Fig. 2. Copyright 2018, Elsevier.

females with testosterone or its aromatized product, estradiol, will induce masculinization if the dose is high enough. This is a highly useful experimental tool for studying the process of masculinization because, in males, it begins *in utero*, and once begun is very difficult to block (McCarthy et al., 2018). This does create a conundrum, though, in that females treated with exogenous hormone to study masculinization are several days older than their male siblings that underwent normal masculinization. In rodents, every day counts, particularly in neonates, but there is no easy way around this confound.

Androgens versus estrogens and the "aromatization hypothesis"

The shift in timing of the critical period in males versus the sensitive period in females is just one of the many challenges associated with studying sexual differentiation and deciding how to design and interpret experiments appropriately. Early in the days of defining the parameters mediating sexual differentiation, scientists recognized the need to use negative controls for the treatment of females with testosterone to be sure they were not observing some nonspecific effect of treatment that masqueraded as masculinization. Cholesterol was one obvious negative control since it carries all the same properties as steroid hormones but does not bind to steroid receptors. But estradiol was considered an even better control because it is a potently active steroid in its own right, and generally considered a female hormone, but does not activate androgen receptors. To the investigators' surprise, estradiol proved to be an even more effective inducer of masculinization when given to females. More importantly, blocking estrogen activity in newborn males, either with an estrogen receptor antagonist or inhibitors of aromatase, effectively blocked masculinization in males. These effects were formulated as the "aromatization hypothesis," which is now established fact and incorporates several properties that are essential for understanding sexual differentiation of the brain (McEwen et al., 1977).

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The first property is that testosterone is a precursor (or prohormone) that is aromatized to estradiol in a rate-limiting step by the enzyme Cyp19a, or aromatase (Fig. 4). The second is that neurons in the brain express the aromatase enzyme and thereby locally synthesize estradiol from the androgen precursors in circulation synthesized by the fetal testis. The distribution of aromatase expression is not random, but is concentrated in specific nuclei and regions, with levels varying between brain areas and the sexes (Fig. 4). The distribution of estrogen receptor(s) expression and aromatase is overlapping but not identical, and the degree to which steroid is synthesized in one cell and released to act on an adjacent cell is not entirely clear but certainly plausible. The third property is that estrogens from the maternal circulation must be excluded from gaining access to the fetal brain, or all the pups will be masculinized. This conundrum is solved by the nifty trick of a steroid-binding globulin called alpha-fetoprotein, found at very high levels in the fetal circulation, which binds estrogens but not androgens. Alpha-fetoprotein creates a sponge, or trap, in the fetal circulatory system exclusive to estrogens but allows androgens to gain access to neurons and be locally converted to estrogens. How precisely androgens access the interior of neurons or other brain cells is not known, but there is reason to believe it is a somewhat regulated process and may play an important but undetermined role. There is also lingering evidence that alpha-fetoprotein may itself be a signaling molecule and may even deliver estrogens to specific cells (McCarthy, 2008). Finally, not all masculinized endpoints are the result of estrogen action; some are regulated directly by androgens. The most well characterized endpoints are the number of motor neurons in specific spinal cord nuclei and the number of cells in the amygdala (Morris et al., 2004). Still other endpoints appear to involve both estrogen and androgen action (Waddell et al., 2013).

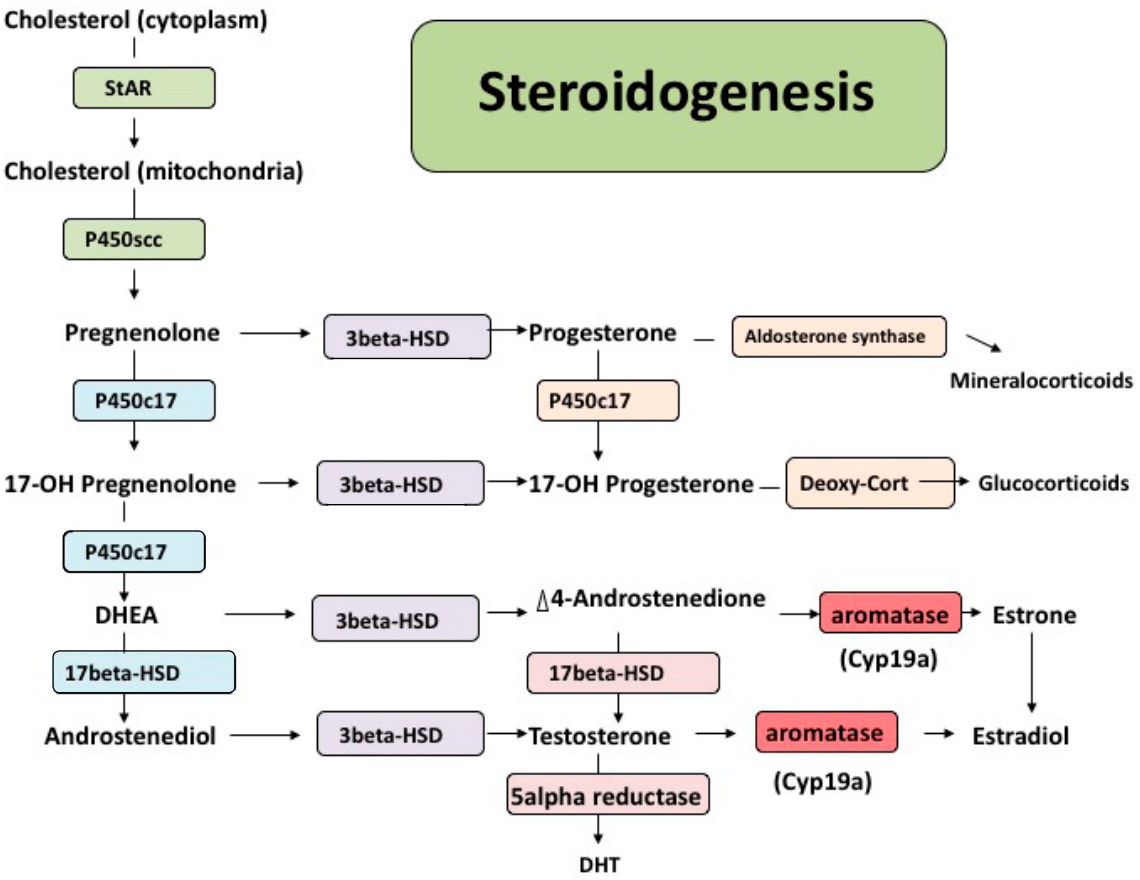

Figure 4. Steroidogenesis. All steroids begin life as cholesterol and, through a series of enzymatic reactions that mostly remove hydroxyl groups and carbons, become either progestins, glucocorticoids, mineralocorticoids, androgens, or estrogens. Testosterone is a precursor to estradiol, which requires aromatization by the enzyme Cyp19a. DHT is also an enzymatic by-product of testosterone following 5-alpha reduction; however, it cannot be converted into estradiol and is therefore called a nonaromatizable androgen. Deoxy-Cort, deoxycorticosterone; DHEA, dehydroepiandrosterone; HSD, hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein.

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Experimental Approaches

How to determine which steroid is driving sexual dimorphism

There are two general approaches to the question of whether a particular endpoint is sexually differentiated by estrogens, androgens, or some combination thereof. The first is the historically more recent approach of using genetically modified mice, which have proven highly effective at confirming the central role of the alpha form of estrogen receptor 1 (Ers1) to masculinization (Ogawa et al., 2000) and revealed an unexpected role for the beta form in defeminization (Kudwa et al., 2005). Mice lacking the aromatase enzyme or alpha-fetoprotein further confirm the centrality of estradiol to most hormonally mediated differentiation events (Bakker et al., 2003, 2006), whereas the androgen receptor–deficient mouse highlights the importance of testosterone and other androgens (MacLusky, 1988).

The genetic mutability of the mouse is a powerful tool, but a fundamental and unavoidable shortcoming is that once the gene is inactivated in development, it remains so for life, making it impossible to differentiate developmental (often called "organizational") effects from those exerted in adulthood (referred to as "activational"). So the second approach for investigating sexually differentiated endpoints is the use of steroids. Fortunately, steroids are fairly easy to administer exogenously, can be administered as early as the day of birth, and are structurally the same across all species. That said, steroids generally do not follow lawful dose-response curves, and their action is complicated by the need for cofactors, nuclear as well as membrane receptors, rapid and enduring effects, and much more that is beyond the scope of this discussion. However, one does not need to be expert in endocrinology to properly administer hormones during development, and some general guidelines follow.

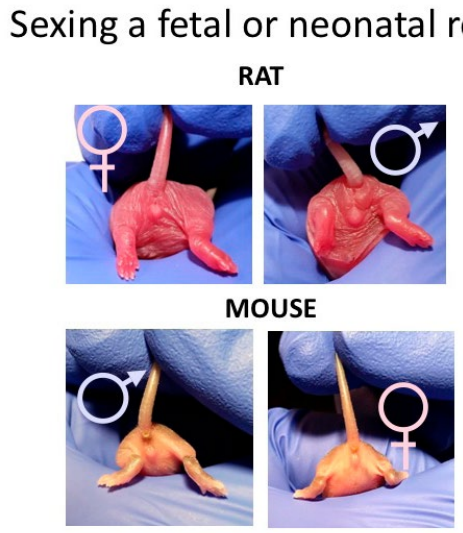

Figure 5. Sexing rat and mouse pups. The anogenital distance is longer in males than in females and can be either measured with calipers or assessed visually with experience. Moreover, the male usually has a slight swelling and sometimes pigmentation in the anogenital area, indications of the future scrotum.

Sexing newborn rodent pups

Distinguishing male and female pups from each other is a simple task for the trained eye but a daunting one for those not familiar with working with such small animals (newborn mice and rat pups weigh on the order of a few grams). Even my institution's animal care and use committee once returned my proposal with the question, "How will you tell the boys from the girls?" I restrained myself from replying that it was easy—the boys are blue and the girls are pink—and instead explained how one could simply look at the genitalia and distinguish male from female with 99.9% accuracy. As shown in Figure 5, males have a longer distance between the anus and the urethra, and there is a slight swelling (future scrotum), often accompanied by some pigmentation.

Although this approach is easily used on animals at the time of birth and can be effective for very late-stage embryos, dissection of the abdomen to identify the presence of testes provides stronger assurance. The testes of male rodents do not descend until well after birth and can be readily found in the lower body cavity, where they appear as two small pearly grains of rice, one on each side. The female ovary can also be seen but is much more difficult to detect, and therefore less reliable.

The testes develop very early in pregnancy, but the further one goes back toward conception, the more difficult they are to see. However, sex can also be confirmed genetically using PCR for Y-chromosome-specific genes, and this is an excellent solution when immediate identification is not required. It is tempting to think that one should conduct PCR for *Sry*, but this is actually not ideal, as there can be multiple copies of this gene in some species, with only one being functional. Instead, it is recommended to measure the gene *Jared*, which is found on both the X and Y chromosome but is smaller on the Y chromosome, therefore producing two PCR bands in males but only one in females (Clapcote and Roder, 2005). An alternative approach is to measure repetitive sequences on the Y chromosome as a proxy for *Sry* (Itoh et al., 2015).

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Treatment with exogenous steroids

Determining whether a sexually differentiated endpoint is mediated by androgens, estrogens, or some combination is fairly simple to achieve by exogenous treatment with steroids, but there are some caveats. As noted earlier, estrogens are sequestered in the fetal blood by alpha-fetoprotein; thus, an exogenous dose must overwhelm the binding capacity of this steroid-binding globulin. We find that in rat pups, a dose of 100 μg estradiol benzoate given subcutaneously in a sesame oil vehicle once on the day of birth and again the next day fully recapitulates any estrogen-mediated sexual differentiation processes. Although this dose is very large compared with the 10 μg used to induce sexual receptivity in an adult (and so tends to alarm reviewers on occasion), we have confirmed that it increases brain levels of estradiol in females only to the level of males in the most sexually dimorphic region: the preoptic area. This finding indicates that we are inducing physiological levels in the CNS (Amateau et al., 2004).

Treatment of a newborn pup with estradiol is an effective approach, but some argue it circumvents the normal process of aromatization and precludes any additional or supplementary effects that might involve direct androgen action. Thus, if you desire to be fully confident that an effect is mediated solely by estradiol, additional groups should include testosterone treatment, in which you expect to see the same endpoints, and dihydrotestosterone (DHT) treatment (the latter is a nonaromatizable androgen and therefore activates only the androgen receptor). To compare across groups, we keep the doses of each steroid the same. In order to ensure the steroid is long lasting in the circulation, we use estradiol benzoate, testosterone proprionate, and DHT proprionate. The benzoate and proprionate moieties do not impact steroid action but slow the release from the oil depot and muscle, thereby extending the half-life and more closely mimicking endogenous steroids.

When treating neonates, the preferred vehicle is sesame oil and the preferred injection volume is 0.1 cc given subcutaneously. We use a 30 ga needle and inject under the skin at the scruff of the neck or over the rear haunches. To prevent the oil from flowing back out the injection site, the skin is pinched between thumb and forefinger as the needle is withdrawn and then the site is dabbed with either New Skin or Super Glue to seal the hole. This step is essential, as steroids are highly lipophilic: if the oil escapes, there will be an easy transfer of steroid to littermates via absorption through the skin.

We generally inject pups without anesthesia, but they can be cryoanesthetized if desired. This is a good practice when first learning the technique, as it eliminates the squirm factor. Cryoanesthesia is a fancy word for chilling. Pups are placed on top of tin foil over ice in a standard lab ice bucket and then placed in a 4°C refrigerator for 10–20 min, depending on age. When the pups are blue and motionless, they are anesthetized. Do not place multiple pups together, as they will huddle for warmth. Recovery from anesthesia is achieved by placing them under a mild heat lamp (or just a light bulb) or on a very low heating pad. More pups die from being overheated than overchilled, so be careful, although it is possible to overchill as well. Only when pups are pink and wiggly should they be returned to the dam.

Antagonizing or blocking endogenous steroids

Treating with exogenous steroids is easy, but blocking endogenous steroids is not. The difficulties arise from the timing of administration, effectiveness, solubility, and specificity.

Timing

The goal of blocking endogenous steroids is often to interfere with or prevent naturally occurring masculinization. But this process begins *in utero* with the surge in androgen production around embryonic day (E) 16 through E18. Blocking steroid synthesis in the pregnant dam would compromise the pregnancy, and it is not technically feasible to selectively treat the developing fetuses. The critical period for males does extend postnatally, but elevated steroid levels last for only ~2 hours after birth, which essentially means one must observe pups being born and treat them within an hour or so. Anyone who works with pregnant rats and mice appreciates that this is easier said than done, as they do not commence delivery on command or to suit your work schedule. However, not all actions of endogenous steroids appear to be restricted to those first few hours. We have antagonized estrogen production and receptor action in the hippocampus of neonates on the day of birth and one day later to good effect (Bowers et al., 2010).

Effectiveness

Highly effective aromatase inhibitors have been available for some time because of their potential therapeutic use against metastatic estrogen-dependent breast cancer (Blakemore and Naftolin, 2016). Aromatase inhibitors come in two types. The first are analogs of the androgen precursors that bind irreversibly to the enzyme. Formestane is

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the most commonly used inhibitor of this type and is beneficial for its high solubility in biologically tolerable vehicles, such as sesame oil. It can readily cross the blood-brain barrier (BBB), but it is only ~75–80% effective at reducing estradiol production. Given the need for complete inhibition in cancer treatment, this led to the development of the second type: nonsteroidal aromatase inhibitors. The most effective one is letrozole, which has ~99% effectiveness. However, it is difficult to dissolve in standard vehicles and does not reliably cross the BBB. For this reason, we have relied on Formestane (100 μg), which we find reduces brain estradiol in a neonate to near undetectable levels (Konkle and McCarthy, 2011).

Specificity

The most commonly used estrogen receptor antagonist is tamoxifen, which has advantages in terms of solubility and penetrance of the BBB. We have found tamoxifen to be an effective blocker of endogenous estrogen actions when given at a dose of 100 μg (notice the pattern here, as mentioned above: there is not a strong dose-response curve with steroids). The concern with tamoxifen is its well-known capacity to occasionally act as an agonist, not an antagonist. However, its agonist effects seem to be restricted to its actions in bone or after an extended period of exposure. A purer antagonist is ICI 182,780 (Fulvestrant), though its shortcoming is poor penetration of the BBB, so it is a trade-off. Both tamoxifen and ICI have affinity for the alpha and beta forms of the estrogen receptor, and thus cannot be used to distinguish between these isoforms. If one needs to distinguish between ERα and ERβ (and I encourage anyone to ask themselves first whether the distinction is really important), then there are relatively specific agonists and some more recently developed ones that can be employed. However, the limitations of pharmacology are in strong evidence here. If one is working with mice, it may be far better to use a genetic approach.

As difficult as antagonizing estrogens is, blocking androgens is even messier. First, selectively blocking synthesis is more challenging, as this class of steroids is further up the chain of steroidogenesis (Fig. 4) and the synthetic enzymes are less specific than is the obligatory aromatase for estrogen synthesis. The most commonly used androgen synthesis inhibitors target the 17-a lyase (CYP17) enzyme, but these have many off-target effects, and often, residual androgen receptor activity (Stein et al., 2014). In general, my recommendation is to avoid trying to inhibit androgen synthesis as an experimental approach.

Second, the only readily available androgen receptor antagonist is flutamide, and it is a lousy one at that. We have consistently found (and been told anecdotally by others) that flutamide is an effective androgen receptor antagonist only when used to block exogenous androgen action. Using flutamide to block endogenous steroid simply doesn't work. Nonetheless, it can be useful for confirming an androgen effect by giving testosterone to females with or without flutamide with the assumption that flutamide will effectively block any effects of testosterone.

Masculinization, Feminization, and Defeminization

Up to this point, we have been emphasizing the process of masculinization because it is the process by which the male is sexually differentiated from the female, which is the default. In other words, feminization of both brain and body is the developmental trajectory that occurs in the absence of testis and androgen production. That is, an ovary is not required for female development, although it is essential to adult reproductive capacity. This does not mean that feminization of the brain is not an active process (it surely is), but it is much harder to discern what it is in the absence of some third "neither male or female" phenotype. There is evidence of a later critical period in female brain development (during PN week 2) that may involve estrogen production by the ovaries (Bakker and Baum, 2008), but unfortunately, this concept is not fully developed. Perhaps the best angle from which to discern feminization is its polar opposite: defeminization, an active process whereby the female phenotype is removed. This phenomenon is best illustrated (and perhaps limited to) the sex-typic mating behavior seen in rats and mice, wherein males show mounting behavior toward sexually receptive females, which respond with lordosis, a posture that allows the male to intromit his penis. Because feminization is the default, the neural circuitry of lordosis comes as "preinstalled software." Removing that programming is achieved by defeminization, which is also driven by androgens aromatized to estrogens in developing males but via distinct cellular mechanisms (Schwarz and McCarthy, 2008). Why such a system has evolved is a mystery, and whether it applies outside the context of sex behavior is debatable. However, it does tell us that multiple independent processes occur simultaneously in the developing brain that ensure as little overlap as possible between males and females in certain key reproductive functions. Notably, no parallel process of demasculinization exists in females, and when masculinization is blocked in

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males, it is best referred to as "dysmasculinization," since it is the disruption of a normal process rather than a normal process itself.

Nonhormonal and Nongenomic Factors: The Environment

The advent of the "four core genotypes" mouse model described elsewhere in this course has irrefutably demonstrated the impact of sex chromosome complement on brain and behavior. Given that genetics is a constant, and is not exclusive to development, we will not review it further here. However, any discussion of sex differences must consider nonhormonal and nongenomic factors: i.e., the environment. In humans, the gender of a child affects everything, including the way it is dressed, handled, and even spoken to, all of which occurs during major epochs of brain development. Even in rodents, the maternal dam interacts with her male and female pups differently. Beginning with anogenital licking and grooming (which is essential to pup survival, as they cannot urinate or defecate on their own), the dam performs this function on male pups more frequently than on female pups. The extra attention the males receive provides vital stimulation to the developing motor neurons that innervate the penis, promoting myelination and ultimately enhancing adult reproductive functioning (Moore, 1984). Males also receive preferential treatment if separated from the nest. For very young pups that cannot see, locomote, or thermoregulate, being isolated from the dam is an alarming circumstance to which they respond with vigorous and frequent ultrasonic vocalizations. Interestingly, the males are more frequent and more vigorous in their distress calls, and this motivates the dam to retrieve them back to the nest more quickly than she does the females (Bowers et al., 2013). Although not directly tested, this rapid response can be inferred to result in less stress to the male pups after a separation.

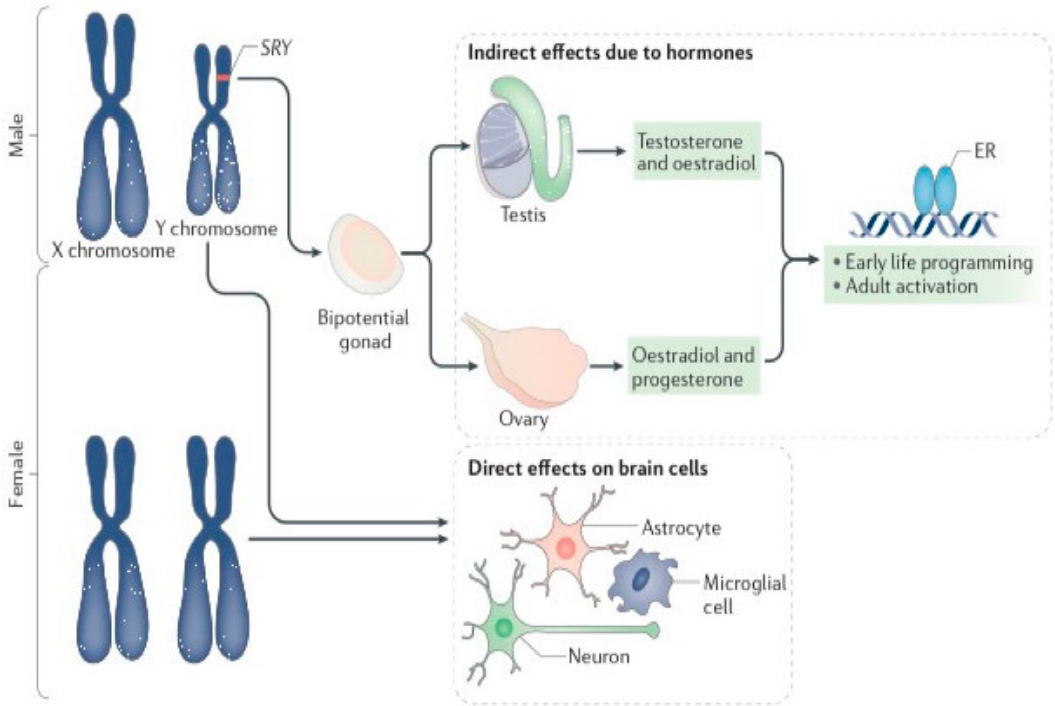

Figure 6. Epigenetic sources of brain sex differences. Several critical epigenetic mediators are X and Y linked, such as the histone lysine demethylases Kdm6a and Kdm5c, which escape X inactivation in the brain. The Y-linked homologue to kdm6a (UTX), kdm6c (UTY), is expressed at higher levels in the male brain (Xu et al., 2008). Sex-specific expression of epigenetic modifiers such as these has the potential to establish widespread sex differences in the chromatin landscape, gene expression, and thus structural and functional sex differences in the brain. X-linked chromatin-binding proteins such as MeCP2 have also been shown to be important for establishing brain sex differences. Male gonadal hormones reduce the expression of the methyl-binding protein MeCP2 in the amygdala. They have also been shown to reduce DNA methyltransferase activity and methylation genomewide in the preoptic area and alter methylation on specific promoters related to brain masculinization, such as the estrogen receptors (ERs) and progesterone receptors. Hormonal modulation at the level of histone methylation and acetylation has also been demonstrated in the preoptic area and bed nucleus of the stria terminalis, potentially mediating both activational and repressive chromatin states. Reprinted with permission from McCarthy et al. (2017), Fig. 2. Copyright 2017, Nature Publishing Group.

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The final issue I want to raise is that, even *in utero*, male and female fetuses can respond profoundly differently to stress that occurs to the pregnant dam, and this can have life-long consequences. Stress as early as the first week of pregnancy impacts both the placenta and the brain of male pups, but has seemingly no effect on the females (Howerton et al., 2013). Effects on males can be so profound that they persist to the next generation via epigenetic modifications (Morgan and Bale, 2011). Indeed, epigenetic changes occurring during gestation, or perinatally during hormone-mediated sexual differentiation, are likely the foundation on which sex differences persist following either perturbed or normal development (McCarthy and Wright, 2017) (Fig. 6).

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Adolescence and Reward: Making Sense of Neural and Behavioral Changes Amid the Chaos

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Introduction

Adolescence is a time of significant neural and behavioral change with remarkable development in social, emotional, and cognitive skills. It is also a time of increased exploration and risk-taking (e.g., drug use). Many of these changes are thought to result from increased reward value coupled with an underdeveloped inhibitory control, and thus, a hypersensitivity to reward. Perturbations during adolescence can alter the developmental trajectory of the brain, resulting in long-term alterations in reward-associated behaviors. This review highlights recent developments in our understanding of how neural circuits, pubertal hormones, and environmental factors contribute to typical adolescent reward-associated behaviors, with a particular focus on sex differences, the medial prefrontal cortex, social reward, social isolation, and drug use. This research has only begun to elucidate the contributions of the many neural, endocrine, and environmental changes to heightened reward sensitivity and increased vulnerability to mental health disorders that characterize this life stage.

Adolescence can be both an exciting and a tumultuous time. It comprises the formative years during which individuals reach sexual maturity and develop the social, emotional, and cognitive skills needed as they move toward independence and adulthood (Spear, 2000). It is a time of increased exploration, but this exploration often includes increased sensation seeking and the initiation of drug use (Steinberg, 2004; Lipari and Jean-Francois, 2013), which could contribute to the high percentage of preventable deaths among teens (Minino, 2010). It is also a time of increased vulnerability to stress and the emergence of several psychiatric and behavioral disorders (e.g., schizophrenia, depression, and eating disorders) (Kessler et al., 2005). Hence, research into the neurobiological underpinnings of adolescence is important for providing a basic understanding of normative social, emotional, reproductive, and cognitive development as well as the prevention and treatment of health risks and disorders that characterize this life stage.

The prevailing theory underlying adolescent vulnerability to psychiatric disorders proposes a developmental mismatch in accumbal-driven sensation seeking (risk-taking) and prefrontal inhibition of impulsivity (Casey and Jones, 2010). It is thought that this mismatch leads to a greater sensitivity to rewarding stimuli and may explain adolescents' increased vulnerability to drugs of abuse and stress, mentioned above (Casey and Jones, 2010). Sex differences in vulnerability to psychiatric disorders emerge during adolescence, as do important sex differences in the types of disorders displayed by males and females. For example, males are seemingly more vulnerable to externalizing disorders (e.g., bipolar disorder and attention deficit hyperactivity disorder), whereas females are more susceptible to internalizing disorders (e.g., depression and anxiety). Although it is difficult to disentangle how social structures contribute to these vulnerabilities, it is critical to acknowledge that social and biological variables likely act in concert to produce such outcomes. There are striking sex differences in adolescent development, including in the timing of puberty and neural development. These may give rise to sex differences in the vulnerability to psychiatric disorders. Therefore, forming a concrete understanding of these developmental differences is critical for advancing sex-specific treatment strategies for vulnerable populations.

The reorganization of the reward circuitry during adolescence is one factor that is integral to both adolescent development and increased vulnerability to disease (Luciana, 2013; Doremus-Fitzwater and Spear, 2016). This process is driven by complex interactions among neural pathways, endocrine axes, and environmental stimuli to produce a functional mesocorticolimbic reward system in adulthood. Hence, it is imperative to determine how these factors act independently and in concert to shape the mesocorticolimbic reward circuitry during adolescence. This review highlights research on interactions between the mesocorticolimbic dopamine (DA) system, pubertal hormones, and environmental perturbations (drug use and social stress) and their effects on cognitive and social adolescent development.

Puberty-Dependent and Puberty-Independent Adolescent Development

"Puberty" and "adolescence" both refer to the transition from childhood to adulthood, but these terms are not equivalent. Puberty is reserved for physiological and behavioral changes associated with the attainment of reproductive competence (e.g., activation of the hypothalamic-pituitary-gonadal [HPG] axis, appearance of secondary sex characteristics, and onset of sexual interest and mating behaviors), all of which are sexually dimorphic. Adolescence is a broader term that includes puberty as well as nonreproductive traits (e.g., social, emotional, and cognitive development).

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Reproductive hormones, however, can have widespread effects, and the development of several nonreproductive adolescent traits can also be driven by activation of the HPG axis at puberty (puberty-dependent, e.g., ethanol intake, anxiety-related behaviors) (Primus and Kellogg, 1989, 1990; Vetter-O'Hagen and Spear, 2011). The physiological, anatomical, and temporal changes that differ between males and females can also lead to the emergence of sex differences during adolescence (Schulz et al., 2009a). Other adolescent traits, however, develop independently of HPG activation and merely coincide with pubertal development (puberty-independent, e.g., social play, aggression) (Whitsett, 1975; Smith et al., 1996; Wommack and Delville, 2007). Sex differences may also manifest in puberty-independent traits owing to organizational actions of perinatal hormones or direct actions of genes on the sex chromosomes (Arnold, 2017). This puberty-dependent versus puberty-independent distinction is important because many neuropsychiatric and behavioral disorders arise during adolescence, exhibit striking sex differences, and are impacted by pubertal hormones as well as nonpubertal factors (Fombonne, 2009; Graber, 2013; Trotman et al., 2013). Given that the mesocorticolimbic DA pathway is sexually dimorphic (Becker, 2009) and regulated by gonadal hormones in adults (Kuhn et al., 2010; Becker et al., 2012), a central question is whether adolescent development of reward-related behaviors and circuitry is puberty-dependent or puberty-independent.

Sex Differences in Reward and Reward-Related Circuitry

Studies in humans and laboratory animals generally support the notion that adolescents are more sensitive to reward than adults. This is behaviorally manifest in multiple ways, including elevated levels of sensation seeking and risk-taking, as well as reduced inhibitory control, which are all maximal during the early to mid-adolescent period (Burnett et al., 2010; Andrzejewski et al., 2011; Burton and Fletcher, 2012; Urosevic et al., 2012; Collado et al., 2014). In laboratory rodents, heightened reactivity to drug rewards has also been demonstrated (Doremus et al., 2005; Levin et al., 2007; Anker and Carroll, 2010), although this might depend on the drug or other procedural factors (Doremus-Fitzwater and Spear, 2016). When gender or sex is considered, an even more nuanced picture emerges. For example, compared with males, females have a relatively earlier and lower-magnitude peak in sensation seeking during mid-adolescence that is followed by a more rapid decline to stability by early adulthood (Shulman et al., 2015). In this comprehensive, longitudinal study, it was also demonstrated that impulse control improved steadily following early adolescence in both males and females, but males remained more impulsive than females through their mid-20s. In rats, compared with adults, male adolescents exhibit greater intake and motivation for palatable food that is either calorie dense (sweetened condensed milk) (Friemel et al., 2010) or calorie devoid (Marshall et al., 2017). However, this age-dependent difference in reward sensitivity was not apparent in female rats (Marshall et al., 2017). Using food-restricted rats trained to associate a tone with delivery of a sucrose solution, Hammerslag and Gulley (2014) found that the effects of age and sex were dependent on the characteristics of the behavior being measured. Specifically, females exhibited enhanced development of stimulus-directed behavior in that both adult and adolescent females acquired Pavlovian approach more quickly than males. Adolescents of both sexes, however, had weaker expression of goal-directed behavior (i.e., entries into the sucrose delivery trough) and were less sensitive to reward devaluation than adults.

Recent work has also highlighted gender and sex differences in neural development of reward-related brain circuits that may play an important role in these age and gender/sex differences in behavior. In the striatum, adolescent boys lag behind as they reach peak striatal volume at ~15 years of age compared with 12 years of age for girls (Raznahan et al., 2014). Structural development in the cortex also appears to be relatively delayed in boys compared with girls, although exceptions include a more rapid reduction in the thickness of the dorsolateral prefrontal cortex (PFC) in males (Raznahan et al., 2010). Many of these adolescent cortical changes are associated with adrenal and/or gonadal markers of pubertal maturation, often in a sex-dependent manner (Herting et al., 2017). In the rat medial prefrontal cortex (mPFC), there are significant decreases in neuron number (Markham et al., 2007), dendritic complexity (Koss et al., 2014), and synapse number (Drzewiecki et al., 2016) between adolescence and adulthood. At least some of these changes are more pronounced in females than in males and are closely linked to puberty onset (Willing and Juraska, 2015). However, in the core and shell regions of the nucleus accumbens (NAc), these "pruning" processes and the emergence of adult-like morphological features appear to occur much earlier and well before the onset of puberty (Tepper et al., 1998; Lee and Sawatari, 2011).

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Development of the PFC During Adolescence

The mPFC is a crucial regulator of reward-directed behaviors and likely contributes to cognitive development during adolescence. As a major component of the mesocorticolimbic DA pathway, the mPFC receives dopaminergic projections from the ventral tegmental area (VTA) and sends key glutamatergic projections to the NAc, a key integrator of reward processing (Albertin et al., 2000; McGinty and Grace, 2009; Hamel et al., 2017; Morrison et al., 2017). These regions form a larger circuitry (Fig. 1) that includes the basolateral amygdala (BLA) and ventral hippocampus (vHIP), among others. This circuit acts in concert to modulate dopaminergic and glutamatergic tone integrated by the NAc in response to salient stimuli. Loss or reduction of signaling within the PFC in humans has been associated with numerous psychiatric disorders, including anxiety and depression (Ressler and Mayberg, 2007) and substance use disorders (Volkow et al., 2010) in adulthood. Similar effects have been observed in rodent models in which exposure to stress or drugs of abuse can influence signaling between the PFC and NAc, resulting in addiction-related behaviors (MacAskill et al., 2014) or depressive-related behaviors (Covington et al., 2010; Vialou et al., 2014; Bagot et al., 2015). For example, repeated exposure to cocaine in adult mice decreases the PFC inputs to D1 DA receptor–containing medium spiny neurons in the NAc (MacAskill et al., 2014).

One of the most dramatic brain changes occurring during adolescence is the unfolding of DA connectivity in the mPFC. In contrast to DA projections to limbic regions (e.g., NAc) and cortical innervation of other monoamines (e.g., norepinephrine and serotonin) that reach adult density levels early in life (Coyle and Molliver, 1977; Levitt and Moore, 1979; Lidov et al., 1980; Benes et al., 2000; Diamond, 2002), DA projections to the mPFC do not fully mature until early adulthood (Kalsbeek et al., 1988; Benes et al., 2000; Manitt et al., 2011; Naneix et al., 2012). In rodent models of both sexes, the number of dopaminergic fibers in the mPFC increases linearly between the juvenile period (postnatal day [P] 25) and young adulthood, with the most prominent increases occurring between the late juvenile period and early adulthood (Naneix et al., 2012; Willing et al., 2017). Interestingly, this is not a rodent-specific phenomenon, as protracted mesocortical DA development occurs in nonhuman primates and most likely in humans (Rosenberg and Lewis, 1994; Lambe et al., 2000), paralleling cognitive maturation.

In addition to changes in dopaminergic projections in adolescence, changes in dopaminergic receptor expression are prevalent throughout the mesocorticolimbic system, which may underlie the altered sensitivity to rewarding stimuli. In the NAc and dorsal striatum of rats, DA D1 and D2 receptor expression peaks during adolescence (P40), then declines to reach adult levels at ~P80 (Andersen et

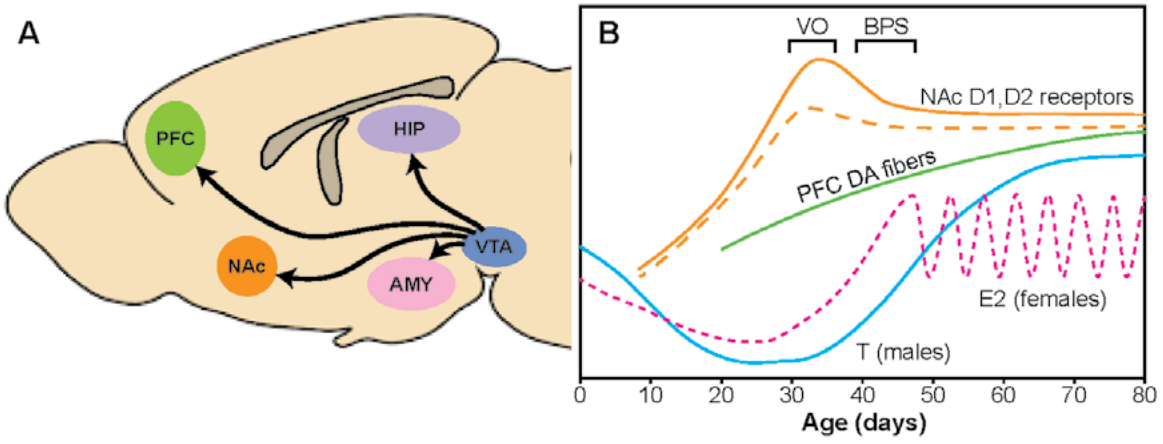

Figure 1. Adolescent development of the mesocorticolimbic DA pathway. ***A***, Major brain areas and projections of the mesocorticolimbic DA pathway. ***B***, Schematic of postnatal development of key c mponents of this pathway along with changes in gonadal steroid hormones and pubertal markers. Hashed lines, Data specific to females. Developmental patterns and markers are based on data from Tarazi and Baldessarini (2000) (NAc D1, D2 receptors); Naneix et al. (2012) and Willing et al. (2017) (PFC DA fibers); and Dohler and Wuttke (1975) and Vetter-O'Hagen and Spear (2012) (gonadal steroid concentrations and pubertal markers). VO, vaginal opening; BPS, balano-preputial separation, E2, estradiol; T, testosterone.

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al., 2000). In the PFC of male rats, there is also a selective decrease of cells expressing D1 receptors that project to the NAc between P44 and adulthood (Brenhouse et al., 2008). However, there may be important species differences in these receptor changes across adolescence (Pokinko et al., 2017), and little is known about sex differences in this developmental milestone.

Measures of functional connectivity in humans have further elucidated the widespread changes between the PFC and subcortical structures throughout adolescence, with some evidence suggesting that a relatively stable network connectivity state does not occur until at least the mid-20s (Dosenbach et al., 2010). The potential relevance of these changes for behavior is not fully understood, but decreases in the functional coupling between subregions of the PFC and the NAc have been linked to decreases in self-reported risky behavior across adolescence (Qu et al., 2015). Interestingly, studies of PFC activation in humans have revealed sex differences in function that go beyond what might be expected from the anatomical correlates. One such functional development is resting-state functional connectivity, which describes the degree of synchrony between two different brain regions or between nearby areas within a brain region. In the dorsolateral PFC, resting-state functional connectivity between the hemispheres tends to increase with age in males but decreases with age in females (Zuo et al., 2010).

These data demonstrate that the mesocorticolimbic DA pathway undergoes vast developmental changes during adolescence both in fiber projections to the PFC and in sensitivity to DA within the NAc, dorsal striatum, and PFC target areas through altered receptor expression. Some of these developmental changes seem to occur independently of the gonadal hormone surge associated with puberty (Andersen et al., 2000; Willing et al., 2017). Although tyrosine hydroxylase immunoreactivity in the PFC increases across adolescence, this increase does not appear to be associated with markers of pubertal status (Willing et al., 2017). Preventing the pubertal rise in gonadal hormones by gonadectomy on P28 does not alter the adolescent (P40) or adult (P80) levels of D1 or D2 receptor expression in the rat striatum (Andersen et al., 2000). Finally, many developmental changes occur before puberty (e.g., adult-like morphological features of striatal neurons) (Tepper et al., 1998; Lee and Sawatari, 2011). For many measures, more research is needed to answer this question. The influence of gonadal hormones on reward-associated behaviors and the mesocorticolimbic pathway in adults (Becker et al., 2012) suggests at least a modulatory role during adolescence, particularly with respect to the emergence of sex differences (Kuhn et al., 2010).

Pubertal Influences on mPFC Adolescent Development

Recent evidence suggests that, within the adolescent period, pubertal onset may be particularly critical in specific aspects of mPFC development and cognition. Previous work in rats has documented a reduction in mPFC volume between the juvenile and adult periods (Van Eden and Uylings, 1985), and this volumetric reduction may reflect a decrease in neuron number. Stereological quantification of the total number of neurons in the mPFC across adolescence revealed that the majority of neuronal losses occur during the period of pubertal onset, particularly in female rats (Willing and Juraska, 2015). Ovariectomy before puberty prevented these neuronal losses, further suggesting a role for pubertal hormones (Koss et al., 2015). Additionally, there are changes in dendritic complexity and synapse number in the mPFC during adolescence. Between P35 and P90, there is a reduction in dendritic spine density in both male and female rats (Koss et al., 2014). In a recent study, Drzewiecki et al. (2016) conducted an immunohistochemical analysis of synaptophysin as a marker for total synapse number in the mPFC in P25, P35, P45, P60, and P90 rats of both sexes. As expected, there was evidence for significant synaptic pruning during adolescence. Interestingly, a direct comparison of prepubertal versus postpubertal females at P35 and prepubertal versus postpubertal males at P45 (corresponding to the average age of pubertal onset) revealed that in both sexes, postpubertal animals had significantly fewer synapses than their prepubertal counterparts.

These structural alterations within the mPFC are associated with changes in cognitive performance during adolescence, which also seem to depend on the timing of puberty. These differences in cognitive performance could reflect differences in reward processing. Indeed, substance use disorder is often described as maladaptive decision-making and reward learning. Given the importance of the entire PFC in reward learning, it follows that structural changes in adolescence result in altered cognitive performance and decision-making with regard to reward. Kanit et al. (2000) found that pubertal onset alters learning strategies in spatial memory tasks. However, there is a paucity of research that accounts for a potential role for puberty, particularly on mPFC-dependent

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tasks. Willing et al. (2016) have recently shown that pubertal onset leads to better performance on an mPFC-mediated cognitive flexibility component of a Morris water maze task in both male and female rats. Path length to the novel platform location was shorter in postpubertal males and females, and prepubertal animals spent a greater amount of time swimming in the quadrant where the platform was initially located, suggesting a deficit in cognitive flexibility that subsides after pubertal onset. In support of these findings, recent evidence suggests that pubertal hormones play a critical role in the maturation of the PFC in female mice. Gonadectomy before puberty blocked the adolescent increase in inhibitory neurotransmission, and prepubertal estradiol treatment accelerated the maturation of inhibitory tone in the PFC and advanced the increase in cognitive flexibility in females (Piekarski et al., 2017). Future studies are needed to determine whether these temporal associations with pubertal status reflect pubertal mechanisms or coincidental timing.

Development of Social Reward During Adolescence

The adolescent transition from childhood to adulthood requires a qualitative shift in the perception of rewarding social interactions (Spear, 2000). In humans, adolescence is characterized by increases in time spent with peers and changes in the quality of social interactions with family and peers (Larson et al., 1996). Adolescents rely on their contemporaries for social support and are increasingly reactive to treatment by their peers (Ladd et al., 2014). These social relationships influence the development and maintenance of maladaptive behaviors in adulthood (Patterson et al., 1992; Hankin et al., 1998). Indeed, peer influence is a strong predictor of adolescent depression (Thapar et al., 2012). This reorganization of social structure during adolescence is necessary for social species to develop appropriate behavioral strategies for survival in adulthood (Gopnik et al., 2017). A close association between adolescent social reorganization and puberty is thought to increase exposure to genetically distinct individuals when sexual behavior emerges, thereby decreasing the chance of inbreeding within a social group (Lawson Handley and Perrin, 2007).

As in humans, adolescent changes in social interactions and social structure are prevalent in rodents. Adolescent male rats place a greater value on peer-directed activities (Pellis and Pellis, 2017) and exhibit a greater preference for social stimuli in a conditioned place preference (CPP) test when compared with adults (Douglas et al., 2004; Yates et al., 2013) and females (Douglas et al., 2004; Weiss et al., 2015). However, this effect is most pronounced in socially isolated males. Additionally, a peer-paired chamber negates CPP induced by cocaine (Zernig et al., 2013) and amphetamine (Yates et al., 2013) in adolescent males but not in females (amphetamine only; Weiss et al., 2015). These data suggest that there are striking sex differences in sensitivity to social reward in adolescent rodents and that males display a greater sensitivity to social reward than females. These differences appear to be influenced by the pubertal hormonal surge and may result in long-term alterations in reward valence, as evidenced by the influence of prepubertal gonadectomy on reward-associated behaviors in both male rodents (Schulz et al., 2009b; Bell et al., 2013a,b) and female rodents (Perry et al., 2013). It is thought that adolescent-specific social experiences result in permanent neural and hormonal changes that coalesce in cognitive strategies that lead to effective coping in adulthood (Spear, 2000). Therefore, these observed sex differences in sensitivity to social reward may profoundly influence the neural circuitry involved in reward and the sex differences in reward-associated behavior seen in adulthood.

The limbic system is a known regulator of social interaction and social reward. In particular, the amygdala is critically important for the integration of emotional stimuli and regulates emotional and motivated behaviors (Wassum and Izquierdo, 2015). The BLA, in particular, has been studied extensively for its role in reward because it is thought to be important in assessing/assigning value to stimuli and is a key regulator of social interactions. Activation of the BLA reduces social interaction (Sanders and Shekhar, 1995), whereas inhibition of glutamatergic or GABAergic transmission within the BLA increases social interactions (Sajdyk and Shekhar, 1997; Paine et al., 2017). Recent evidence suggests that these behavioral effects are likely projection specific, as activating BLA-to-PFC projections decreases social behaviors in male mice (Felix-Ortiz et al., 2016). In addition to its reciprocal glutamatergic projections with the PFC, the BLA projects to the NAc and receives dopaminergic projections from the VTA (Wassum and Izquierdo, 2015). Although sex differences in development have yet to be studied, it is clear that each of these circuits develops at different stages in males (Bouwmeester et al., 2002a,b; Cunningham et al., 2002; Caballero et al., 2014; Wassum and Izquierdo, 2015; Arruda-Carvalho et al., 2017). For example, projections from the PFC to the BLA are established between P10 and

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P15 (Bouwmeester et al., 2002b; Arruda-Carvalho et al., 2017), but the reciprocal projections (BLA to PFC) are established a few days earlier (Bouwmeester et al., 2002b; Cunningham et al., 2002). The amygdalar circuit (including NAc, VTA, PFC, and vHIP) develops during the juvenile/early adolescent period, and synapses are established by the second or third postnatal week. Although projections within the amygdalar circuitry are established before adolescence, recent evidence suggests that the PFC-to-BLA projections undergo significant synaptic strengthening (as measured by the IPSC:EPSC ratio) on P30 (Arruda-Carvalho et al., 2017). Also, there are more PFC-to-BLA projections on P31 compared with P24 and P45 (Pattwell et al., 2016), suggesting that this time point in male adolescence may be a crucial developmental period for limbic structures. The BLA (Trezza et al., 2012; Achterberg et al., 2015) and extended amygdala (Meaney et al., 1981; Meaney and McEwen, 1986; Jessen et al., 2010) are both important regulators of social play, a prominent juvenile social behavior that may be sexually dimorphic (Veenema et al., 2013) and is important for social, emotional, and cognitive development (Pellegrini, 1988; Vanderschuren et al., 1997; van den Berg et al., 1999; Baarendse et al., 2013). Notably, the PFC-BLA synaptic development coincides with the developmental rise in this behavior (Panksepp, 1981). Finally, binding at oxytocin and vasopressin receptors (two social neuropeptides) peaks in the BLA and central amygdala during adolescence (P35) in both males and females (Smith et al., 2017). Given the known sex differences in social reward (Borland et al., 2018), it is imperative that future research determine whether there are sex differences in the development of BLA connectivity with the reward circuitry.

Brain areas outside the canonical reward-associated circuitry are also likely to play a role in sex differences in reward and motivation, particularly those that are hormone sensitive, sexually dimorphic, and send projections to brain areas of the mesocorticolimbic pathway. The medial amygdala (meAMY) is larger in males than in females (Hines et al., 1992; Kerchner et al., 1995). However, unlike most sexually dimorphic brain regions, sex differences in volume of subnuclei within the meAMY do not emerge until adolescence, and the pubertal testosterone surge in males contributes to the organization of this sex difference (De Lorme et al., 2012). This change in meAMY structure co-occurs with changes in rewarding sociosexual behaviors that are in part regulated by it (De Lorme et al., 2012). Additionally, the meAMY is sensitive to stress in adolescence in a sex-dependent manner. For example, adolescent stress demasculinizes the meAMY: meAMY volume and cell number are decreased in males stressed during adolescence compared with their control counterparts, and these stressed males are less efficient at mating (Cooke et al., 2000). Collectively, this literature suggests that the meAMY contributes to the development, initiation, and maintenance of sex differences in reward and motivation. Additionally, the emergence of many sex differences in meAMY during adolescence is affected by social cues and could be crucial for the manifestation of sex differences in motivation and reward in adulthood.

Conclusions

The factors contributing to adolescent reward are many, and we are only beginning to understand the complex interactions among neural networks, endocrine axes, and environmental cues that direct the development of a functioning male- and female-typical mesocorticolimbic reward circuit. The many behavioral changes and neuroendocrine interactions may seem chaotic, but it is clear that adolescent development is a highly regulated and coordinated process. In this review, we have highlighted a few overarching themes that are beginning to emerge from the chaos: (1) There are notable sex differences in adolescent development that might underlie sexually dimorphic reward-associated behaviors in adulthood. (2) The mesocorticolimbic pathway is critical for adolescent changes in social reward and reward learning. (3) Reorganization of the reward circuitry, particularly the PFC, during adolescence relies on social interactions, pubertal hormones, as well as nonpubertal processes. (4) Adolescent reward circuitry is highly vulnerable to social stress and drugs of abuse. Further research is necessary for a comprehensive understanding of the factors that regulate the development of the mesocorticolimbic pathway, those that lead to increased vulnerability to disruption, and how this process drives developmental changes in motivation and reward. This research would benefit from the use of multiple approaches and models to disentangle the neural, endocrine, and environmental influences on adolescent reward. Together, these investigations will provide valuable insight into sex-specific psychiatric and behavioral disorders that arise during adolescence and could lead to novel avenues for treatment and prevention.

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Sex Differences in Behavioral Strategies: Avoiding Interpretational Pitfalls

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Introduction

Despite ample evidence for sex differences in brain structure and function, our understanding of the neurobiological basis of behavior comes almost exclusively from male animals. As neuroscientists move to comply with recent National Institutes of Health (NIH) mandates that biomedical researchers include both sexes in their studies, the ways we interpret outcomes in classic rodent behavioral models deserve closer scrutiny and more nuanced evaluation. In this mini-review, we highlight recent papers on sex differences in learning, decision-making, and spatial navigation paradigms that underscore the distinctions between cognitive capabilities and behavioral strategies that may confer unique benefits to males and females.

Neuroscientists have studied animal behavior in laboratory settings for more than a century, leading to an ever-increasing understanding of the relationships between structure, physiology, and function in the mammalian brain. In particular, rodent behavioral models have provided key insights into the neural basis of dozens of complex processes, including learning, decision-making, stress coping, aggression, and substance abuse. However, because the vast majority of behavioral neuroscience research has been conducted in male animals (Beery and Zucker, 2011), we inarguably (and regrettably) know much more about the male brain than we do about the female brain. In a recent attempt to rectify this imbalance across biomedical research, the NIH implemented a policy that requires funded researchers to consider sex as a biological variable (SABV) and include subjects of both sexes in all experiments (Clayton and Collins, 2014). Despite some resistance (Fields, 2014; Eliot and Richardson, 2016), this initiative is likely to succeed in illuminating aspects of brain function that are common to both sexes, as well as those that are sexually dimorphic (Eliot and Richardson, 2016). Information of either kind can be useful to basic and translational scientists alike, but it is critical—especially in behavioral research—that we interpret potential sex differences in outcome measures thoughtfully.

When we conduct behavioral experiments, we are, in essence, asking animals to tell us what the situation we've placed them in means to *them*. In many cases, the animal might engage in any number of responses, and it is up to us to determine what each one means to *us*. When males and females differ quantitatively in the outcome measures that we've defined, it's important to consider whether these differences reflect true disparities in, for example, cognitive ability or emotional state, or whether they signify a qualitative difference in behavioral strategies that may optimally serve the potentially discrete needs of each sex. In this review, we discuss recent studies that highlight this distinction and emphasize the need for thorough, careful behavioral analyses as more neuroscientists begin to incorporate SABV into experimental design.

Sex Differences in Common Behavioral Models

How do animals process information about threatening environments and stimuli? Although it is not necessarily surprising to learn that males and females might behave differently in response to stressful events, a nuanced understanding of how and why these differences exist is only just starting to emerge. A recent paper from Tronson and colleagues (Keiser et al., 2017) nicely demonstrates that after classical context fear conditioning, female mice are more likely than males to show a generalized freezing response in a novel context; that is, females treat new contexts more cautiously than males, pointing to a potential neural mechanism for this difference in strategy. This was true even with previous exposure to the shock-associated context, a finding that appears to help refine the distinction in male mice (i.e., reduced generalization). These behavioral differences were associated with discrete recruitment of major brain regions: in both contexts, whereas hippocampal activity was greater in males, females selectively showed activation of the basal amygdala. These data could suggest that female mice are unable to discriminate meaningful contexts, or they may instead indicate that after a traumatic experience, treating new environments with extra caution is evolutionarily beneficial to females. Examining this sex difference in a more naturalistic setting will be necessary to appropriately test these hypotheses. In the meantime, experimenters using classic Pavlovian approaches should consider that elevated context generalization in female mice may not reflect a cognitive deficit but instead a strategy to reduce risk to the animal's life.

This latter interpretation is supported by impressive new work by Pellman et al. (2017), who used a 42-day "closed economy" system to examine sex differences in foraging strategies when the foraging environment is risky. Here, male and female rats lived in a two-chamber home cage in which the nesting side was safe, but eating and drinking required traveling to a foraging arena that randomly delivered foot shocks. After two weeks of chamber acclimation without shocks, the authors observed

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the change in animals' behavior as they learned that they would have to endure shocks in order to forage. Although both sexes reduced the time they spent in the foraging chamber during the two-week shock period, males compensated by increasing their meal size, whereas meal size decreased in both intact and ovariectomized females, resulting in reduced overall food consumption and arrested weight gain. In the final two weeks, the shocks were terminated so that the authors could observe extinction. Males rapidly increased time spent in the foraging arena, whereas foraging time in females climbed much more slowly. Together, these data could be interpreted as evidence for impairments in cognitive flexibility or extinction learning in females, but they may instead reflect sex differences in strategy. Specifically, they suggest that females will select general safety over metabolic needs, preferring to avoid a potentially risky environment at the cost of blunted weight gain—even when the risk is no longer there. In contrast, males appear to adapt their feeding efficiency in order to maintain steady weight gain. Although the putative evolutionary value of these sexually divergent strategies is difficult to assess in a controlled laboratory setting, it is clear that males and females assess foraging in risky environments differently. The longitudinal design of this study is noteworthy and laudable because it allows unique insight into complex behavioral strategies over time, rather than capturing a brief snapshot of behavior, as is true of most paradigms.

Our lab has also found that females are more likely to engage in active behaviors to avoid potential threats. As we recently reported (Gruene et al., 2015a), a subset of female rats in a cued fear-conditioning paradigm exhibited escape-like "darting" behavior in response to the conditioned stimulus (CS). This paper suggests that measures of freezing alone are insufficient to quantify fear in females.These animals subsequently demonstrated enhanced extinction retention, suggesting that darting may reflect an adaptive mechanism that promotes cognitive flexibility (Maren et al., 2013). One alternative interpretation is that because they are smaller, female rats perceive the conditioning chamber as larger, and therefore the threshold for "predator imminence" (Fanselow and Lester, 1988) is shifted, thus increasing the likelihood of an escape response instead of freezing. However, this explanation is unlikely for a few reasons. First, within a large cohort (n = 58) of females, no relationship was found between body weight and darting prevalence (Gruene et al., 2015a; author response available at https://elifesciences.org/articles/11352). Second, the observation that animals are more likely to engage in escape responses in larger spaces has been reported only in environments much larger than our chambers (e.g., a hallway, as in Blanchard et al., 1986). A more recent attempt to observe this phenomenon in standard chambers that differed in size by a factor of approximately three (~15 × 23 cm versus 15 × 71 cm) failed to find an effect of chamber size on innate fear behavior (Kabitzke and Wiedenmayer, 2011).

Together, these findings support the idea that darting during classical cued fear conditioning is a sexually dimorphic strategy to promote escape. The fact that it both appears only in females and is advantageous for extinction in the long term may seem contradictory to clinical reports that women are more susceptible to posttraumatic stress disorder (Kessler et al., 1995; Breslau et al., 1999). However, resilient and vulnerable individuals can be found in most populations (Yehuda and LeDoux, 2007), and the absence of darting in males does not necessarily mean that they lack their own strategies and mechanisms for improving long-term outcomes. As we also recently reported (Gruene et al., 2015b), successful extinction retrieval in males (but not females) is correlated with a unique morphology in prefrontal amygdala circuitry. Although the incidence of darting was not associated with the estrous cycle, there is evidence that circulating ovarian hormones can influence fear learning and extinction (Cover et al., 2014). The key message of our work is that if only freezing were measured, darters would have been assumed to be cognitively impaired at forming a CS–US (conditioned stimulus–unconditioned stimulus) association. This is clearly not the case, and therefore freezing by itself is likely an insufficient measure of fear learning and responding, especially in female rats. A more comprehensive examination of animals' behavioral repertoires during classical conditioning tasks will be critical as we move to more thoroughly understand how each sex processes threatening stimuli.

Sex differences in risk evaluation can also be observed in models that more explicitly test decision-making. In an elegant set of experiments, Orsini et al. (2016) used a "risky decision task" to examine how male and female rats weigh reward and punishment against each other. In this task, animals chose between receiving a safe, small food reward or a large food reward that was intermittently punished with a shock. The authors then observed changes in animals' choices as they varied the likelihood of the shock. Although both males and females reliably chose the large reward when there was no chance of receiving punishment, females quickly switched to the small reward as shock probability increased.

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In contrast, males maintained high levels of large-reward choice, even when shock was guaranteed. To rule out the possibility that this effect was the result of greater pain thresholds in males due to their size, the authors recalibrated the intensity of the shock according to each animal's weight, and obtained the same results. Similar to the work described above by Pellman and colleagues (2017), these findings suggest that females will select a behavioral strategy that prioritizes physical safety over metabolic needs. Decision-making work that focuses on the adoption of strategies as the animal learns about outcome contingencies indicates that females take longer to settle on a strategy than males do (van den Bos et al., 2012). Although this delay could be interpreted as a disparity in learning, it is likely that it instead reflects a difference in how males and females use the information presented to them. As Orsini and Setlow (2017) argue in an excellent recent review, it may be more advantageous for males to take a more holistic, swift approach to assessing their situation, whereas females may benefit from a more measured evaluation of each option before settling. They further discuss in detail how this difference applies even in nondangerous scenarios. Here, too, the latter approach in females may indirectly stem from drives related to reproductive success, as mate choice in females is a far more selective process than it is in males (Snoeren and Ågmo, 2014; McCormick et al., 2017). However, it should be noted that this possibility has yet to be directly tested in the lab.

Finally, sexually divergent strategies can be observed in studies of spatial navigation. Although the idea that males and females perform differently in spatially dependent tasks is not new (Gaulin et al., 1990; Galea et al., 1994), recent work has begun to uncover some of the neurobiological mechanisms that determine these differences. Navigation studies are often designed to test whether an animal solves a spatial task using a global, "geometric" strategy or more self-focused or landmark-dependent strategies. Work in both humans and rodents suggests that males prefer the former, whereas females prefer the latter (Blokland et al., 2006; Jones and Healy, 2006). Importantly, this difference appears to depend on circulating ovarian hormones (Korol et al., 2004). More recently, Rodríguez et al. (2013) demonstrated that prepubertal female rats select environment-based strategies in a "snowcone" task and then switch to landmark-guided navigation as adults. They additionally found that ovariectomy caused adult females to adopt a geometric strategy, suggesting that the female preference for nongeometric strategies may develop during puberty in order to aid reproductive success (Jones et al., 2003). Recent work by Yagi et al. (2016) further defined the significance of strategy distinctions by dividing larger cohorts of both male and female subjects by their strategy preference. They found that males that used a geometric spatial strategy displayed enhanced pattern separation and had greater neurogenesis in the dentate gyrus, but neither of these correlations was observed in females that preferred the same strategy. Therefore, as in other models, the divergence of navigational strategy selection between males and females appears to uniquely serve each sex.

Conclusions

The studies discussed here represent just a small subset of the rapidly growing body of behavioral neuroscience literature that considers SABV. Our primary goal was to emphasize the need to be mindful of outcome interpretations and consider alternative explanations for sex differences in common paradigms. As we have argued previously (Shansky, 2015), doing so may be especially important in stress-related and anxiety-related models, like the elevated plus maze and the forced swim test, which were developed using mostly males and therefore may not tap into the same processes in females (Fernandes, 1999; Kokras et al., 2015). Although the scope of this brief perspective chapter did not encompass stress research, exciting recent work points to novel, sex-dependent mechanisms underlying the impact of stress on cognition and physiology (Laredo et al., 2015; Senst et al., 2016; Grafe et al., 2017). In addition, an excellent review on this topic (Bangasser and Wicks, 2017) can be found in the recent double issue of *Journal of Neuroscience Research* entitled "An Issue Whose Time Has Come: Sex/Gender Influences on Nervous System Function" (Journal of Neuroscience Research, 2017). This epic collection of more than 70 reviews and primary research articles should serve as a foundational primer for any neuroscientist interested in sex differences in the brain. For behavioral neuroscientists in particular, it is critical that we be prepared to challenge dogmas about what our models tell us and consider the possibility that even seemingly identical behavioral outcomes in males and females could have discrete underlying mechanisms (De Vries, 2004). This point is especially relevant for those of us whose goal is to inform translational and clinical work: whether or not there are gender disparities in disease prevalence, an understanding of the biological basis of addiction, mental illness, and neurological disorders in each sex will be integral to developing more effective treatments. It is therefore vital that we pay careful attention to behavioral studies and interpret putative sex differences thoughtfully.

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Sex Differences and the Role of Ovarian Hormones in Modulating the Behavioral Effects of Nicotine in Rodent Models

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Overview

Sex differences are pronounced in tobacco use, with females displaying higher rates of smoking and greater susceptibility to the negative health consequences associated with long-term tobacco use (Lombardi et al., 2011; Park et al., 2016). Women are also less likely to quit smoking, and cessation medications appear to be less effective in women than men (McKee et al., 2016; Smith et al., 2016). Unfortunately, our understanding of the factors that promote tobacco use in females is lacking. Thus, more research is needed to provide information that will help reduce health disparities between women and men. Additional work in this area will also help researchers respond to the recent mandate from national funding agencies to address sex differences in various public health problems, such as substance abuse. This chapter summarizes existing preclinical work in rodents examining sex differences and the role of ovarian hormones in modulating the behavioral effects of nicotine, the major addictive compound in tobacco products. We also consider the unique challenges of studying nicotine use in female rats and present new data examining the effects of nicotine on the estrous cycle.

Behavioral Effects of Nicotine in Rodent Models

The most frequently used rodent models for assessing the rewarding effects of nicotine involve operant responding for intravenous self-administration (IVSA) or an increase in time spent in an environment paired previously with nicotine in conditioned place preference (CPP) procedures. IVSA involves operant learning, in which the reinforcing properties of a drug are assessed based on its ability to increase a behavioral response. CPP involves classical conditioning, in which the drug serves as the unconditioned stimulus and the environmental context serves as the conditioned stimulus. During conditioning, the animals are administered nicotine and are then confined to a compartment with distinct environmental cues. On intervening days, the rats are given vehicle and confined to the alternate compartment. After conditioning, rats are allowed to explore both compartments in a drug free state. CPP is operationally defined as an increase in time spent in the drug-paired side versus the neutral location.

During abstinence from chronic nicotine exposure, a withdrawal syndrome emerges in rodents that includes physical signs and negative affective states. A common method for inducing nicotine dependence in rodents involves surgical implantation of osmotic pumps that deliver nicotine for at least 5–7 days. Nicotine withdrawal has been studied following the removal of the nicotine pump (spontaneous withdrawal) or administration of a nicotinic receptor antagonist (precipitated withdrawal). A common behavioral model to study negative affective states produced by withdrawal involves avoidance of a chamber paired previously with nicotine withdrawal in conditioned place aversion (CPA) procedures. An array of measures can be used to assess anxiety-like behavior produced by withdrawal. These include an increase in time spent in the closed arms of an elevated plus maze or the dark side of the light/dark transfer apparatus.

The sections below describe sex differences and the role of ovarian hormones in modulating nicotine reward and withdrawal. The chapter is organized following a prescribed approach that first assesses whether sex differences exist (Becker et al., 2005). If differences exist between female and male rats, then the role of ovarian hormones can be assessed in ovariectomized (OVX) female rodents. If ovarian hormones appear to modulate a particular effect, then subsequent studies might examine the effects of hormone replacement in OVX rats and/or examine whether behavioral effects fluctuate across the estrous cycle.

Sex differences

Differences between female and male rodents result from a complex interplay of biological and developmental factors. The results from studies of sex differences in nicotine IVSA are mixed, with some reports showing greater nicotine intake in females and other reports showing greater intake in males. To more clearly understand the nature of sex differences in nicotine IVSA, we recently conducted a meta-analysis that combined effect-size values from studies that compared nicotine IVSA in female and male rats under various experimental conditions (Flores et al., 2017). Overall, the analysis revealed that female rats display greater nicotine IVSA than males. A subsequent moderator-variable analysis also revealed that female rats also display greater nicotine intake in procedures involving extended access to IVSA, a cue light that signals nicotine delivery, and higher reinforcement requirements for nicotine administration. This finding suggests that certain experimental parameters can influence the magnitude of sex differences in nicotine IVSA. Consistent with findings from IVSA studies, females also display more robust CPP produced by nicotine compared with male rats (Torres et al., 2009) and mice (Kota et al., 2007, 2008).

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With regard to nicotine withdrawal, females display greater CPA produced by nicotine withdrawal compared with male rats (Torres et al., 2009) and mice (Kota et al., 2007, 2008). Subsequent work has revealed that female rats display larger increases in anxiety-like behavior, corticosterone levels, and changes in the expression of stress-associated genes in the brain as compared with males (Torres et al., 2013). Consistent with work from other laboratories, female rats display greater increases in plasma corticosterone levels during nicotine withdrawal than males (Gentile et al., 2011; Skwara et al., 2012). Together, these studies suggest that both the rewarding effects of nicotine and the aversive effects of withdrawal are greater in female versus male rodents. These findings provided the foundation for our hypothesis that greater sensitivity to nicotine reward and withdrawal contributes to enhanced vulnerability to nicotine use in females (O'Dell and Torres, 2014).

Studies comparing sex differences in nicotine reward and withdrawal involve unique challenges. In procedures involving extended access to nicotine IVSA, we have noted that catheter patency is generally longer in male versus female rats. This may be related to greater tissue growth that envelops the catheter entry port into the jugular vein, which likely produces greater insulation of the catheter in males. Also, female rats are smaller, with thinner skin, and these characteristics appear to contribute to more abrasions and greater shifting of the catheter port on the ventral surface of female rats. The latter effects may also be related to the fact that nicotine produces a greater suppression of body weight in female versus male rats (Grunberg et al., 1985). We have also noticed that a greater percentage of female rats self-administer high doses of nicotine, sometimes to the point of death. Female rats may be less sensitive to the aversive effects of nicotine, a hypothesis that is consistent with the finding that high doses of nicotine produce less CPA in female versus male rats (Torres et al., 2009). A final point to consider is that nicotine administration increases locomotor behavior to a larger extent in female than male rats, an effect that has been observed across different strains (Faraday et al., 2003). Thus, strong stimulant effects have the potential to alter behavioral assessments of sex differences in nicotine IVSA in rats.

Ovarian hormones

The role of ovarian hormones in modulating the behavioral effects of nicotine has been assessed following ovariectomy procedures that remove the major source of ovarian hormones. Previous studies have revealed that OVX rats display a reduction in nicotine IVSA (Flores et al., 2016) and CPP (Torres et al., 2009) as compared with intact females. Also, estradiol replacement in OVX rats returned nicotine IVSA to intact-female levels (Flores et al., 2016). Estradiol appears to have intrinsic rewarding properties, as this hormone produced CPP in OVX rats (Frye and Rhodes, 2006), and conversely, administration of an estrogen receptor antagonist inhibited the formation of CPP produced by estradiol in OVX rats (Walf et al., 2007).

With regard to withdrawal, previous work has shown that OVX female rats display less anxiety-like behavior produced by nicotine withdrawal as compared with intact females (Torres et al., 2015). This report also showed that the expression of stress-associated genes was reduced in the brains of OVX versus intact females. OVX female rats also displayed a reduction in dopamine and estradiol receptor gene expression, suggesting that estradiol regulates gene expression in female rats. To our knowledge, the role of progesterone in modulating nicotine reward and withdrawal has not been examined in OVX rodents.

There are several issues to consider when employing ovariectomy procedures. First, ovariectomy can be done either early in development to study the organizational effects of hormones or following puberty to examine the activational effects in a developed rodent. This presents a challenge because there is no clear agreement about how early to perform the ovariectomy to study the organizational effects of ovarian hormones. The onset of puberty has also been shown to be species dependent (Gillies and McArthur, 2010; Sengupta, 2011). Another challenge in ovariectomy studies is that hormone replacement procedures vary with regard to doses and the frequency of the injection procedure. Some studies administer estradiol in a 2 day on, 2 day off procedure that mimics the two peak increases in estradiol that occur across the estrous cycle. Progesterone is typically administered via injections or silastic implants that attempt to mimic reduced peak changes and the steady rise in this hormone across the estrous cycle.

Estrous cycle

The estrous cycle in rodents is categorized into the luteal and follicular phases. The luteal phase is characterized by a gradual rise in progesterone and decreasing levels of estradiol. During the follicular phase, estradiol levels peak, and progesterone levels slowly increase, leading to ovulation. The luteal phase can be subdivided into metestrus and diestrus, and the follicular phase comprises proestrus and

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estrus. The fluctuations in ovarian hormones change the cellular cytology of the vaginal wall in a manner that allows researchers to identify particular phases of the estrous cycle in female rodents using vaginal lavage procedures.

With regard to the rewarding effects of nicotine, an early report revealed that female rats displayed high levels of nicotine IVSA that did not fluctuate across the estrous cycle (Donny et al., 2000). Consistent with this, a subsequent report revealed that the magnitude of CPP produced by nicotine was similar in rats that were tested during different phases of the estrous cycle (Torres et al., 2009). Both studies used lavage procedures. However, another report revealed that adolescent female rats displayed high levels of nicotine intake that were negatively associated with progesterone levels but positively associated with the ratio of estradiol to progesterone (Lynch, 2009). This latter finding suggests that plasma levels of estradiol and progesterone may influence nicotine reward in a manner that may not be evident in studies utilizing vaginal lavage procedures. To our knowledge, no studies have compared nicotine withdrawal in female rats during different phases of the estrous cycle.

When using vaginal lavage procedures, several factors should be considered. First, a previous report found that repeated lavage can induce a pseudo-pregnancy involving constant diestrus (Goldman et al., 2007). Thus, repeated lavage may alter the vaginal cytology in a manner that makes it difficult to assess whether behavioral effects fluctuate across the estrous cycle. Second, the classification of estrous cycles based on cellular cytology is subjective, and there is overlap in the cell cytology across stages. Third, nicotine exposure possibly alters the estrous cycle in female rats. To address the third issue, the following section examines whether nicotine alters the estrous cycle in freely cycling female rats.

Does nicotine exposure alter the estrous cycle?

We conducted repeated vaginal lavage procedures in 54 intact-female adult Wistar rats. The cytology was classified into one of four phases of the estrous cycle. Briefly, a sterile plastic pipette was filled with 0.9% saline to collect epithelial cells, which were then transferred to a glass microscope slide. The cells were then fixed with methylene blue stain and viewed under a microscope to examine their shape. Each phase was classified using the following criteria: proestrus (presence of round nucleated epithelial cells), estrus (presence of cornified, nonnucleated epithelial cells), metestrus (limited presence of epithelium cell and leukocytes), and diestrus (presence of leukocytes) (Cora et al., 2015). The lavage procedures were conducted for 8 days before and after implantation of an osmotic pump that delivered nicotine (3.2 mg/kg/day; expressed as base). The rats were pair-housed in a colony room that was kept on a 12 hour reverse light/dark cycle with lights off at 8:00 A.M. The lavage procedures were conducted every 24 hours at approximately 9:00 A.M.

First, we assessed the effects of nicotine on the estrous cycle by computing the frequency at which each stage was sampled before and then during nicotine exposure. The resulting pie chart (Fig. 1) displays the percentage of times the rats were sampled during each phase of the estrous cycle. During the luteal phase, the rats were sampled less frequently in metestrus pre-nicotine (31%) than during nicotine exposure (37%). Also, the rats were sampled less frequently in the diestrus phase pre-nicotine (25%) than during nicotine exposure (27%). The increase in sampling frequency within the luteal phase during nicotine exposure resulted in a concomitant reduction in the follicular phase. Specifically, the rats were sampled more frequently in estrus pre-nicotine (32%) than during nicotine exposure (25%). Also, there was a small decrease in sampling frequency in diestrus pre-nicotine (12%) compared with during nicotine exposure (11%). These results suggest that nicotine exposure produced a lengthening of the luteal phase and a concomitant decrease in the follicular phase.

To better understand how nicotine altered the estrous cycle, we conducted an analysis that quantified the transitions between each individual phase of the estrous cycle. The results of this analysis are depicted in the heat map shown in Figure 2. Our assessment of the heat map revealed that nicotine exposure produced an increase in transitions in the luteal phase and a decrease in transitions in the follicular phase. To assess whether the latter observations reached statistical significance, we compared the average number of transitions pre-nicotine versus during nicotine exposure ($p \leq 0.05$). We observed a significant increase in transitions from metestrus ~ metestrus and from metestrus ~ diestrus. We also noted a decrease in transitions from estrus ~ estrus. Altogether, our results imply that nicotine extended the luteal phase, as evidenced by an increase in the number of transitions in this phase. We also observed a concomitant shortening of the follicular phase that appears to be caused by a decrease in transitions during nicotine exposure. In general, we observed a greater number of changes in transitions in the luteal phase (significant changes in two green cells) as compared

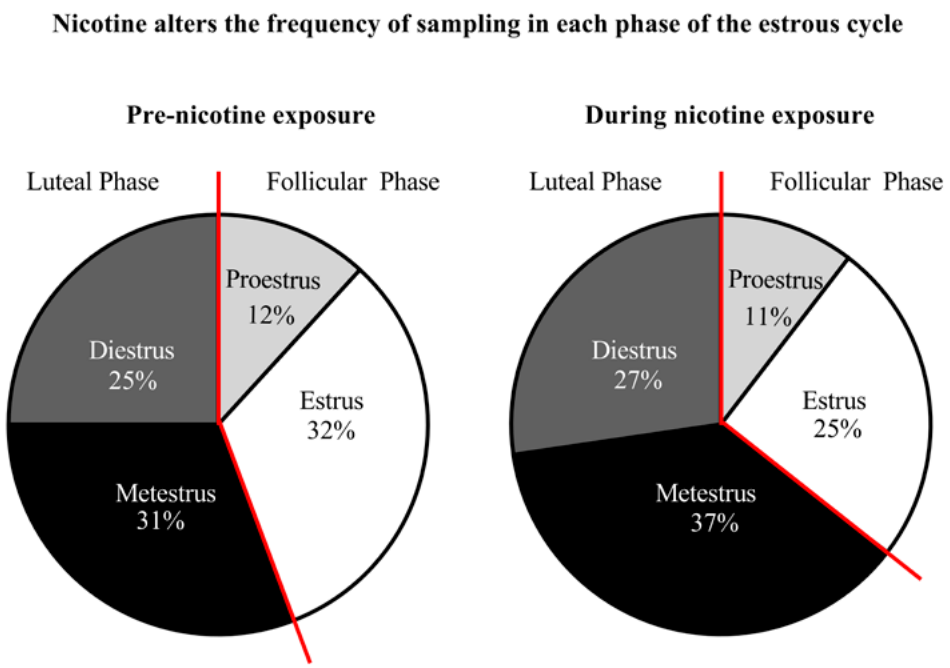

Figure 1. Pie charts denoting the frequency of sampling in each phase of the estrous cycle. During the luteal phase, the rats were sampled less frequently in metestrus pre-nicotine than during nicotine exposure. The rats were sampled less frequently in the diestrus phase pre-nicotine than during nicotine exposure. The increase in sampling frequency within the luteal phase during nicotine exposure resulted in a reduction in the follicular phase. These results suggest that nicotine exposure lengthened the luteal phase and concomitantly decreased the follicular phase.

with the follicular phase (significant change in one red cell). This might have been expected given that the rats were sampled once every 24 hours, and one might expect a higher incidence of transitions in the longer luteal phase (metestrus ~21 hours; diestrus ~57 hours) relative to the shorter follicular phase (proestrus ~12 hours; estrus ~12 hours). These time estimates are based on a publication using lavage procedures in female Wistar rats (Paccola et al., 2013).

In conclusion, it appears that nicotine exposure might have expanded the luteal phase and shortened the follicular phase of the estrous cycle. One possible explanation is that the antiestrogenic effects of nicotine blunt peak increases in ovarian hormones. If so, this would result in a cytology phenotype that reflects a shortened follicular phase and a longer luteal phase during which hormone levels are relatively lower. Consistent with these findings, clinical reports have shown that heavy smokers display a shorter follicular phase as compared with nonsmokers (Windham et al., 1999). Our hypothesis that nicotine lowers hormone levels is also consistent with the finding that women who smoke display increased breakdown of estradiol (Michnovicz et al., 1986) and excrete less estradiol during the luteal phase, suggesting a decrease in estrogen production (MacMahon et al., 1982). Moreover, epidemiological reports suggest that women who smoke are relatively estrogen deficient and reach menopause at an earlier age compared with female nonsmokers (Midgette and Baron, 1990). The limited literature on the effects of nicotine on the estrous cycle have yielded mixed results, with one study showing that nicotine does not alter the sampling frequency across the estrous cycle (Halder et al., 2015) and another study showing that nicotine increases sampling frequency in estrus (Wenning et al., 2017). Future studies are needed to more clearly resolve the influence of nicotine on the estrous cycle.

Neural circuitry

Preclinical studies have shown that the neural circuitry that governs the behavioral effects of nicotine is mediated largely by dopamine in the mesocorticolimbic pathway, which originates in the ventral tegmental area (VTA) and terminates in several forebrain structures, including the nucleus accumbens (NAc) (Dani et al., 2011; Pistillo et al., 2015). Following nicotine administration, dopamine levels in the NAc are increased, and during nicotine withdrawal, dopamine levels in this region are decreased. NAc dopamine release from VTA projections is regulated by a balance

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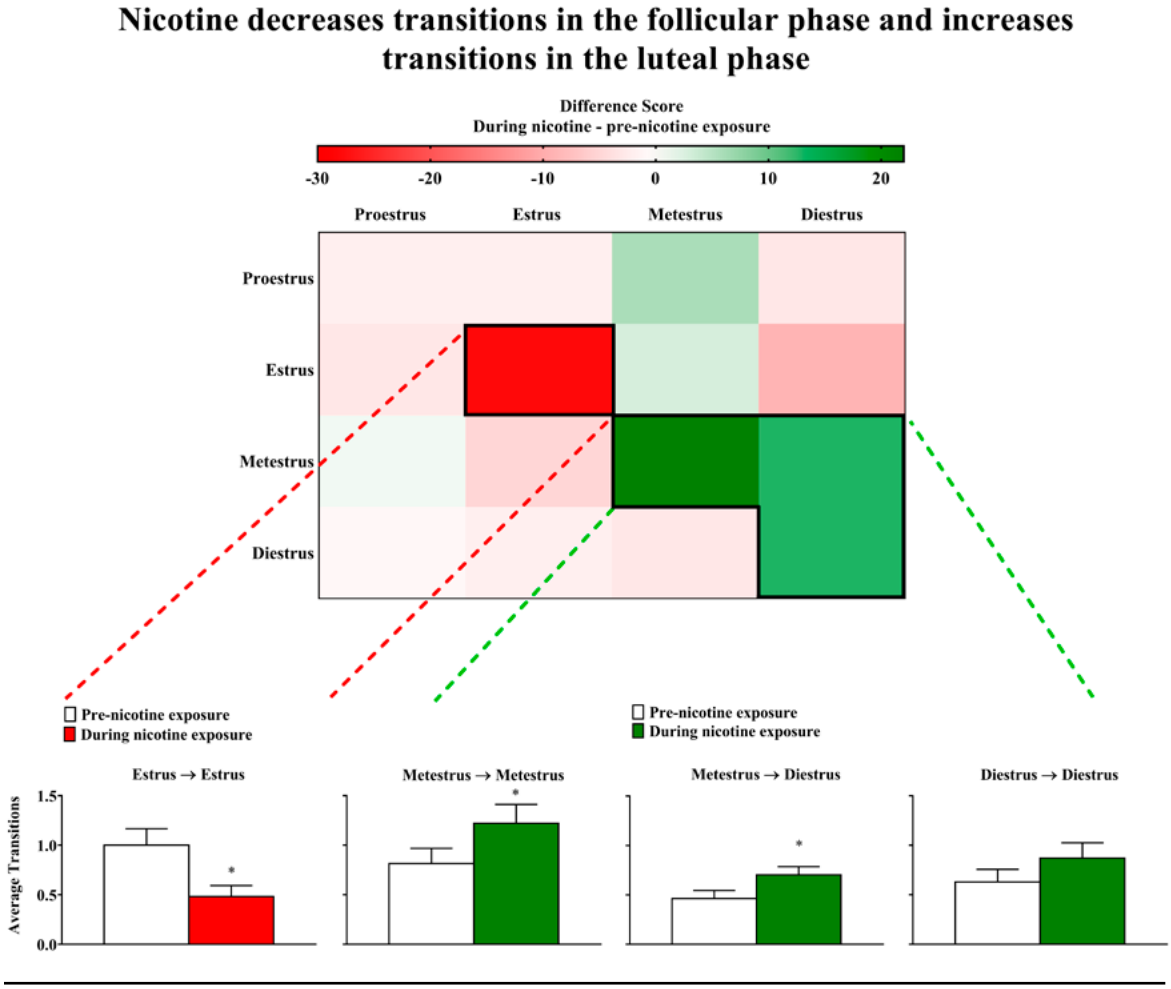

Figure 2. Heat map showing the frequency of transitions from each phase of the estrous cycle (vertical axis) to the other phases of the cycle (horizontal axis). To illustrate changes in the number of transitions produced by nicotine exposure, a difference score was calculated by subtracting the frequency of transitions during nicotine exposure from pre-nicotine values. The green shade reflects an increase, and the red shade denotes a decrease in the frequency of transitions produced by nicotine exposure. White boxes denote a lack of change in transitions following nicotine exposure. A greater number of changes in transitions were observed in the luteal phase (significant changes in 2 green cells) than in the follicular phase (significant change in 1 red cell).

between excitatory and inhibitory inputs on VTA dopamine neurons. To our knowledge, nicotine-induced dopamine release in the NAc has not been directly compared in female and male rodents. Work in our laboratory has shown that, during nicotine withdrawal, the decrease in dopamine release in the NAc is larger in female than in male rats (Carcoba et al., 2017). This large decrease in dopamine appears to be caused by greater GABAergic inhibition of dopamine release in the NAc of female versus male rats. The notion that the NAc modulates sex differences produced by nicotine withdrawal is consistent with the finding that nicotine withdrawal produces a larger upregulation of stress-associated genes in the NAc of female versus male rats, an effect that was not observed in the amygdala or hypothalamus (Torres et al., 2013). A subsequent report found that the upregulation of stress-associated genes in the NAc is blunted in female rats lacking ovarian hormones (Torres et al., 2015). These studies suggest that the NAc plays a key role in modulating sex differences in nicotine withdrawal.

In addition, a growing body of literature suggests that the aversive effects of nicotine withdrawal are also modulated via the habenula-interpeduncular nucleus (Hb-IPN) pathway (Dani and De Biasi 2013; Fowler and Kenny, 2014; Molas et al., 2017). In our assessment of this literature, studies that have examined the role of the Hb-IPN pathway in the behavioral effects of nicotine have used male rodents. Thus, future studies are needed to better understand how different brain pathways (e.g., mesocorticolimbic and Hb-IPN) modulate sex differences in the behavioral effects of nicotine.

Stimulant drugs other than nicotine

Previous studies using drugs of abuse other than nicotine have found that female rats display greater cocaine-induced IVSA (Lynch and Caroll, 1999; Swalve et al., 2016) and CPP (Zakharova et al., 2009). Similarly, female rats display greater methamphetamine IVSA (Reichel et al., 2012) and CPP (Chen et al., 2003) than males. With regard to the contribution of ovarian hormones, previous studies have found that OVX females display a reduction in the rewarding effects of cocaine that returns to control levels following estradiol replacement (Lynch et al., 2001; Russo et al., 2003, 2008; Larson et al., 2007). Moreover, female rats also display higher levels of cocaine-induced reinstatement than do males (Kerstetter et al., 2008). Interestingly, the latter effect was more pronounced in female rats that were tested during estrus.

Conclusions and Remaining Questions

A novel finding presented here is that nicotine exposure lengthened the luteal phase and shortened the follicular phase of the estrous cycle. This conclusion is based on an increase in sampling frequency during metestrus and diestrus, and a decrease in sampling during estrus. Consistent with this pattern, previous studies in female rats have shown that repeated exposure to methamphetamine (Siato et al., 1995) or cocaine (King et al., 1990) increases sampling frequency in diestrus. Our pattern of changes is also consistent with studies showing that chronic alcohol exposure increases sampling frequency in metestrus and diestrus and decreases sampling in estrus (Sanchis et al., 1985). Together, these studies suggest that females display strong rewarding effects across a number of drugs of abuse and that proper estrous cyclicity is altered by chronic drug exposure.

In the present assessment of the literature, many questions remain to be addressed in order to better understand sex differences and the role of ovarian hormones in the behavioral effects of nicotine. Below we offer a few remaining questions that might be addressed in future studies using preclinical models:

- Do compounds other than nicotine in tobacco products promote the behavioral effects of nicotine in females?
- Are there sex differences in nicotine metabolism that promote the behavioral effects of nicotine?
- Are there sex differences in nicotine withdrawal? If so, are these effects ovarian-hormone dependent?

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Neuroinflammation and Neurosteroidogenesis: Reciprocal Modulation During Injury to the Adult Zebra Finch Brain

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Introduction

Steroids organize, reorganize, and activate the developing, juvenile, and adult CNS and are thus considered critical modulators of brain and behavior throughout the vertebrate lifespan (Gurney and Konishi, 1980; Breedlove and Arnold, 1981; MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; McEwen, 2002; McEwen and Milner, 2017). Estrogens like 17β-estradiol (E2) are known organizers of masculine and feminine sexual behavior (Gurney and Konishi, 1980; MacLusky and Naftolin, 1981; Adkins-Regan and Ascenzi, 1990), although they also activate juvenile and adult behaviors in both sexes. Indeed, the range of physiological and behavioral endpoints affected by E2 has increased considerably and now includes, but is not limited to, the regulation of copulation, aggression, mood, balance, learning, and memory. Included in this list is a more recently discovered role for this steroid in the regulation of neuroplasticity and the preservation of neural circuits.

Influence of E2 on the Injured Brain

We have now learned that, in addition to E2-mediated plasticity of the normal brain, the injured brain is profoundly affected by this steroid. Indeed, premenopausal women have a lower risk of stroke compared with age-matched men (Barrett-Connor and Bush, 1991), and hormone replacement has been reported to decrease the risk of neurotrauma associated with cardiovascular disease (Grady et al., 1992). Interestingly, following traumatic brain injury (TBI), although there is no sex difference in the duration of unconsciousness following injury, the predicted outcome and recovery of females are better than for males (Groswasser et al., 1998). Taken together, these data suggest the possibility that endocrine factors may be responsible for some aspects of protection associated with neurotrauma.

In support, animal studies strongly suggest a role for E2 in neuroprotection and brain injury. Hall and colleagues (1991) reported lower levels of necrosis in females relative to males following experimental ischemia in gerbils, and embolic infarcts in rats (Nagpal et al., 1996). This bias also holds true for mice following medial carotid artery occlusion (Delpy et al., 2005; Brown et al., 2010; Fairbanks et al., 2012; Liu and McCullough, 2012). Further, infarcts from strokelike injuries are larger in metestrus rodents compared with those in estrus (high E2) and are inversely related to circulating E2 levels (Sohrabji and Williams, 2013). Additionally, ovariectomy increases subsequent infarct size relative to sham surgeries, and infarct sizes increase further the longer the animal is deprived of ovarian estrogens (Selvamani and Sohrabji, 2010).

Thus, the data reveal a neuroprotective effect of circulating E2 following brain trauma in multiple species that may involve several cellular mechanisms, including cell turnover. In many vertebrates, E2 is a well-established regulator of adult neurogenesis, neuronal survival, and neuronal death. E2 is an effective protectant across a broad range of neural insults. As many *in vitro* and *in vivo* studies have found, E2 protects neurons against cell damage and death such as that caused by serum deprivation (Green et al., 1996), glutamate (Mize et al., 2003), excitotoxicity (Garcia-Segura et al., 1999a), or mechanical injury (Peterson et al., 2001). Indeed, E2 is neuroprotective in several experimental models, including stroke and multiple sclerosis (Brown et al., 2010), and involves the action of estrogens on apoptotic and inflammatory pathways (Delpy et al., 2005). We are now beginning to learn that the source of this steroid is not limited to the periphery, but also involves an increase in neural synthesis of E2, particularly following damage to the brain.

Induction and consequences of injury-associated aromatase expression in reactive astrocytes

E2 is synthesized in many tissues, including the ovaries, adipose tissue, and placenta (Simpson et al., 2002). The brain also synthesizes E2 via the neuronal expression of aromatase (*E-synthase*) (MacLusky and Naftolin, 1981; Peterson et al., 2004). Neuronal aromatization has been intensely studied in many vertebrates because of its pivotal role in the organization and activation of reproductive behaviors (MacLusky and Naftolin, 1981; Balthazart et al., 1983; Balthazart and Schumacher, 1984; Adkins-Regan and Ascenzi, 1990). However, the previous decade has taught us much about aromatization in nonneuronal cells and the role that glial-derived E2 plays in neuroprotection (Garcia-Segura and McCarthy, 2004; Saldanha et al., 2010, 2013).

In mammals and birds, various forms of neural insult result in a dramatic upregulation of aromatase in reactive astrocytes at the site of damage (Azcoitia et al., 2002; Garcia-Segura and McCarthy, 2004; Saldanha et al., 2010). Specifically, excitotoxic damage to the hippocampus, a stab wound to the cerebral cortex, or a penetrating wound to the entopallium all induce astrocytic aromatase expression in rats and birds (Garcia-Segura et al., 1999b; Peterson et al., 2001, 2004; Azcoitia et al., 2002; Rau et al., 2003; Wynne and Saldanha, 2004; Saldanha et al., 2005; Wynne

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et al., 2008). The upregulation of aromatase (and resultant E2 provision) is neuroprotective, as site-specific aromatase inhibition increases (Wynne and Saldanha 2004) and E2 administration decreases (Saldanha et al., 2005) the extent of damage after mechanical injury and other neural insults (Saldanha et al., 2013). More specifically, injection of the aromatase inhibitor fadrozole results in larger injuries and more apoptosis relative to the vehicle alone (Azcoitia et al., 2002; Wynne and Saldanha 2004; Wynne et al., 2008). In the zebra finch, the inhibitory influence of local aromatization on apoptosis is potent enough to completely mask the wave of secondary degeneration consistently observed in the injured mammalian brain (Benkovic et al., 2006). This wave of secondary degeneration, however, is clearly observable upon aromatase inhibition in the injured songbird brain (Wynne et al., 2008). The influence of induced aromatization on indices of degeneration is similar but not identical in the rodent brain. Aromatase expression is induced in astrocytes following various forms of insult in the rodent brain (Azcoitia et al., 2003; Garcia-Segura and Melcangi 2006; Arevalo et al., 2015). Further, pharmacological or genetic inhibition of aromatase results in greater neuropathy following mechanical brain damage in rodents (Azcoitia et al., 2001). These data suggest that in multiple species, the induction of aromatase is key in controlling brain damage following neural insult.

In contrast, aromatase inhibition with concomitant replacement with E2 dramatically reverses the aforementioned effects in songbirds with corresponding decreases in the size of injury and lower levels of cell death, including apoptosis (Saldanha et al., 2005). In agreement, peripheral or central administration of E2 has been found neuroprotective in rats and mice (Garcia-Segura and McCarthy, 2004). The protective effects of E2 provision also involve mechanisms that may repair damaged tissue, as evidenced by the observation in the songbird that E2 replacement around sites of a penetrating central injury increases cytogenesis and neurogenesis (Walters et al., 2011).

This influence on multiple indices of cell turnover (most if not all of which may preserve and/or rebuild neural circuits) provides a promising target for therapies that seek to limit neurodegeneration and promote recovery following TBI. In fact, understanding the specific insult-dependent signal that is responsible for rapidly inducing aromatase expression and E2 provision may be key to developing such therapies.

The aforementioned studies have laid the foundation for a recent expansion in the literature about the role of sex, steroids, and their mechanism of function following TBI (Gibson et al., 2008; Berry et al., 2009; Herson et al., 2009; Chakrabarti et al., 2015; Brotfain et al., 2016). In general, the data all point to increased resilience following TBI in women compared with men (Ponsford et al., 2008; Yeung et al., 2011), a pattern also reflected in several studies in rodent models (Sarkari et al., 2010; Shahrokhi et al., 2010; Day et al., 2013). Indeed, neuroprotective estrogens during TBI appear to work via the more recently discovered membrane form of the receptor, G-protein coupled estrogen receptor-1 (GPER1), which provides a mechanism for rapid effects of these steroids on various aspects of neuroplasticity (Day et al., 2013; Wang et al., 2017). The neuroprotective effects of E2 are echoed by similar effects of progesterone in rodents (Feeser and Loria, 2011; Stein, 2013), although these patterns are not well supported by more recent clinical trials in humans (Lin et al., 2015; Stein, 2015; Zeng et al., 2015).

Brain injury may induce aromatase expression in astrocytes via alternative transcripts

What is responsible for aromatase expression in reactive astrocytes following neural insult? Our laboratory first decided to take a molecular approach to answering this question by entertaining the hypothesis that the aromatase transcript expressed in astrocytes following brain damage could be a novel transcript variant induced only by factors associated with neurotrauma. The number of genes for aromatase varies across vertebrates. Humans, mice, and zebra finches have one gene that, owing to variance in promotors and/or splice events, is expressed differentially across tissues (Ramachandran et al., 1999). For example, Yague and colleagues (2006) reported at least four different isoforms of exon 1 in humans. Zebrafish, goldfish, and pigs have multiple copies of the *cyp19* (aromatase) gene, and these are also differentially expressed in tissues, including ovary and brain (Robic et al., 2014). Importantly, all these genes, splice variants, and tissue-specific promotors make a single, well-conserved protein product that varies between 50 kD and 55 kD in size.

Given that multiple aromatase isoforms could produce the same protein product, Wynne and colleagues (2008) tested the hypothesis that the single zebra finch gene was alternatively spliced in ovarian follicular cells, neurons, and astrocytes. In the zebra finch, a single aromatase gene at Exon 1 is alternatively spliced and is expressed differentially

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in the brain (exon 1a) and ovary (exon 1b) (Ramachandran et al., 1999). After successfully discriminating between the two known transcripts using PCR, we then used overlapping primers along with 5' and 3' RACE (rapid amplification of cDNA ends) to isolate the entire product of the aromatase transcript specifically upregulated by injury (Wynne et al., 2008). Upon sequencing, this product was found to be exactly the same as the known brain transcript (containing exon 1a). These data suggest that the neural expression of aromatase occurred via the expression of identical transcripts in both neurons and astrocytes.

Brain injury is accompanied by a host of neural responses including, but not limited to, cell death and neuroinflammation. Either (or both) these processes could involve signaling molecules that may also serve as inducers of aromatase in astrocytes. Importantly, the almost invariable coincidence of these processes makes it very difficult to separate them. However, inducing inflammation in the absence of substantial cell death proved to be a more rewarding avenue of pursuit in our search for factors that induce astrocytic aromatase expression.

Inflammation induces aromatase expression

In very general terms, brain damage is characterized by two phases, the first of which results in tissue damage and cell death from the force of injury. The second phase involves inflammatory signals, including increases in cytokines, chemokines, and prostaglandins, that can occur within minutes of injury and last for months (Rothwell and Strijbos, 1995; Ghirnikar et al., 1998; Marciano et al., 2002). Although the initial activation of inflammation is neuroprotective, the chronic activation can lead to increased brain damage via breakage of the blood-brain barrier, production of reactive oxygen species, or the amplification of proinflammatory signaling.

Inflammatory processes themselves may play an inductive role in the expression of aromatase following penetrating brain injury. Major proinflammatory signals, which include cytokines, prostaglandin E2 (PGE2), and NF-κB, have been shown to regulate aromatase expression in the periphery. More specifically, inflammatory signals regulate aromatase in normal and malignant breast tissue (Purohit et al., 1995; Singh et al., 1997; Purohit et al., 2005; Morris et al., 2011). It is hypothesized that cyclooxygenase-2 (COX-2)–derived PGE2 stimulates PKA (protein kinase A) production, which results in *cyp19* transcription and thereby increases in aromatase expression. The proinflammatory cytokine interleukin-6 (IL-6) has been shown to regulate aromatase expression and E2 synthesis within tumors in endometrial cancer cells (Che et al., 2014). IL-6 has also been shown to increase aromatase expression in other forms of cancer, including cervical and non-small-cell lung carcinoma (Irahara et al., 2006; Veerapaneni et al., 2009; Miki et al., 2010).

Although much evidence focuses on the regulation of aromatase by inflammatory signals in the periphery, further evidence suggests that central inflammation is capable of regulating central aromatase expression. In the neonatal rat, administration of PGE2 increases aromatase and E2 content in the developing rat cerebellum, and treatment with the COX inhibitor indomethacin prevents this effect, with dramatic effects on dendritic morphology and neurophysiology (Dean et al., 2012a,b). Thus, local COX activity and consequent PGE2 synthesis can regulate aromatase activity in the developing mammalian brain.

Inflammation also induces glial aromatase expression in brain injury models. An experiment done in our lab found that application of the toxin phytohemagglutinin (PHA) induces glial aromatase expression in the absence of detectable cell death (Duncan and Saldanha, 2011). However, because PHA stimulates multiple components of the inflammatory pathway, including the stimulation of macrophages, T-cells, cytokines, and prostaglandins, the specific signal that induces glial aromatase remained unclear (Phillips et al., 1978; Duncan and Saldanha, 2011). Given the previous data in neonatal rats, we hypothesized that in zebra finches, COX activity may be necessary for the induction of glial aromatase and consequent E2 synthesis following a penetrating brain injury.

The induction of aromatase following brain injury: the influence of sex

To test this hypothesis, we administered indomethacin, a nonspecific COX-1/2 inhibitor, during a penetrating brain injury in adult male and female zebra finches (Pedersen et al., 2017). We found that COX activity is necessary for injury-induced E2 and is detectable in temporally distinct patterns between sexes. First, we measured central PGE2 content at 6 h or 24 h after injury. At both time points, PGE2 was decreased in the hemisphere treated with indomethacin, suggesting that our treatment was effective at both time points. However, the temporal pattern of aromatase induction following brain injury appears to differ between the sexes. More specifically,

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6 h after injury, there is no evidence of injury-induced aromatase expression or a change in local E2 levels in males. However, females at the same time point displayed robust increases in E2. This induction of local E2 is severely curtailed by the administration of indomethacin, suggesting that COX activity is necessary for injury-induced aromatization (Mehos et al., 2015; Pedersen et al., 2017). The effect of indomethacin on aromatase expression and central E2 content is evident in males, however, at 24 h postinjury. Indeed, in males, COX inhibition prevents the increase of aromatase and E2 content following brain injury at this time point. Interestingly, also at this time (and despite lower PGE2 levels), in females the E2 content around injuries injected with indomethacin did not differ from E2 levels around injuries treated with vehicle. These data strongly suggest that aspects of injury-induced inflammatory signaling are in part responsible for the induction of E2 following brain damage. The factors that sustain injury-induced aromatase expression in either sex is unknown.

The temporal difference in the COX-dependent induction of aromatase expression may reflect a basic sex difference in the induction patterns of glial aromatase. Previous reports from our lab found that females induce glial aromatase faster than males following a penetrating brain injury to the entopallium (Saldanha et al., 2013). Females have inductions of glial aromatase as soon as 2 h postinjury, whereas they are not evident in males until 24 h. Interestingly, by 24 h, the sex difference disappears, and both males and females have similar numbers of aromatase-expressing cells around the site of damage. A similar female-biased sex effect occurred following penetrating injury to the zebra finch cerebellum (Mirzatoni et al., 2010). The result of indomethacin preventing the induction of E2 in a temporally distinct, sex-specific manner may be a reflection of a sex difference in the time course of aromatase induction. Current work in our lab is exploring mechanisms underlying this sex difference.

Multiple reports from our lab have found basal and injury-induced sex differences in cytokine expression (Saldanha et al., 2013; Pedersen et al., 2016). For example, following injury to the entopallium, females induced glial aromatase faster than males while having larger increases in IL-1β (Saldanha et al., 2013). It is difficult to dissect the time course of proinflammatory signals, such as cytokines and PGE2, after brain injury. However, these sex differences in the time course of cytokine and PGE2 induction following injury may be important to investigate. This is of special significance given the sex difference between basal and induced inflammatory signals following injury, and given that inflammation seems to regulate aromatase and E2 expression. We have now begun to understand that the inductive role of inflammatory signaling on aromatization appears to be part of a reciprocal relationship as local increases in estrogens are responsible for decreases in chronic neuroinflammation.

Astrocytic aromatization decreases indices of neuroinflammation

Sex steroids can dramatically influence inflammation, and the evidence strongly suggests that E2 can exacerbate or inhibit several indices of inflammation in a diverse set of tissues. Indeed, the chronic inflammatory conditions that accompany several human diseases, including rheumatoid arthritis, osteoporosis, asthma, endometriosis, and obesity, are strongly influenced by E2. However, although E2 exacerbates inflammation in endometriosis and asthma (Bulun et al., 2012; Keselman and Heller, 2015), the data suggest a strong anti-inflammatory role for this steroid in rheumatoid arthritis and osteoporosis (Sapir-Koren and Livshits, 2017). Hypotheses explaining this differential influence across tissues abound and are beyond the scope of this review; however, across many species, there appear to be consistent reports of an anti-inflammatory role for E2 in the acute regulation of several components of the immune cascade.

We have found that the anti-inflammatory effect of E2 extends into the traumatized brain. As mentioned earlier, mechanical damage to the finch brain increases local E2 by about fourfold (Mehos et al., 2015). We queried the role of induced aromatization following brain damage by performing bilateral injuries in adult birds. One hemisphere received the aromatase inhibitor fadrozole, whereas the contralateral hemisphere received vehicle (Pedersen et al., 2016). Twenty-four hours later, we found exaggerated levels of IL1-β and COX-2 transcription in the hemisphere injected with fadrozole relative to vehicle. These data suggest that the inhibition of induced aromatization during brain damage results in a sustained level of inflammation. In agreement, levels of prostaglandin E2 were elevated in the hemisphere injected with fadrozole relative to the vehicle-treated contralateral hemisphere, suggesting that local aromatization may be responsible for the anti-inflammatory effects observed.

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To test the E2 dependency of the effect above, we inflicted bilateral penetrating injuries and injected the aromatase inhibitor fadrozole into adult zebra finches of both sexes. In one hemisphere, however, we concurrently injected E2 to assess the potential local influence of this steroid on multiple indices of inflammation. In the hemisphere injured in the presence of E2, the expression of COX-2 was lower relative to the contralateral side (Pedersen et al., 2016). This expression apparently influenced prostaglandin levels, as hemispheres with E2 also had lower levels of PGE2 (Pedersen et al., 2016). These data strongly support an anti-inflammatory role for E2 during brain injury.

Sex differences in E2 modulation of neuroinflammation following brain injury

Previous studies have revealed a strong interaction between estrogens and the innate immune system. Resident macrophages isolated from female mice are more plentiful and express higher levels of toll-like receptors compared with those in males (Scotland et al., 2011), perhaps suggesting a higher sensitivity of the female immune system. Indeed, tumor necrosis factor alpha (TNF-α) and IL-1β increase in women with low circulating E2 as a result of either natural (Pfeilschifter et al., 2002) or surgical menopause (Pacifici et al., 1991). These sex differences appear to be caused by differences in circulating E2; for example, ovariectomized mice have higher neural cytokine expression following peripheral endotoxin treatment relative to sham controls (Brown et al., 2010), suggesting an anti-inflammatory role for circulating E2. In agreement, indices of inflammation are higher in postmenopausal women and ovariectomized mice compared with premenopausal, age-matched controls and intact animals, respectively. Specifically, the expression and secretion of TNF-α, IL-1β, and IL-6 are higher at times of low circulating E2 relative to controls, as is the expression of their cognate receptors (Pfeilschifter et al., 2002). The present data extend these findings to the brain by demonstrating a role for injury-induced aromatization within the CNS—one that involves a potent inhibition of multiple components of the inflammatory cascade within neural tissue.

There appear to be differences in the anti-inflammatory effects of E2 between the sexes. Upon aromatase inhibition, injury-associated levels of TNF-α and IL-1β are higher in females than in controls, but only TNF-α remains high in males. In partial agreement, replacement with E2 lowers TNF-α in males and IL-1β in females, but not vice versa. These data suggest that the initial portions of the inflammatory cascade may be influenced by aromatization differently between sexes. It is important to point out, however, that regardless of these differences in cytokine expression, downstream inflammatory signaling does not appear to be sex-specific. Indeed, the inhibition of aromatase and E2 replacement exaggerate and mitigate injury-associated COX expression in both sexes (Pedersen et al., 2016).

Previous studies have hypothesized that cytokines may serve different biological functions in men and women (Lynch et al., 1994). Thus, it is likely that E2 manipulation may affect inflammation in a sexually differentiated manner. Experiments that vary the severities of injury and time points of injury need to be explored in order to increase confidence in this interpretation. However, therapies that seek to control injury-associated inflammation may need to be tailored to these important sex differences in the temporal and cytokine-specific pattern of neural changes following various types of insult.

Conclusion

Although much work had focused on the neuroprotective role of glial aromatase and consequent E2 synthesis, the mechanisms regulating this influence were unknown. We have presented a novel relationship between immune and endocrine systems in the brain, which appears to be sexually differentiated. These latent sex differences, however, ultimately achieve the same result: the induction of astrocytic aromatization of E2 and consequent anti-inflammatory effect of E2, via the decrease in PGE2.

This feedback between neuroimmune and neuroendocrine signaling may serve as a unique model of neuroprotection. The release of inflammatory factors following brain injury can exacerbate neurodegeneration (Rothwell and Strijbos, 1995; Ghirnikar et al., 1998; Denes et al., 2010). However, these inflammatory factors have the ability to shift from neurodegenerative to neuroprotective via PGE2-dependant increases in aromatase and E2, which decrease inflammatory signaling. This ability may be important to exploit in a therapeutic context, given that chronic elevation of inflammatory signaling is notable in many disorders, including depression, Alzheimer's disease, Parkinson's disease, stroke, and TBI (Perry, 2004; Turgeon et al., 2006; Perry et al., 2007).

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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)

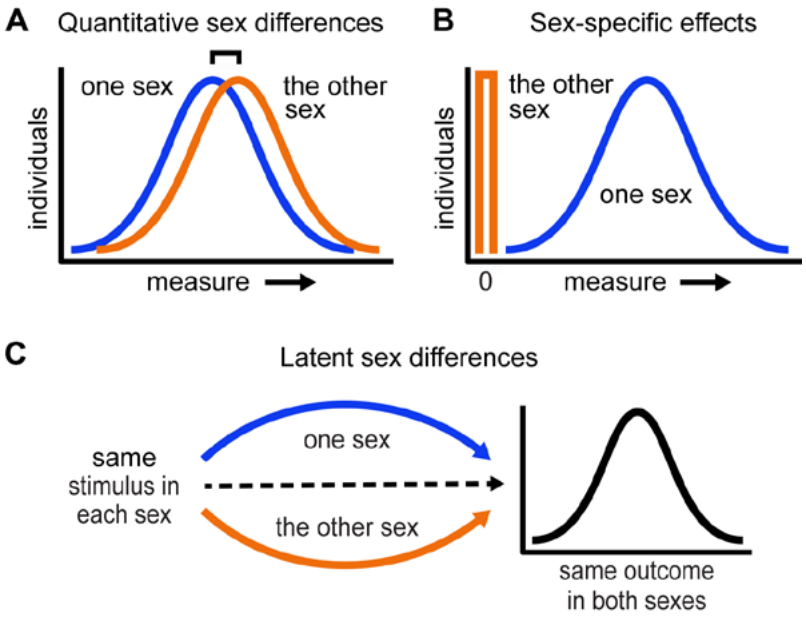

Figure 1. Types of sex differences. ***A***, Quantitative sex differences are evident as differences between the sexes in the distributions of an experimental measure. ***B***, Sex-specific effects are evident when an experimental variable can be measured in one sex but the other sex scores zero for 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

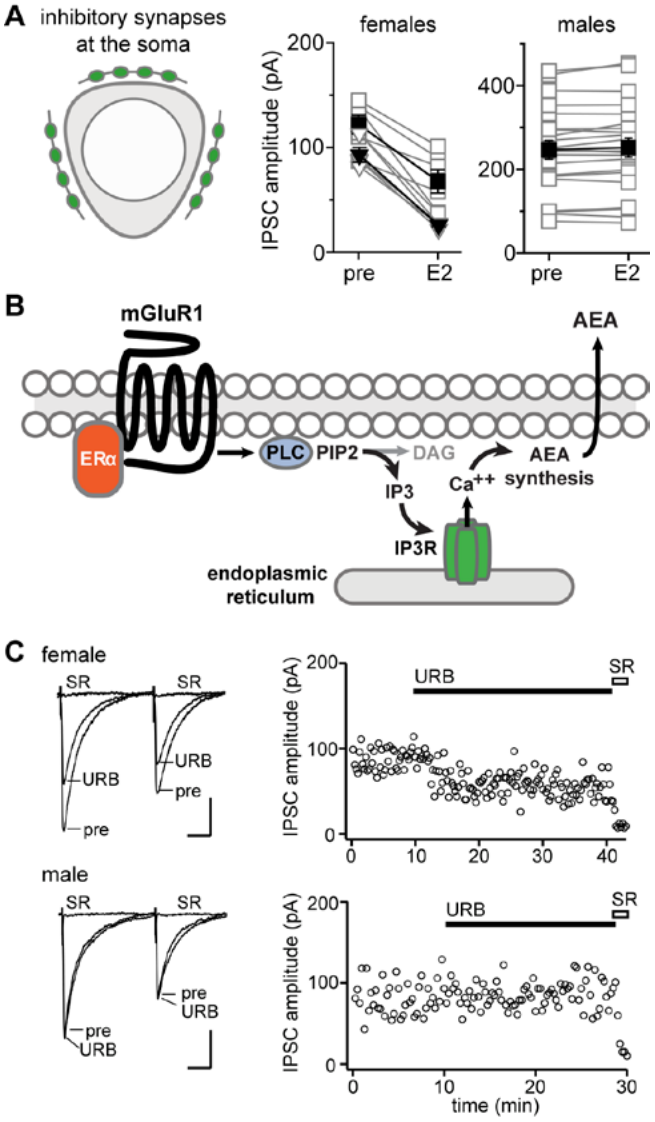

Figure 2. Sex-specific mechanisms of inhibitory synaptic modulation in the hippocampus. ***A***, Inhibitory postsynaptic currents (IPSCs) evoked by stimulation of perisomatic synapses are suppressed by E2 in females, with no effect in males. Squares represent compound IPSCs; triangles represent unitary 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

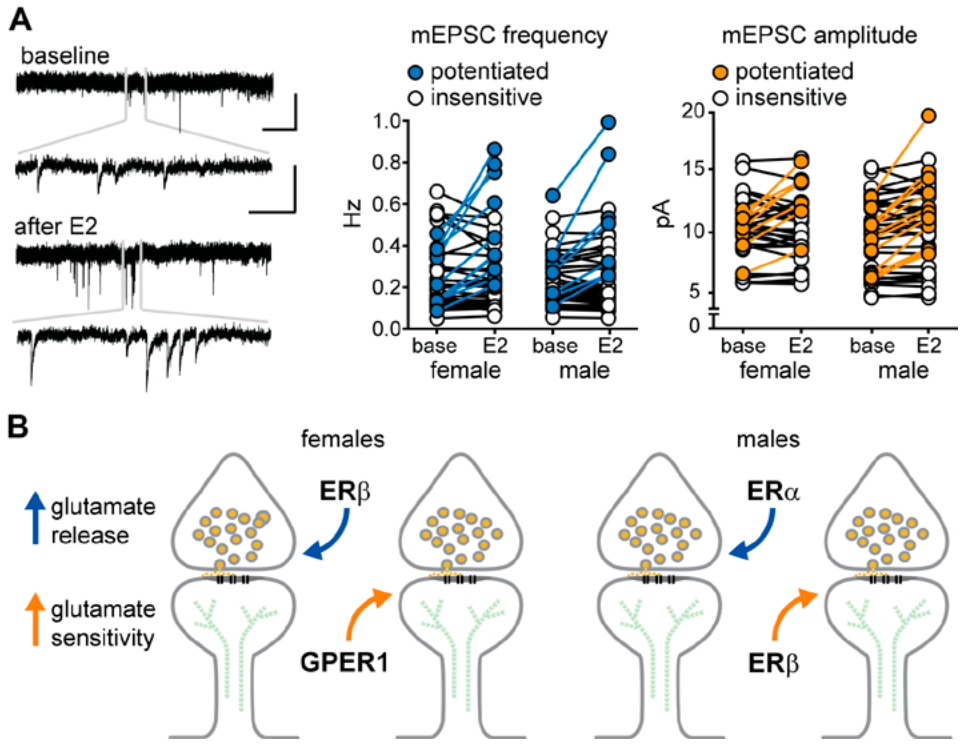

Figure 3. Latent sex differences in excitatory synaptic modulation in the hippocampus. ***A***, E2 increases both mEPSC frequency (blue) and mEPSC amplitude (orange) in a subset of hippocampal neurons in both females and males with no apparent sex differences. Colored symbols show cells with an effect 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.

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The Stress Response: Sex-Specific Neural Mechanisms

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Introduction

Stressor exposure can precipitate and/or increase the symptom severity of psychiatric disorders including posttraumatic stress disorder (PTSD), major depression, attention deficit hyperactivity disorder (ADHD), and schizophrenia (Newman and Bland, 1994; Melchior et al., 2007; Hirvikoski et al., 2009; Holtzman et al., 2013). These disorders are also sex biased, such that rates of PTSD and depression are higher in women (Breslau, 2009; Kessler et al., 2012), whereas rates of ADHD and schizophrenia are higher in men (Ramtekkar et al., 2010; Mendrek and Mancini-Marïe, 2016). Symptoms of these disorders also present differently in the sexes. For example, depressed women report more sleep disturbances (Plante et al., 2012), and men with schizophrenia exhibit more negative symptoms (e.g., social withdrawal, flattened affect) (Mendrek and Mancini-Marïe, 2016). This link between sex-biased psychiatric disorders and stress has led to investigations of whether sex differences in stress responses predispose males and females to different psychopathology. Here we will review sex differences in one key orchestrator of the stress response—corticotropin-releasing factor (CRF)—and consider how these sex differences can lead to sex-biased pathology. We will first detail the range of sex differences that have been found in CRF function, from its presynaptic regulation to its postsynaptic efficacy. Then we will link sex-specific sensitivities to CRF within specific brain regions to differences in male versus female physiology and behavior. Finally, we will review how sex differences in CRF function are established. By using CRF as a model system, we hope to highlight principles that can be more broadly applied to the investigation of sex differences in the brain.

Molecular Sex Differences in the CRF System

CRF produced in the paraventricular nucleus (PVN) of the hypothalamus is best known for its ability to activate the hypothalamic-pituitary-adrenal axis response to stress, resulting in glucocorticoid secretion from the adrenal glands. However, CRF is also produced in other areas, such as the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST), and is released into brain regions where it acts as a neuromodulator (Valentino and Van Bockstaele, 2002). Sex differences have been found in CRF's central and endocrine effects.

We have previously reviewed sex differences in CRF (Bangasser and Wiersielis, 2018). (Excerpts from that review are provided for the rest of this section without explicit citation.) CRF-producing neurons are regulated by a variety of afferents, including glutamate. Glutamatergic regulation of CRF neurons via NMDA receptors alters fear expression and social withdrawal in male but not female mice, revealing sex differences in the inputs controlling CRF neurons (Gilman et al., 2015). CRF neurons themselves can produce different amounts of CRF in males versus females. For example, in contrast to males, it is reported that female rodents typically have higher CRF expression in the PVN (Viau et al., 2005; Iwasaki-Sekino et al., 2009; but see Sterrenburg et al., 2012). This increased CRF expression in the PVN of females may explain why levels of glucocorticoids are higher in female than in male rodents (Kitay, 1961). Outside of the PVN, increased CRF expression in females is found in the CeA and the fusiform but not the oval division of the BNST (Iwasaki-Sekino et al., 2009; Sterrenburg et al., 2012). Functionally, excess CRF expression in females has been linked to increased anxiety (Li et al., 2016). Specifically, oxytocin interneurons in the medial prefrontal cortex of both male and female mice release CRF-binding protein (CRFBP), which binds free CRF, reducing its bioavailability and thereby inhibiting CRF's effect on its receptors (Van Den Eede et al., 2005). Despite the release of CRFBP in both sexes, oxytocin interneurons mitigate the anxiogenic effect of CRF only in males. This lack of an effect in females is attributed to their higher levels of CRF expression, which are thought to exceed the capacity of CRFBPs to prevent CRF from inducing anxiety. Notably, in the pituitary, CRFBP expression is higher in females than in males, perhaps to compensate (at least in part) for higher levels of CRF in the PVN (Speert et al., 2002). When considered together, these studies implicate sex differences in CRF regulation, expression, and CRFBP efficacy as important contributors to sex differences in stress responses.

At the postsynaptic level, there is evidence for sex differences in CRF receptor density, expression, distribution, trafficking, and signaling in certain brain regions (Fig. 1). Evidence for sex differences in CRF receptors first comes from binding studies. Specifically, CRF_1 receptor binding, in regions of the amygdala and cortex, is higher in adult female rats, whereas CRF_2 receptor binding is higher in regions of the amygdala and hypothalamus in male

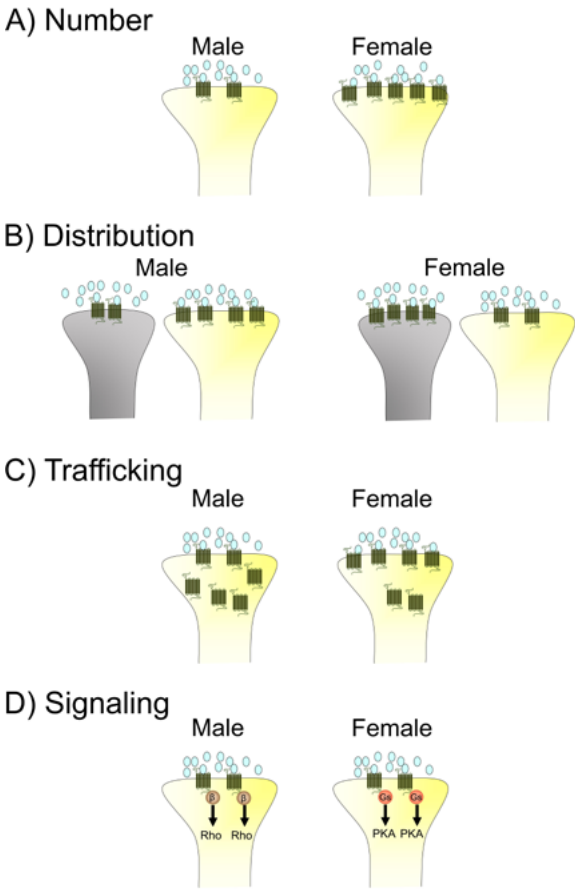

Figure 1. Depiction of sex differences in CRF receptors in rodents. CRF receptors are in green and CRF is in blue. ***A***, Sex difference in CRF receptor expression. ***B***, Sex difference in the localization of CRF receptors on different cell types. ***C***, Sex difference in CRF receptor trafficking. ***D***, Sex difference in CRF receptor coupling and signaling. β, β-arrestin-2; PKA, protein kinase A. Adapted with permission from Bangasser and Wiersielis (2018), Fig. 1. Copyright 2018, the Hellenic Endocrine Society and Springer Nature.

rats (Weathington and Cooke, 2012; Weathington et al., 2014). Interestingly, many of these changes in binding emerge following puberty, implicating pubertal hormone surges in these sex differences (Weathington and Cooke, 2012; Weathington et al., 2014). Sex differences in receptor binding can be driven by changes in receptor number. Although the regions in the binding study were not directly assessed for sex differences in receptor levels, the dorsal raphe (DR) has been. In the dorsal and ventrolateral portions of the DR, CRF_1 receptor expression is increased in female compared with male rats, and in the ventrolateral DR, CRF_2 receptor expression is also higher in females than in males (Fig. 1*a*) (Lukkes et al., 2016). Unlike in rats, sex differences in CRF_1 receptor expression are not found in the DR of mice, but sex differences in CRF_1 receptor distribution are (Howerton et al., 2014). Specifically, the CRF_1 receptor colocalizes with DR parvalbumin neurons more in male than in female mice (Fig. 1*b*). Given that the levels of CRF_1 receptor mRNA are comparable in both sexes, CRF_1 receptors must colocalize with a cell type different from parvalbumin neurons in females, although the identity of that cell type remains unknown. Sex differences in the types of neurons preferentially regulated by CRF could lead to different behaviors. In fact, this sex difference in CRF_1 receptor distribution is associated with increased anxiety in males following local administration of CRF into the DR (Howerton et al., 2014). Sex differences in the distribution of CRF receptors are also found in hippocampal CA1 dendrites, where female rats have more CRF receptors in δ-opioid receptor–containing dendrites than do males (Williams et al., 2011). These structural sex differences could lead to sex differences in the interactions between CRF and endogenous opioids.

In addition to sex differences in CRF receptor distribution on different types of neurons, we identified sex differences in CRF_1 receptor localization within neurons in the locus ceruleus (LC) arousal center. During a stressful event, CRF is released into the LC, where it binds to CRF_1 receptors (Page et al., 1993; Valentino et al., 1998). This receptor activation causes LC neurons to increase their firing rate, thereby releasing norepinephrine into the forebrain to increase arousal (Page et al., 1993; Valentino et al., 1998). Typically, activation of this circuit increases alertness to facilitate responding to stressors. However, overactivation of this circuit can lead to the dysregulated state of hyperarousal, which is characterized by restlessness, lack of concentration, and disrupted sleep (Gold and Chrousos, 2002). One cellular mechanism to compensate for excessive CRF release is receptor internalization. During internalization, β-arrestin-2 binds to the CRF_1 receptor, initiating its trafficking from the plasma membrane to the cytosol, where the receptor can no longer be activated (Hauger et al., 2000; Oakley et al., 2007). In male rats, acute swim stressor exposure causes β-arrestin-2 to bind to the CRF_1 receptor, an effect accompanied by CRF_1 receptor internalization in LC dendrites (Reyes et al., 2008; Bangasser et al., 2010). However, β-arrestin-2 binding and internalization are not observed following exposure to swim stress in female rats (Bangasser et al., 2010). Further, studies in CRF-overexpressing (OE) mice, with overexpression throughout their

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lifespan, revealed a similar pattern of CRF_1 receptor internalization in the LC dendrites of males, but not females (Fig. 1c) (Bangasser et al., 2013). This lack of internalization in females may render their LC neurons more sensitive to conditions of excessive CRF release. In fact, LC neurons of CRF-OE females fire three times faster than those of males (Bangasser et al., 2013), an effect that would lead to increased arousal in CRF-OE females.

CRF_1 receptors also activate different intracellular signaling pathways in male and female rodents (Bangasser et al., 2010, 2017). CRF_1 receptors are G-protein (guanine-nucleotide binding protein) coupled receptors (GPCRs) that preferentially bind Gs (a type of G-protein) to activate the cAMP–protein kinase A (PKA) signaling pathway (Grammatopoulos et al., 2001). CRF_1 receptors are more highly coupled to Gs in females than males (Bangasser et al., 2010). Accordingly, overexpression of CRF induces greater cAMP-PKA signaling in female than in male mice (Bangasser et al., 2010, 2017). In the LC, this increased CRF_1 receptor signaling through the cAMP-PKA pathway in females is associated with increased sensitivity to CRF. Thus, a stressful event could increase arousal more in females than in males, because female CRF_1 receptors signal more through the cAMP-PKA pathway that activates LC neurons.

Interesting to note, male CRF_1 receptors may preferentially signal through a different pathway. Recall that their CRF_1 receptors more readily bind β-arrestin-2 than those of females (Bangasser et al., 2010). In addition to initiating internalization, β-arrestin-2 can activate signaling cascades that are often distinct from pathways activated by G-proteins (Lefkowitz and Shenoy, 2005; DeWire et al., 2007; Violin and Lefkowitz, 2007). Using a phosphoproteomic approach in CRF-OE mice, we found increased phosphorylation of β-arrestin-2–mediated signaling pathways (e.g., Rho signaling) in CRF-OE male mice (Bangasser et al., 2017). Collectively, these results suggest a model of sex-biased CRF_1 receptor signaling, such that this receptor signals more through β-arrestin-2–mediated pathways in males, and more through Gs-mediated pathways in females (Fig. 1*d*) (Valentino et al., 2013). Different signaling pathways induce distinct cellular consequences, leading to different physiological responses, some of which may increase the risk for certain types of pathology. Therefore, sex differences in signaling could predispose males versus females toward different diseases. In fact, an unexpected finding from our phosphoproteomic studies was that overexpression of CRF increased the phosphorylation of proteins in Alzheimer's disease pathways more in female than in male mice (Bangasser et al., 2017). Using a mouse model of Alzheimer's disease pathology, we found that CRF overexpression increased amyloid plaque formation to a greater degree in females than in males (Bangasser et al., 2017). Taken together, these results suggest that sex-biased CRF receptor signaling is an important yet underexplored mechanism by which sex differences in risk factors for diseases, ranging from psychiatric to neurodegenerative, are established.

Male versus Female Sensitivity to CRF Is Region Specific

In many of the above examples, females appear more vulnerable to CRF's effects on anxiety and arousal. Yet emerging evidence suggests that sex differences in sensitivity to CRF are region specific and that males tend to be more vulnerable to the effects of CRF on cognition (Bangasser et al., 2018). For example, unlike the LC, in which CRF_1 receptors are internalized by stress in males, in the male CA1 region of the hippocampus, stress causes CRF_1 receptors to move toward the plasma membrane (McAlinn et al., 2018). This alteration in CRF_1 receptor trafficking is not observed in female rats. This receptor sex difference could increase male hippocampal sensitivity to stress and may contribute to the hippocampal-dependent learning impairments observed following chronic stress in male, but not female rats (Luine et al., 2017).

Compared with female rats, male rats also appear to be more sensitive to the effects of CRF on the basal forebrain cholinergic system. This system is critical for mediating sustained attention, which is the ability to monitor situations for rare and unpredictable events (Sarter et al., 2001). Central CRF impairs sustained attention in both sexes (Cole et al., 2016). However, unlike males (as well as females in estrous-cycle stages with low levels of ovarian hormones), females in the stages of the estrous cycle with high levels of ovarian hormones are resistant to the negative effects of CRF on attention. Because males do not have elevated levels of these hormones, they do not benefit from their protection. New findings on the effects of CRF in the medial septum (MS) on spatial learning are also revealing male vulnerability (Bangasser et al., 2016). Although a high dose of CRF in the MS disrupts spatial learning in both sexes, the low dose is disruptive only in male rats. The mechanisms contributing to this male vulnerability to CRF

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in the MS are, at this time, unknown. Clinically, this male vulnerability to the disrupting effects of CRF on cognition may contribute to their higher rates of disorders with cognitive features, such as schizophrenia and ADHD. Importantly, these studies highlight the regional specificity of sex differences in sensitivity to CRF.

How Sex Differences in CRF Function Are Established

We previously discussed that how these sex differences in CRF function are established remains mostly unknown (Bangasser and Wiersielis, 2018). There is evidence that, in some cases, circulating ovarian hormones play a role (Atkinson and Waddell, 1997; Viau et al., 2005; Cole et al., 2016; Wiersielis et al., 2016). These hormones may directly regulate the expression of CRF because its promotor contains putative estrogen response elements (Vamvakopoulos and Chrousos, 1993). Membrane estrogen receptors (ERs) that initiate intracellular signaling cascades also can regulate CRF neurons. For example, estradiol increases the excitability of CRF neurons in the PVN via the activation of the putative Gq-coupled membrane ERs (Hu et al., 2016). The effect of CRF on postsynaptic neurons can also be regulated by membrane ERs, such as the G-protein-coupled ER-1, which can form a heterodimer with CRF receptors (Akama et al., 2013). Although the cellular consequences of this interaction remain unknown, this receptor heterodimerization likely alters intracellular signaling. It is important to note, however, that not all sex differences are regulated by circulating ovarian hormones. For example, sex differences in CRF_1 receptor function in the LC remained in gonadectomized males and females (Curtis et al., 2006; Bangasser et al., 2010). This result indicates that circulating gonadal hormones do not play a role; rather, this receptor sex difference results from the organizational effects of hormonal surges on development or the different complement of genes on sex chromosomes. In fact, not only can circulating levels of estradiol regulate CRF in the hypothalamus (Roy et al., 1999), but perinatal estradiol exposure masculinizes adult hypothalamic CRF gene expression (Patchev et al., 1995). This result highlights how organizational effects of gonadal hormones can lead to the sex differentiation of CRF circuits. As more sex differences are identified, additional studies will be needed to determine the factors that establish sex differences in CRF function.

Implications

These studies on CRF highlight three main findings: (1) sex differences occur at every aspect of CRF function, (2) sex differences are region specific, and (3) a variety of hormonal mechanisms can establish sex differences in CRF. Compared with other neuropeptide systems, much research has gone into investigating sex differences in CRF. However, it is unlikely that CRF is unique; rather, CRF is similar to other neuropeptides and binds to GPCRs, a very common receptor class. Therefore, it is likely that as more researchers include sex as a biological variable, similar molecular sex differences will be found in other systems. Thus, principles learned about CRF can be applied more broadly to the study of sex differences in the brain. Most significantly, these studies highlight that by comparing male and female brains, we can gain insight into the multitude of mechanisms that can predispose males and females toward different pathologies.

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