How to Study Female and Male Rodents

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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

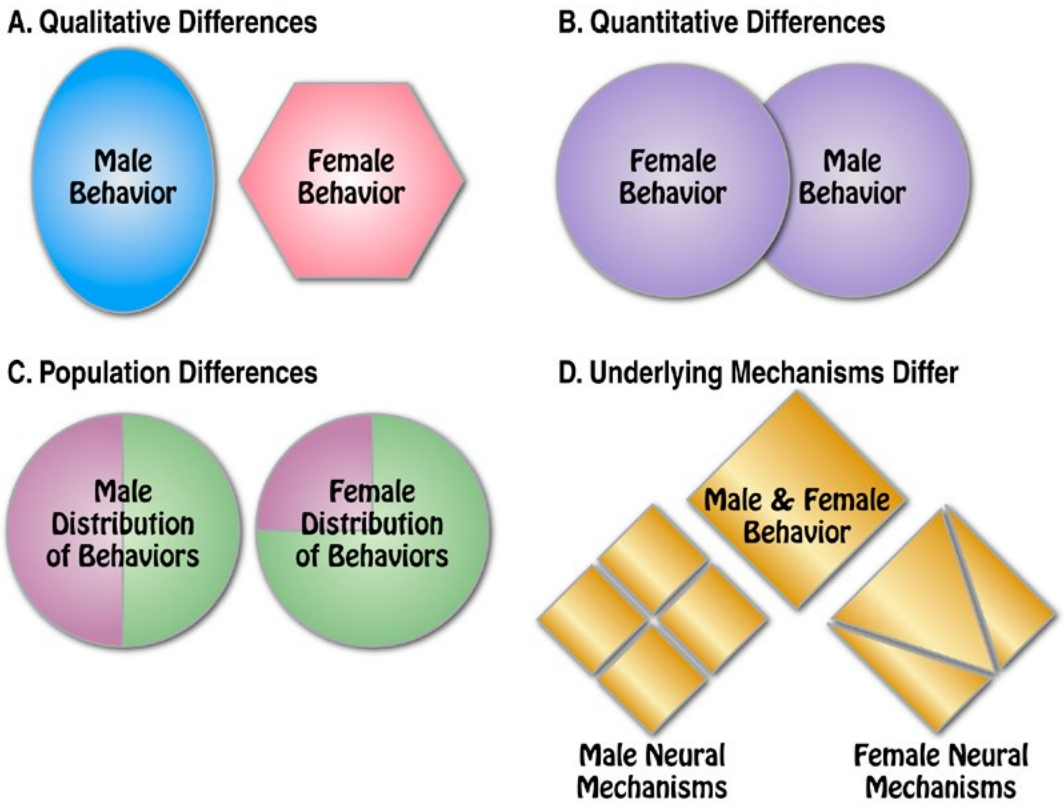

Figure 1. Four types of sex differences that can be observed: qualitative, quantitative, population, and mechanistic, also referred to as compensatory, divergent, or latent sex differences. Reprinted with permission from Becker and Koob (2016), Fig. 1. Copyright 2016, American Society for Pharmacology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

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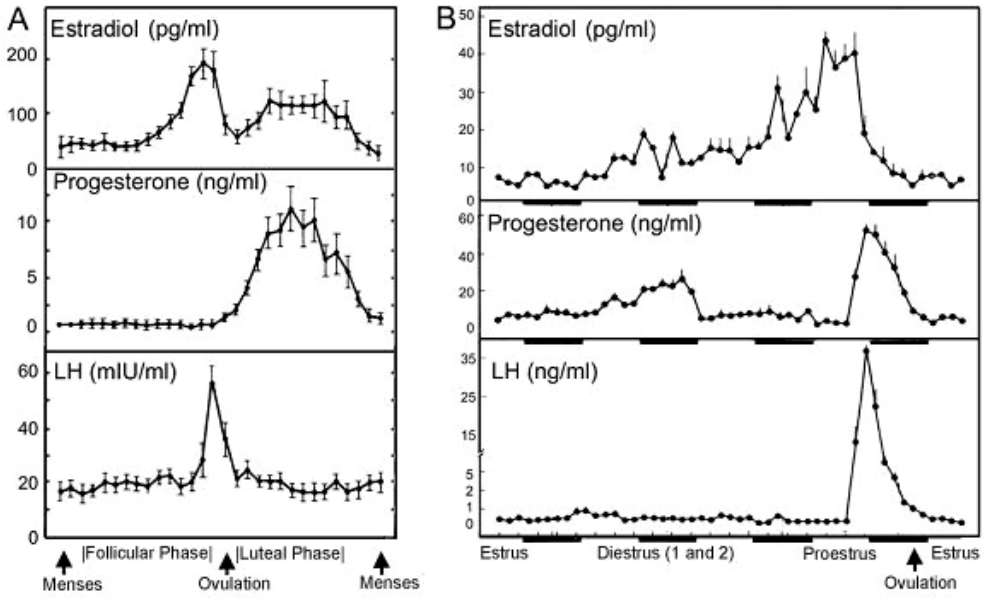

Figure 2. Patterns of estradiol, progesterone, and luteinizing hormone (LH) in the female human (***A***) and rat (***B***) during the reproductive cycle. Time unit of the *x*-axis in ***A*** is days; in ***B***, *x*-axis is hours. ***B***, Dark bars, Dark period of the day/night cycle. Note that during 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.

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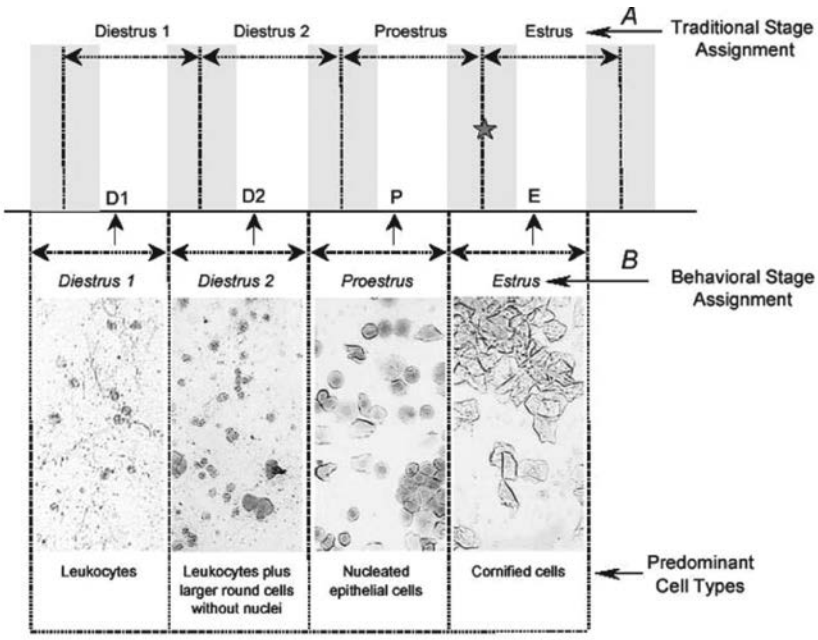

Figure 3. Stage assignment across a rat's 4 d estrous cycle in relation to a 12 h light/12 h dark cycle and samples of vaginal cytology. Top, Shaded bars denote successive 12 h dark periods. Vertical arrows, Time (early-to-middle light phase) when vaginal cytology is typically sampled. Gray star, Time 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.

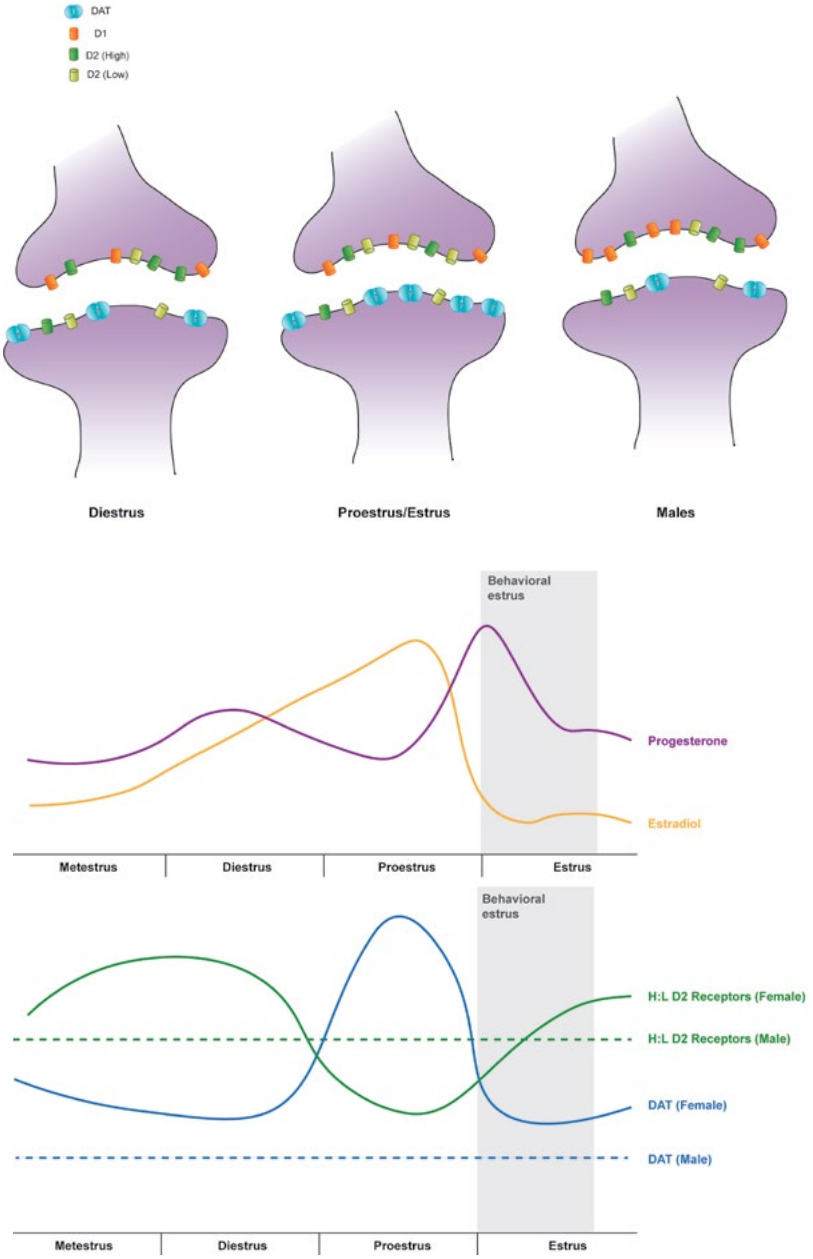

Figure 4. Sex differences and dynamic changes in dopamine (DA) receptors and dopamine transporter (DAT) across the estrous cycle. Expression of DAT (top, blue ovals) is higher in females (solid blue line) than males (bottom, dashed blue line) overall and increases the morning of proestrus, when levels 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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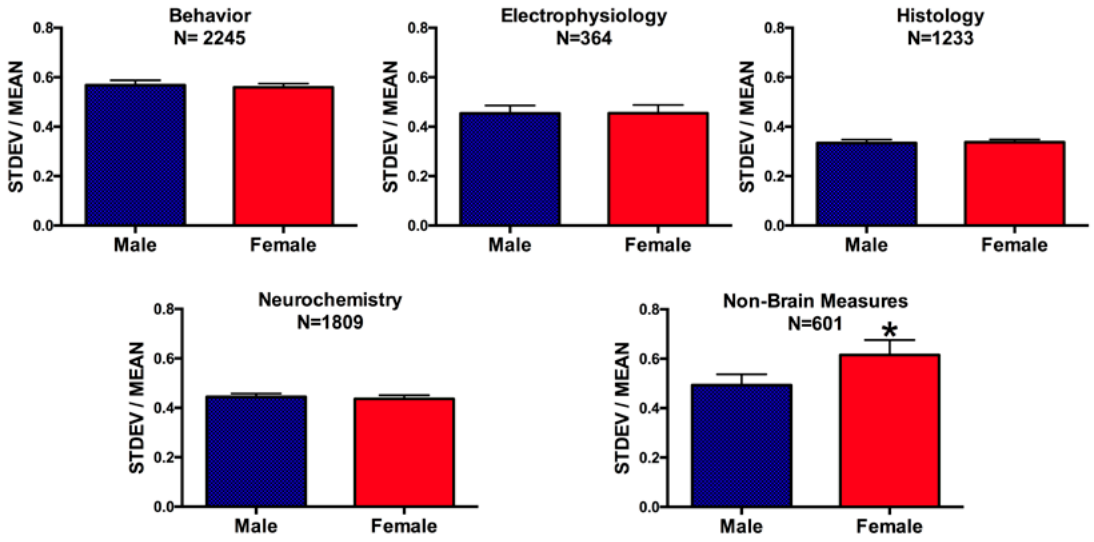

Figure 5. Trait variance as indicated by the SD (STDEV) divided by the mean for behavioral measures, electrophysiological measures, histological measures, neurochemistry, and nonbrain measures. *N*, number of data points each for males and females. For nonbrain measures, greater variability was seen 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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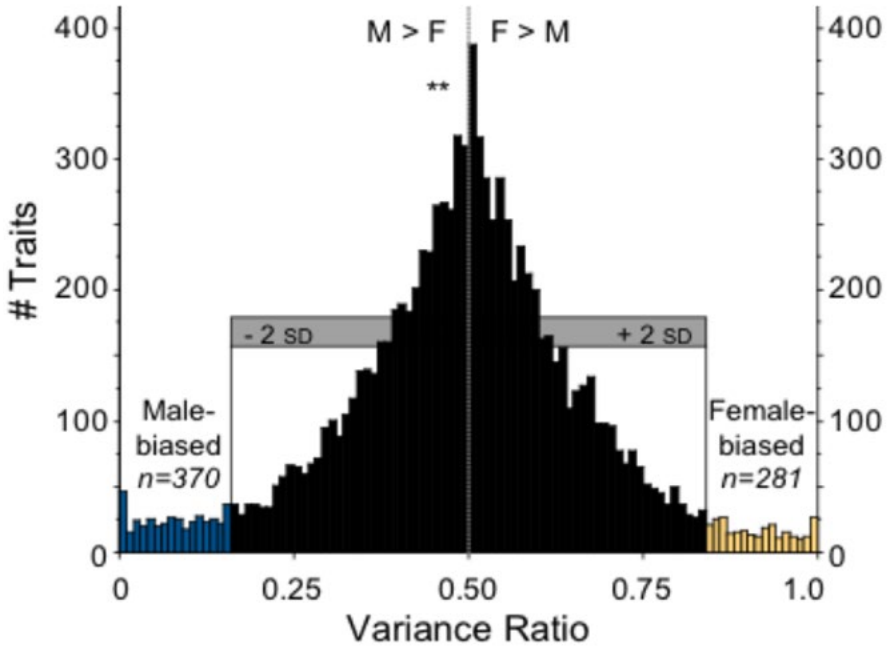

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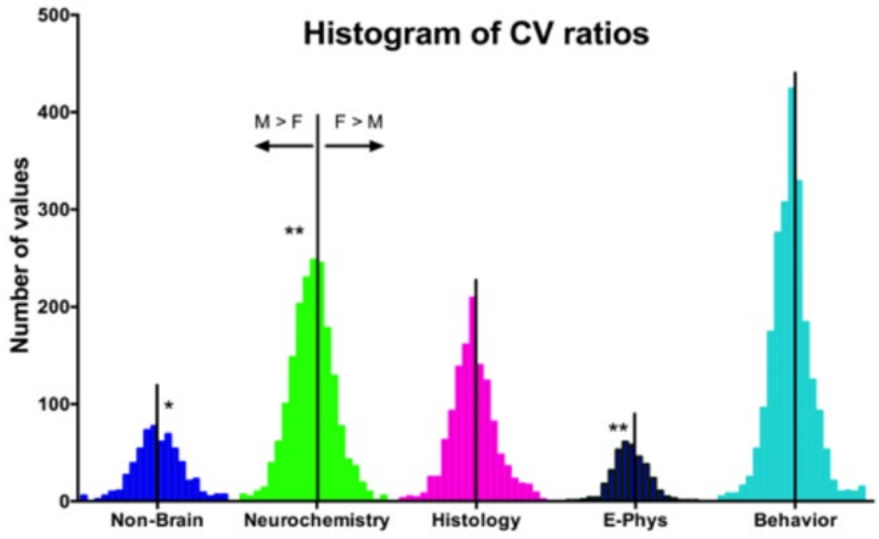

Figure 6. Distribution of CV ratios in mice (left) and rats (right). ***A***, CV ratios were assessed in male and female mice across >9900 measurements of traits. Variability was similar in males and females, with more male-biased than female-biased traits, and a mean variance ratio significantly lower 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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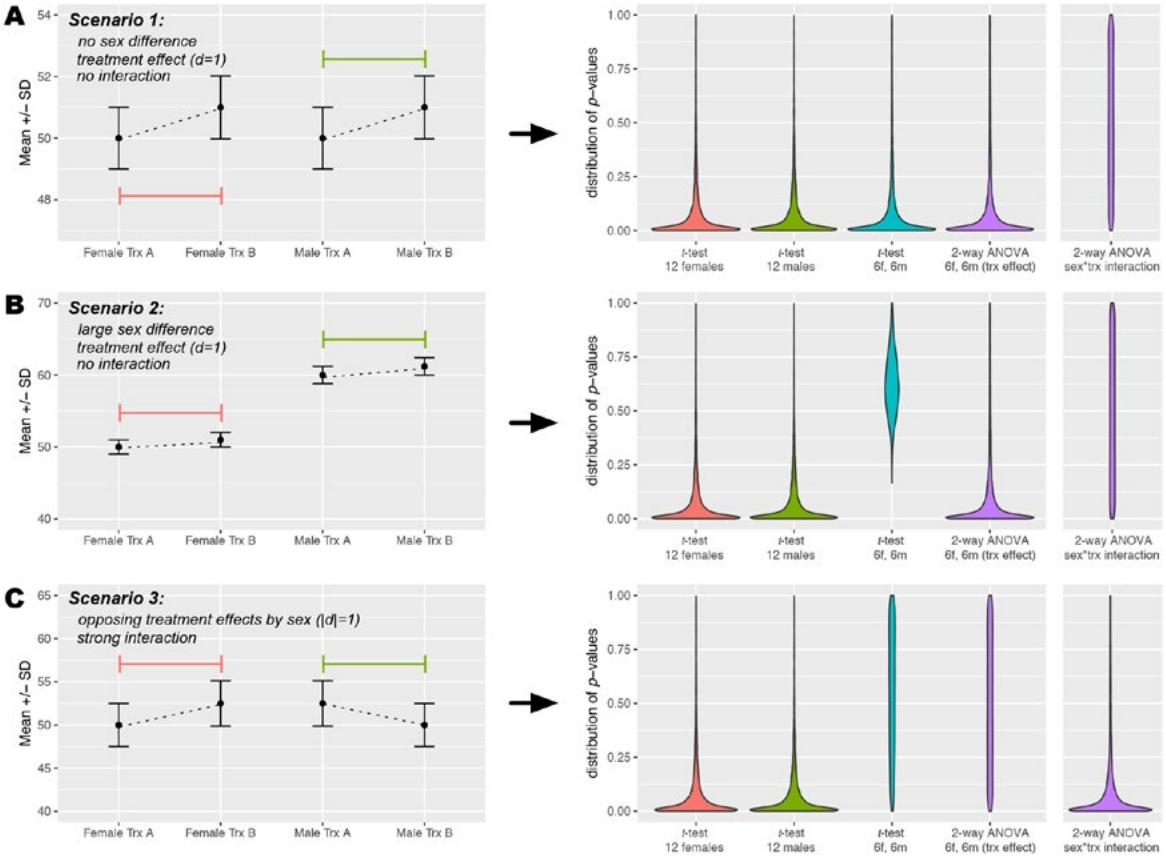

Figure 7. Simulated *p*-value distributions for different group compositions and treatment effects. The statistical outcomes of two-group and factorial tests used simulated data. Consider an experiment with two treatments ("A" and "B," e.g., hot vs cold room temperature) and an outcome measure (e.g., 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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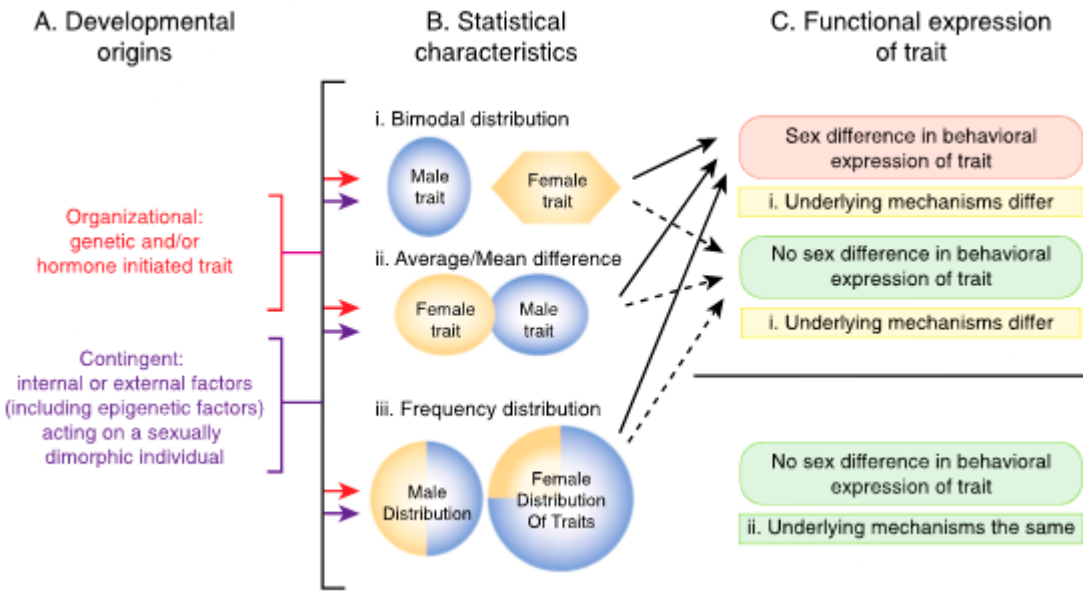

Figure 8. Developmental origins, statistical characteristics, and functional expression of sex differences in the brain. ***A***, Developmental origins of sex differences may arise from organizational influences or be contingent on interaction with internal or external factors. Organizational origins 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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