This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

Module 2, Video 13: Study Design Toolbox and Reporting Considerations for Incorporating Sex as a Biological Variable (Part II)

Studying sex-based differences has many nuances. In this video, we will delve deeper into understanding the underlying basis of sex differences and how to think about and approach studying them if you want to.

Once you decide to pursue studying a sex difference further, it is important to begin to understand the potential sources of the sex effect and how to characterize it. In terms of the source, sex effects can arise from two biological sources that differ between males and females. 1) The first is sex hormones, either secreted in adulthood or as a consequence of developmental exposure. 2) The second is the sex chromosome compliment.

To determine which of these two biological sources is responsible for your observed effects, it is best to first focus on sex hormones released from the gonads during adulthood as this strategy is easier to employ and accounts for most of the observed sex differences in adult animals.

You can employ several strategies to design a study to examine this source. The first strategy is to surgically remove the gonads of males and females in adulthood. If the effect persists when all gonadal hormones are removed, then the source is likely developmental or chromosomal. The second strategy is to try to mimic the hormonal profile of the sex you believe is biasing the results, for example, females. For this, you would remove the gonads of the female animals and administer exogenous testosterone at levels similar to intact males. If this strategy of creating similar hormone levels across males and females abolishes the sex effect, then again, adult gonadal steroid hormones are likely the source of the sex effect. The third strategy would be to focus on the effects of hormones in a single sex, such as only males or only females. In this case, you would again remove the gonads and then compare animals with or without hormone replacement.

In all three strategies, however, you must hold off on your experiments long enough to allow circulating steroids to clear from the blood stream as well as fat or other tissue deposits after gonad removal. Long term gonadectomy can also downregulate steroid hormone receptors, changing the sensitivity of reintroduced steroids. Additionally, the strategy you choose should be based on a literature review and your desired outcome measures.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

Alternatively, you can also track hormone levels to determine if your observed sex effects are specific to a particular stage of the female estrous cycle or due to variation in male sex hormone levels. This is often one of the single biggest factors that deters many researchers from incorporating sex as a biological variable. Yes, there are many documented sex effects that are enhanced or masked by variations in hormone levels across the estrous cycle. It is also true that assessing hormone levels in an experiment can be more labor intensive and require more animals in each group to provide sufficient power for comparisons across the estrous cycle. But this option may be more translatable than gonadectomy because the natural cycle is maintained. There are two options to address cycling in females in an experiment. One is to simply monitor the phases of the estrous cycle during your experiment. In this instance, the phase of the cycle would be included as a covariate in your statistical analysis. The other option is to test animals on a specific phase of the cycle or have a group of females at each phase of the cycle. However, as previously mentioned, cyclic hormone levels do not always need to be assessed as a first step and should only be done after careful examination of data from an experiment conducted in females independent of the cycle stage.

If you have excluded adult gonadally synthesized hormones as the source of your sex effect, the next step is to determine whether the sources of your sex effects is exposure to gonadal hormones during sensitive development periods or a sex chromosome effect. In this video, we will not dive deeply into these two potential sources. These points have been nicely reviewed elsewhere and these resources are included as an accompaniment to this video [6, 9-10]. However, a few points should be noted. First, development effects of gonadal hormones on the organization and function of the brain have been observed across the life span. Thus, it should be assumed that sex effects can always be observed, dispelling the common myth that sex effects are only an adult animal problem. Second, the dose and route of hormones need to be carefully considered when used in neonates due to differences in the specific mechanisms of steroid hormones across the life span. This information has been extensively reviewed elsewhere [6].

In terms of chromosome-related sex differences, a relatively new genetic tool has emerged. Termed the Four-Core-Genotypes Model, this tool involves the use of a genetically modified mouse line in which the testis-determining gene, Sry, has been moved from the Y chromosome to an autosome. As a result, XX mice that develop testes and XY mice that develop ovaries are produced. This model allows for sex effects caused by chromosomal differences to be distinguished from those sourced from sex hormones.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

Correctly reporting sex effects is also important to provide guidance for both the design of subsequent experiments as well as the interpretation and reporting of current experimental findings. There are four operational categories of sex effects:

The first category, qualitative differences, refers to traits exhibited by males and females that do not look the same. This also includes traits that are present in one sex but absent in another, many of which are associated with reproduction, such as maternal aggression, lordosis or male-specific courtship behaviors.

The second category, quantitative differences, is when an endpoint exists upon a continuum in both sexes. However, when compared between the sexes, the mean value for the endpoint would be different for males vs. females (or vice versa). There are many well-studied examples of quantitative sex differences including stress and anxiety responses, pain thresholds, social behavior and learning and memory.

The next category, population differences, is when the incidence or distribution differs between males and females. One example can be found in cocaine addiction studies, where more females tend to choose cocaine over palatable pellets than males, but the behaviors exhibited during cocaine taking do not differ between males and females [12, 13]. Sometimes, population differences only emerge under certain conditions, such as after exposure to a stressor, a pharmacological compound or environmental toxin. In this case, males and females may have initially demonstrated a similar magnitude on an endpoint but the stressor causes an increase in the endpoint in females and a decrease in the endpoint in males, or vice versa. In other cases, the event causes the endpoint to change in a similar direction for both males and females, but the magnitude of the effect is greater in one sex than the other. In this case, the inclusion of both sexes allows the researcher to capture the full picture of responses that are possible and not make erroneous conclusions of X exposure on Y endpoint.

Finally, sex differences in the underlying neural mechanism refers to an endpoint that is similar in males and females but the underlying mechanisms are different. In this case, the two sexes converge to the same endpoint, which might appear to suggest that no sex effect exists. However, a further exploration of the mechanism shows that the underlying neurophysiology is vastly different. We already covered two examples of this category in Video 3.

Regardless of which category of sex effect you find in your experiment, these distinctions should be noted when interpreting AND reporting your results. This can improve future research.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

In these two videos, we provided practical study design guidance for incorporating sex as a biological variable, regardless of whether your ultimate goal is to intently study sex differences or you want to only explore using mixed sex groups in your experiments. Importantly, we outlined how incorporating both sexes does not necessitate doubling your group sizes, but should be based on your desired outcome measures and a power analysis. We further provided guidelines for communicating results to avoid overinterpretation or misinterpretation of sex effects. All these points highlight the importance of careful and thoughtful experimental and statistical study design.

Combined References

1. Zakiniaeiz, Y., et al., *Balance of the Sexes: Addressing Sex Differences in Preclinical Research.* Yale J Biol Med, 2016. **89**(2): p. 255-9. DOI
2. Beery, A.K., *Inclusion of females does not increase variability in rodent research studies.* Current opinion in behavioral sciences, 2018. **23**: p. 143-149. DOI
3. Percie du Sert, N., et al., *The Experimental Design Assistant.* PLoS biology, 2017. **15**(9): p. e2003779-e2003779. DOI
4. van Wilgenburg, H., P.G. van Schaick Zillesen, and I. Krulichova, *Sample Power and ExpDesign: tools for improving design of animal experiments.* Lab Anim (NY), 2003. **32**(3): p. 39-43. DOI
5. Pfister, R., et al., *Good things peak in pairs: a note on the bimodality coefficient.* Frontiers in psychology, 2013. **4**: p. 700-700. DOI
6. Becker, J.B., et al., *Strategies and methods for research on sex differences in brain and behavior.* Endocrinology, 2005. **146**(4): p. 1650-73. DOI
   Developmental effects of sex hormones.
7. Percie du Sert, N., et al., *The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research.* PLOS Biology, 2020. **18**(7): p. e3000410. DOI
8. McCarthy, M.M., et al., *Sex differences in the brain: the not so inconvenient truth.* J Neurosci, 2012. **32**(7): p. 2241-7. DOI
9. Arnold, A.P. and X. Chen, *What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues?* Front Neuroendocrinol, 2009. **30**(1): p. 1-9. DOI
   Review of the Four-Core Genotype model.
10. Büdefeld, T., et al., *Sex differences in brain developing in the presence or absence of gonads.* Developmental neurobiology, 2008. **68**(7): p. 981-995. DOI
    Review of the Four-Core Genotype model.

This video series was made possible through a generous grant from the National Institute of General Medicines (Grant Number: 5 R25 GM133017-03), awarded to Cohen Veterans Bioscience (Principal Investigator: Chantelle Ferland-Beckham, PhD)

11. Becker, J.B. and G.F. Koob, *Sex Differences in Animal Models: Focus on Addiction.* Pharmacol Rev, 2016. **68**(2): p. 242-63. DOI
12. Perry, A.N., et al., *The Roles of Dopamine and α1-Adrenergic Receptors in Cocaine Preferences in Female and Male Rats.* Neuropsychopharmacology, 2015. **40**(12): p. 2696-2704. DOI
13. Perry, A.N., C. Westenbroek, and J.B. Becker, *The development of a preference for cocaine over food identifies individual rats with addiction-like behaviors.* PLoS One, 2013. **8**(11): p. e79465. DOI