Journal of Neuroscience Methods 401 (2024) 110003

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Practical solutions for including sex as a biological variable (SABV) in preclinical neuropsychopharmacological research

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ARTICLE INFO

Keywords:
Sex as a biological variable (SABV)
Sex differences
Hormones
Behavior
Pharmacokinetic
Pharmacodynamic
Therapeutics
Preclinical research
Cell lines

ABSTRACT

Recently, many funding agencies have released guidelines on the importance of considering sex as a biological variable (SABV) as an experimental factor, aiming to address sex differences and avoid possible sex biases to enhance the reproducibility and translational relevance of preclinical research. In neuroscience and pharmacology, the female sex is often omitted from experimental designs, with researchers generalizing male-driven outcomes to both sexes, risking a biased or limited understanding of disease mechanisms and thus potentially ineffective therapeutics. Herein, we describe key methodological aspects that should be considered when sex is factored into in vitro and in vivo experiments and provide practical knowledge for researchers to incorporate SABV into preclinical research. Both age and sex significantly influence biological and behavioral processes due to critical changes at different timepoints of development for males and females and due to hormonal fluctuations across the rodent lifespan. We show that including both sexes does not require larger sample sizes, and even if sex is included as an independent variable in the study design, a moderate increase in sample size is sufficient. Moreover, the importance of tracking hormone levels in both sexes and the differentiation between sex differences and sex-related strategy in behaviors are explained. Finally, the lack of robust data on how biological sex influences the pharmacokinetic (PK), pharmacodynamic (PD), or toxicological effects of various preclinically administered drugs to animals due to the exclusion of female animals is discussed, and methodological strategies to enhance the rigor and translational relevance of preclinical research are proposed.

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https://doi.org/10.1016/j.jneumeth.2023.110003
Received 29 August 2023; Received in revised form 13 October 2023; Accepted 27 October 2023
Available online 31 October 2023

1. Introduction

The goal of incorporating sex as a biological variable (SABV) into experimental design is to promote rigorous, reproducible and responsible biomedical research and improve the generalizability and translational potential of preclinical findings for clinical discovery. However, researchers have traditionally been biased toward using only one sex (generally males) for experiments but generalized the findings to both sexes, without evidence of validity. This sex bias is prominent across disciplines, with some fields making better strides than others (Beery and Zucker, 2011; Kokras and Dalla, 2014). In neuroscience and pharmacology, the historical bias towards the use of males only in experiments has created a particularly negative effect for understanding disorders that affect women more than men, such as major depression and anxiety, potentially leading to the lack of development of effective therapeutics that are personalized to the individual patient (Butlen--Ducuing et al., 2021; Eid et al., 2019; Dalla et al., 2010; Pavlidi et al., 2023; Kokras et al., 2019; Hodes and Kropp, 2023). Additionally, the underrepresentation of females in preclinical research has been linked to a higher incidence of adverse drug reactions in women, possibly due to a lack of understanding of sex-specific differences in pharmacokinetics and pharmacodynamics (Zucker and Prendergast, 2020; Heinrich, 2001).

The underrepresentation of female subjects has alarmed funding agencies such as the US National Institutes of Health (NIH), the European Commission and the Canadian Institutes of Health Research (CIHR), all of which decided to implement new policies to spur equal representation of both sexes in preclinical research. The editorial policies of prominent scientific journals have also requested researchers to factor SABV in the design of studies and report sex differences as appropriate (Clayton, 2018a, 2016; Miller and Reckelhoff, 2016; Heidari et al., 2016; Pawluski et al., 2020; Will et al., 2017; Docherty et al., 2019).

These policies, however, do not mandate the inclusion of both sexes in preclinical research, only that the inclusion of both sexes is considered, and have raised many questions about how researchers should design studies that include both male and female animals. Although some resources provide guidance on these new recommendations (Ritz et al., 2014; Becker et al., 2005), most of these resources focus on why sex is a critical biological variable rather than how to practically implement SABV into preclinical research design, analyses, and reporting (McCarthy et al., 2017). Consequently, the bias toward the predominant use of male animals remains present.

Here, we introduce a guideline that provides the practical knowledge necessary for researchers to incorporate SABV into their current and future research, mainly for preclinical studies. This guideline was created through funding from the NIH (Grant Number: 5R25GMI33017–03) under the leadership of Cohen Veterans Bioscience (CVB) and in consultation with a Scientific Advisory Board comprised of experts in sex differences, study design, statistics, and drug discovery and development. An accompanying open-access video series (link) was also developed, which serves (1) to explain the rationale behind each item in the guidelines, (2) to clarify key concepts, and (3) to provide illustrative examples. We anticipate that this guideline will empower researchers across career stages to conduct rigorous and reproducible research on both sexes by providing the practical knowledge and guidance they might need, specifically in preclinical and clinical research.

2. Hormonal fluctuations: debunking some myths

One argument for excluding females from preclinical research is that circulating ovarian hormones make data from female animals more variable than data from males (Wald and Wu, 2010). Additionally, some researchers were concerned that, especially in acute studies, they would need four times the number of experimental animals to assess the effects of estrogen across the different stages of the estrous cycle, which would be both costly and time consuming. But are these concerns really justified? Herein, we discuss how hormone variations occur across both sexes, contributing to behavioral and physiological variability in both females and males. We also describe examples of when experimental outcomes may necessitate tracking hormone fluctuations in both males and females, and best practices for how to track hormone levels when appropriate.

Variations in hormone levels (estradiol and progesterone) across the female infradian rhythm are well understood (Fig. 1a). However, testosterone levels also vary throughout the day and across the lifespan (Coquelin and Desjardins, 1982; Ellis and Desjardins, 1982) (Fig. 1b). Furthermore, factors related to group housing can affect within-cohort hormone variability in both sexes. For example, in mice, circulating testosterone levels can be (on average) five times higher in dominant versus subordinate males (Machida et al., 1981), leading to high variability among mice of the same age and strain housed under identical conditions (Bartke et al., 1973). Thus, there is no a priori reason why data variability should be larger in females than in males.

Indeed, two comprehensive meta-analyses of large numbers of studies in mice (Prendergast et al., 2014) and rats (Becker et al., 2016) showed that data variability was comparable for males and females across a range of common measures. Thus, in general, sex-dependent variability should be accepted as natural biological variability (Levy et al., 2023), and estrous cycle assessment is not a necessity. This does not mean that gonadal hormones should never be accounted for. However, hormone variability should be considered equally across both sexes based on the potential to influence experimental outcomes (Levy et al., 2023; Graham, 2023).

In males, systematic studies regarding the impact of male hormonal fluctuations on study outcomes are more limited because historically, hormone fluctuations in males were not considered a source of data variability. However, as awareness has increased, some papers have begun to stratify male animals according to their hormone status. Preliminary evidence of the effects of testosterone level variations have been observed for anxiety (Fernández-Guasti and Martínez-Mota, 2005; Aikey et al., 2002), depression, spatial abilities, and memory (Celec et al., 2015). A highly cited paper (Aikey et al., 2002) analyzed the effects of testosterone on anxiety in mice, showing that testosterone — either endogenous or exogenous — increases the amount of time spent in the open arms and the number of open-arm entries in the elevated plus maze in a dose-dependent manner, suggesting an anxiolytic-like effect (Aikey et al., 2002). Thus, male behavioral variations may also be related to fluctuations in hormone levels. The impact of fluctuations in hormonal levels seen in acute/single timepoint studies could be reduced with observations performed over a longer period of time.

3. Tracking hormone levels in both males and females

When tracking hormones is necessary, best practices should be followed. In females, swab and lavage are the two most commonly used methods (Fig. 2a). With swab, a cotton swab is moistened, gently inserted into the animal's vagina, and then turned and rolled against the vaginal wall before being removed. Lavage involves flushing cells from the vaginal lining by introducing a small amount of saline or distillated water into the vagina using a rounded tip disposable pipette that is gently placed at the opening of the vaginal canal. The fluid spontaneously aspirates into the vaginal canal without tip insertion. The pressure is controlled by pressing or releasing the pipette bulb. With both techniques, extracted cells are then examined with a microscope to determine the cycle stage based on the types and morphology of the cells.

Each of these methods has advantages and disadvantages and requires different levels of expertise and time (Fig. 2b). The decision of which method to use is highly dependent on the study design, but swabbing is generally preferred because it is the quickest and produces high quality smears (Gonzalez, 2016; Byers et al., 2012; Caligioni, 2009). It should also be noted that vaginal samples need to be taken at

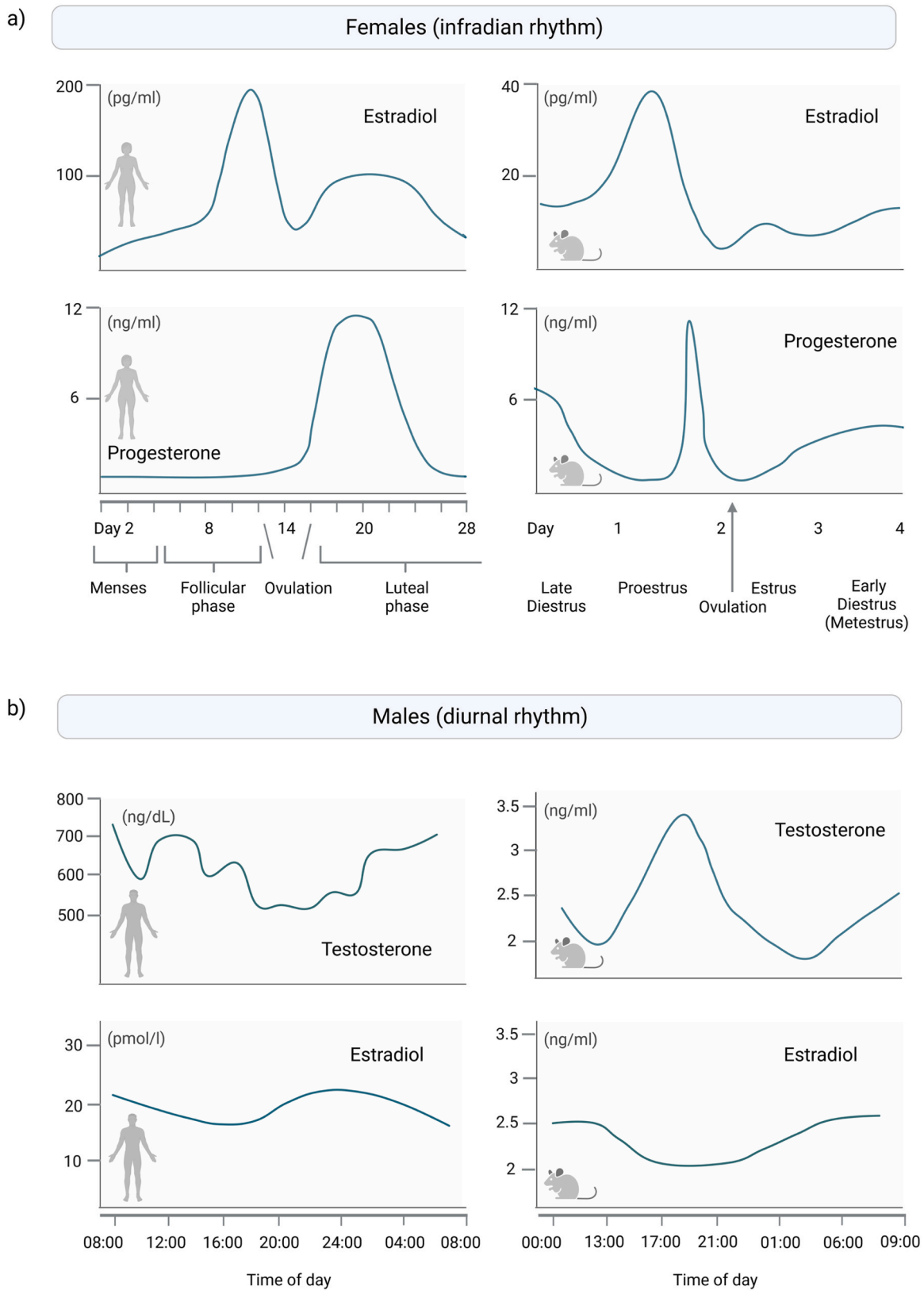


Fig. 1. Variations in estradiol, progesterone and testosterone in **a)** female and **b)** male humans and rodents during the reproductive period. Figure adapted from (Donner and Lowry, 2013), (Wu et al., 2008) and (Esquifino et al., 2004).

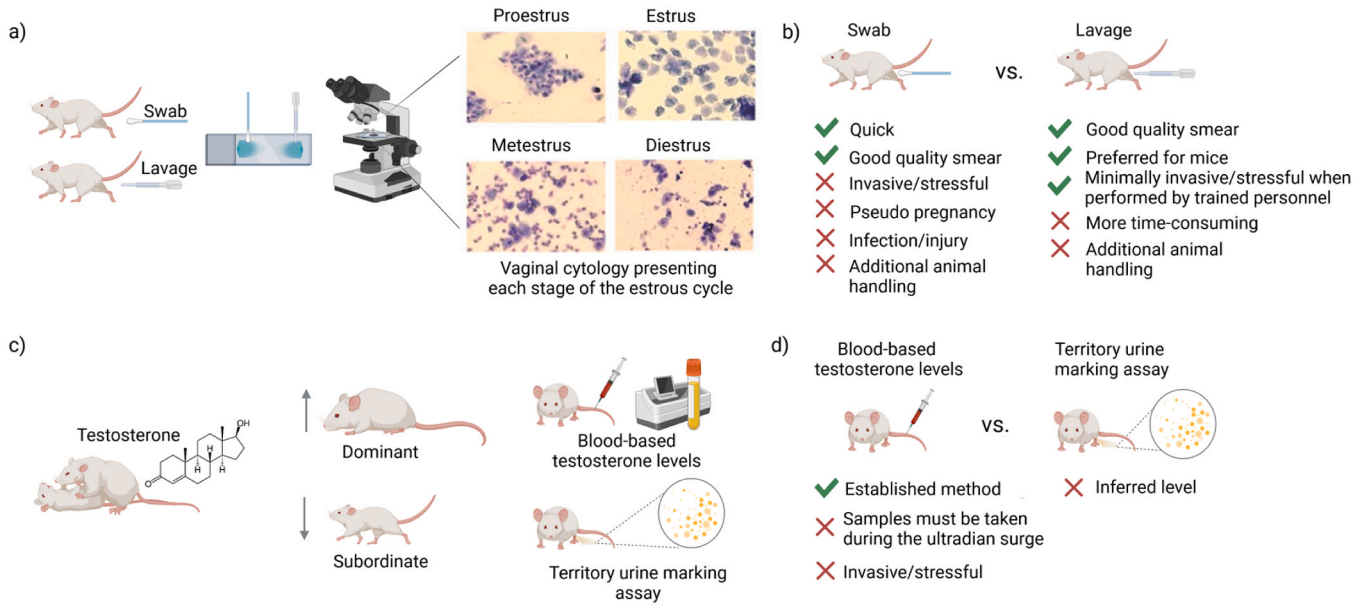


Fig. 2. Tracking hormone fluctuations. a) In females, swab and lavage are the two most used methods. With both techniques, the extracted cells are then examined with a microscope to determine the cycle stage based on the types and morphology of the cells. b) Both methods have advantages and disadvantages, and the selection of the right method should depend on the study design. c) In males, circulating testosterone levels vary depending on the dominance hierarchy, but there is no validated model to track hormone-influenced hierarchy patterns. Testosterone levels can be tracked by using blood samples or the territory urine marking assay, **d)** each of which has advantages and disadvantages.

the same time each day on consecutive days over a period of time to provide detailed information about the estrous cycle. If an experiment requires cycle tracking or must be performed during a certain stage of the estrous cycle, cycle assessment should start about a week ahead of the experiments to ensure accuracy and eliminate animals that fail to show a regular cycle. Though less commonly used, it is also possible to use a visual identification method, which involves gently lifting the tail of the animal while holding it, and then examining and evaluating the appearance of the vulva based on the criteria described by Champlin et al. (Byers et al., 2012; Champlin et al., 1973). While generally considered to be non-invasive, simple, cheap, fast, and less stressful to animals and researchers, this method eliminates the possibility of detecting transitional stages, or the intersection of two consecutive cycle stages, and is susceptible to variations in lighting. It is also important to note that the estrous cycle in rodents is affected by various environmental factors such as the light/dark cycle, temperature changes, seasonal variations, social interactions, odor cues, diet and nutrition, as well as stress caused by experimental manipulations or housing conditions. However, the impact of these factors may differ among different rodent species and strains, which should be taken into account when planning new experiments and/or comparing outcomes across experiments (Peña et al., 2019; Kokras et al., 2015, 2012; Meziane et al., 2007; Manzano Nieves et al., 2019).

In males, tracking hormones is much more difficult. There is no proper method for monitoring hormone levels in males without collecting blood (Fig. 2c). Studies that have tracked male testosterone levels did so using blood samples, which has its own inherent challenges. Namely, only males sampled during the ultradian surge will have detectable testosterone levels (Wu and Tollkuhn, 2017; Heywood, 1980). Other studies have inferred higher or lower levels of hormones by assessing hormone-influenced hierarchy patterns in mice via assays, such as the territory urine marking assay (Lehmann et al., 2013; Fulenwider et al., 2022). However, this method has not been reliably compared to blood-based testosterone levels (Fig. 2d). For experiments sensitive to these effects researchers should reconsider the experimental unit (for recommendations see part 4 and (Mähler Convenor et al., 2014)).

4. Behavioral experiments: logistical considerations and sex-specific behavioral readouts

Laboratory animal behavior has been studied for many years since Thomas Wesley Mills's work in the 1890's and Pavlov's work in the early 20th century (Miles, 1930; Mills, 1890, 1898a, 1898b; Pavlov, 2010). However, much of this early work was exclusively conducted in males or did not specifically determine the effects of sex when both males and females were included (Beery and Zucker, 2011). In preclinical research, behavioral tests are mainly used to improve understanding of the central nervous system and to test treatments for psychiatric and neurological disorders. Unfortunately, many behavioral tests (Crawley, 1981; Lister, 1987; Pellow et al., 1985) were developed using only male rodents and thus the results need to be interpreted more cautiously when females are used.

Logistically, there are some important factors to consider when designing a behavioral experiment:

1. First, as mentioned above, it is not always necessary to measure gonadal hormones. However, if the experiment necessitates it, hormone levels should be determined after behavioral testing to avoid stress to the animal. One exception is when the behavior only occurs at a specific point in the animal's cycle, such as for lordosis (Hardy, 1972; Molina-Jiménez et al., 2018; Guttman et al., 1975).
2. The size of the equipment may also need to be adjusted because males are larger than females. Typically, adult male rats weigh between 300 and 500 g, whereas adult female rats weigh between 250 and 300 g, and body size is proportionally different. An example of adjustment in the size of equipment is during the Open Field test where in some cases the height of the infrared beams should be different between sexes in order to detect animals' movement. Similar dimorphic size differences are found in other species and should be considered across multiple logistical aspects of experimental design.
3. Food restriction is commonly employed in neuroscience to motivate performance in behavioral tasks that offer a food-based incentive. But due to their different sizes, males and females may require

different levels of food restriction. The maximum body weight loss should not exceed 15%, when compared to an age- and sex-matched, ad libitum fed control animal. Moreover, food restriction should be age-dependent, since young or growing animals are sensitive to food restriction and their health and minimum growth requirements should be of paramount importance (National Institutes for Health, 2023).

4. Female rats are also more active than males. Tests that depend on the animal's activity may detect fewer or more behavioral changes in females than in males depending on the direction of the effect (Kokras and Dalla, 2014; Fernandes et al., 1999).

5. When using both males and females, cleaning behavioral equipment between animals becomes particularly important as the odors left behind on the apparatus from conspecifics may change the behavioral outcome. In cases where cleaning the behavioral equipment is not possible or restricted, e.g., operant boxes, using different sets for each sex is advised.

6. The odors of conspecifics are also important inside the testing room. For example, female mice show more rearings in proximity to male urine (Redaelli et al., 2014). Similarly, male mice will explore and vocalize more in the presence of female urinary scents (Matsumoto and Okanoya, 2018; Roullet et al., 2011). Two exceptions are operant testing and food motivation tasks where reward/motivation factors overcome these effects (Wahlsten, 2011; Harb et al., 2014; Karlsson and Cameron, 2023).

When using both males and females, behavioral tests can be organized in blocks containing both sexes alternated throughout the testing day. This contributes to controlling for non-gonadal circulating hormones that fluctuate throughout the day. Males and females can also be tested on different days, but this could introduce potential environmental changes that cannot be statistically controlled for. It is recommended to run a subgroup of each sex on each day of behavioral testing, which will be described in the study design section below.

A number of behavioral tests show sex-specific differences including fear conditioning (Shansky, 2018), spatial learning (Korol and Kolo, 2002; McElroy and Korol, 2005; Pisani et al., 2016), running behavior, and pain sensitivity (Nicotra et al., 2014). Interpreting animal behavior in the laboratory requires considering both the situation the animals are placed in and how their response reflects differences in the needs of each sex.

One example highlighting sex-specific behavioral responses involves how male and female rodents may differentially respond to fear. In the typical rodent fear-conditioning task, animals are presented with a tone paired with an aversive electric shock to the foot. This pairing normally induces a fear response, usually measured by freezing behavior (Fadok et al., 2017). However, emerging evidence from Rebecca Shansky's lab suggests that females also have the capacity to show fear by another behavior, called darting, which is a brief, high-velocity movement. In her experiments, most males displayed the typical freezing behavior, with only 10% of males exhibiting darting, whereas approximately 40% of females exhibited darting. Further, both behaviors could be extinguished over time, and females that darted exhibited better extinction memory than non-darters (Gruene et al., 2015a). Darting was also not associated with the estrous cycle while freezing-based extinction was more robust during proestrus when estrogen levels are higher (Gruene et al., 2015b). Morphology changes in the prefrontal amygdala circuitry were observed in males with successful extinction retrieval (high freezing vs. low freezing) but not in females even though both males and females split into high versus low freezing groups (Gruene et al., 2015b). This example highlights how two different behaviors can both indicate fear learning and memory, but possibly through distinct underlying mechanisms.

Spatial learning strategies are another example of differences in an underlying mechanism but with the same outward behavior. Rats can be trained on a T-maze to find a reward located in a specific arm. Once they have learned the place of the reward during training, the maze is rotated 180 degrees and their ability to find the reward during a probe trial is assessed. Males predominantly use a place strategy to find the reward (reviewed in (Goodman, 2020)). When the ability of males and females to utilize a place strategy was compared on the T-maze, females were judged to perform worse, suggesting females had poorer learning and memory on spatial tasks such as the T-maze. However, subsequent studies showed that females predominantly find the reward using another strategy, called response strategy, which involves distinct anatomical regions and employs unique neural circuitry (Korol and Kolo, 2002; McElroy and Korol, 2005; Hawley et al., 2012). Comparing females to the male standard — place strategy — caused researchers to incorrectly conclude that females did not learn well in the T-maze tasks. The discovery of the response strategy in females showed that males and females both learn in spatial tasks, but through different strategies. Similar observations have been observed in the Morris Water maze (Perrot-Sinal et al., 1996).

5. Sex differences across the lifespan: experimental design considerations

Most research is done with young adult animals. However, experiments using animals outside of young adulthood or spanning multiple ages is also important but requires special considerations, particularly when both males and females are used in the same experiment. Dramatic changes in gonadal hormones across the lifespan can affect experimental outcomes (Bell, 2018). These changes occur on different timelines for males and females across species. Herein, we cover logistical considerations for designing experiments to study males and females across the lifespan.

Puberty occurs later in males than in females and this could be problematic when this time period is studied. So, how can timing differences in puberty be addressed, while keeping the experimental variables controlled? In other words, should animals be age-matched even though one sex will not be undergoing puberty or should a span of time that covers puberty in both sexes be assessed? The answer depends upon the experimental questions and desired outcomes. Puberty is accompanied by hormonal changes, switches in how the brain reacts to certain proteins, and changes in brain structure that are both dependent and independent of hormones (Yasuda et al., 2003; Juraska and Willing, 2017; Andersen, 2003). Thus, the timing of animal testing can have profound effects on behavioral and other outcomes. To track puberty onset, physical, hormonal, and behavioral changes can be observed. For female rodents, vaginal opening is a reliable indicator of puberty onset, which can be observed by gently lifting the tail and looking for a visible opening (Sergio, 2006; Gaytan et al., 2017). For male mice, balanopreputial separation, the separation of the prepuce from the glans penis, is a reliable indicator of puberty onset (Yoshimura et al., 2005; Hoffmann, 2018). However, the method can be distressing to animals and thus, regular observation and recordings of physical and behavioral changes such as increased body weight, growth of reproductive organs, and appearance of the external genitalia, as well as increased exploratory behavior and scent marking, can be used as alternative methods. Urine samples can also be collected, if necessary, to measure the levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as they significantly increase during puberty onset (Korol and Kolo, 2002).

It is essential to note that puberty onset timing can vary across rodent species (Bell, 2018) and strains (James et al., 1990) and be influenced by experimental factors such as stress (McCormick et al., 2017) or diet (Tingbei Bo et al., 2021; Engelbregt et al., 2002). It is also important to consider how exposure to stress or endocrine-disrupting chemicals during critical windows of development may contribute to subsequent alterations in puberty onset. For example, in females, early-life stress has been shown to accelerate sexual maturation in both humans (Belsky et al., 2015; Mendle et al., 2007) and rodents (Cameron et al., 2008; Cowan and Richardson, 2019; Honeycutt et al., 2020). In males,

however, early-life stress has either no effect (Biagini and Pich, 2002) or delays puberty onset (Cowan and Richardson, 2019; Bodensteiner et al., 2014), although inconsistencies could be due to difficulties in measuring puberty onset in male rodents (Tremblay and Frigon, 2005).

Another important example is the timing of shipping animals. Shipping animals of both sexes during puberty or pregnancy can have short-term and long-lasting consequences on behavioral outcomes and hormone responses (Laroche et al., 2009; Bowman et al., 2004; Herrenkohl, 1979, 1983; Herrenkohl and Politch, 1978; Sachs and Lumia, 1981; Holliday et al., 2020), likely due to the stress of the shipping process. These two aforementioned examples highlight the importance of considering how shifts in pubertal timing are important to consider when planning experiments.

Studying aging in rodents also requires special considerations. Female rodents, like women, undergo regular reproductive cycles during adulthood, and they also experience dysregulation of this cycle and the hypothalamic–pituitary–gonadal (HPG) axis, as well as changes in their ovaries and fluctuations in their gonadal hormones, as they age. However, it is important to note that unlike humans, rodents do not menstruate and therefore do not naturally experience menopause (Lu et al., 1979). Menopause, by definition in humans, occurs when menstruation has ceased, ovarian follicular activity is lost, and hormone levels fall (Bacon, 2017; Santoro, 2005; Utian, 2004). In rodents, however, following the onset of irregular estrous cycles, some rodents will transition directly to an anestrous state. This state is similar to menopause in humans, including no ovulation and low levels of gonadal steroids, but mature ovulatory follicles can still exist (Lu et al., 1979). Before the onset of anestrous, rats more often than mice can enter a pseudopregnancy phase that can continue for the rest of their lives, meaning that these animals never mimic the physiological conditions necessary to study menopause in humans (Finch, 2014). There are also surgical and non-surgical options as well as some newly developed genetic models to model menopause in rodents (Danilovich and Ram Sairam, 2006; Bimonte-Nelson et al., 2008a). The right model will depend on the experimental question and outcome measures (Table 1).

It should be noted that male rodents also have varying levels of testosterone across their lifespan, with the highest concentrations observed in adulthood/middle age (Bimonte-Nelson et al., 2008b). Gonadectomy in male animals has good translatability to andropause syndrome in men (Table 1).

The age of the animal may also impact specific types of methodologies that might be employed. One example is hippocampal long-term potentiation (LTP). LTP is an extensively studied phenomenon in neuroscience and shows significant changes across the lifespan (Barnes, 1979; Foster, 1999; Lynch et al., 2006; Morris et al., 2003). Many of these changes are attributed to gonadal hormone fluctuations across the lifespan, which might lead to different levels of hippocampal neuron excitability. Cyclic changes in hippocampal LTP across the estrous cycle have also been observed (Good et al., 1999; Warren et al., 1995).

However, a 2009 review study found that 59% of hippocampal LTP studies were performed in animals that had not yet reached adulthood (McCutcheon and Marinelli, 2009). Furthermore, 30% of these studies also pooled together animals whose ages varied by up to 3–4 weeks. Thus, the effects of the experimental manipulation cannot be distinguished from the effects of hormonal variations when the results of animals spanning critical periods of development are analyzed together. Designing experiments that take into account factors that can change with both age and sex is crucial. This can be achieved by including appropriate control groups, such as a gonadectomy group, sham group, and so on.

Another important topic related to steroid hormones is the use of the estrogen modulator tamoxifen, which has become a critical tool for investigating gene function in mice. It allows researchers to temporally control gene deletion and/or genetic inducible fate matting (lineage tracing) using the Cre/loxP system to determine whether a gene is required in an adult animal. A point mutation is introduced to the ligand-binding domain of estrogen receptor alpha (ERα), resulting in a receptor that selectively binds the synthetic selective estrogen receptor modulator tamoxifen, but not endogenous estrogens (Hsieh et al., 2007; Badea et al., 2003).

Tamoxifen shows mixed agonist/antagonist activity for ERα, depending on the tissue and cell type. Tamoxifen has also been used to delay precocious puberty (Eugster et al., 2003), and thus may delay puberty onset when used to delete a gene before puberty. Even its use in adult animals can confound some experimental results because, similar to estrogens, tamoxifen treatment causes an acute drop in food intake and body weight (Wade and Heller, 1993; Kiermayer et al., 2007). Transient treatment with tamoxifen also has short-term effects on glucose tolerance and insulin secretion (Ceasrine et al., 2019), and strikingly persistent effects on lipid metabolism and fat mass (Liu et al., 2018). These factors highlight the importance of including tamoxifen-only controls in all behavioral analyses when it is used at any age to induce gene deletion.

6. SABV and in vitro experiments: cell and tissue considerations

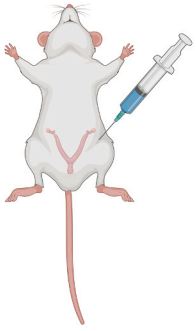
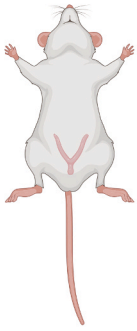
The use of females in in vitro research has faced greater resistance than in other areas of preclinical research. While the FDA no longer requires animal studies for drug approval when a suitable alternative exists (Wadman, 2023), the reliance on in vitro models (cell cultures, organoids, co-culture and microfluidic systems and bioreactors) raises concerns about potential sex-specific biases that may be overlooked in experimental design and analysis.

Although cells may respond differently once removed from the body (Ritz et al., 2014; Penaloza et al., 2009), sexually dimorphic differences between male- and female-derived cells have been observed in many cell populations (Shah et al., 2014; Perego et al., 2022), suggesting that SABV is indeed relevant for in vitro research. But many researchers argue that the availability of tissues or cells is a limiting factor to considering SABV in in vitro research.

In vitro experiments use either primary cells harvested directly from the tissues of humans or animals or commercially available immortalized cell lines. Both show inherent advantages and challenges, particularly in the context of studying sex differences. Immortalized cell lines are always derived from a single donor source of a single sex, which is very often not reported (James et al., 2021). When a similar cell line derived in both sexes can be found, the cells may have different demographic profiles. A sex difference should not be claimed, even in cases when male and female cell lines with similar demographic characteristics are available. The reason is that the two lines were established from a single male and single female, which is the equivalent of a N of one (Ritz, 2011; De Souza Santos et al., 2018). In this case, it is important to accurately report that the cells were derived from a single source (donor) as well as to include the number of cells per treatment used in the experiment. Cell lines also demonstrate chromosomal instability so authentication of the sex chromosome configuration is necessary (De Souza Santos et al., 2018). Primary cells also face challenges of demographic variability but it is possible to recruit male and female donors with similar demographic profiles. However, establishing human cell lines is labor, skill and time intensive and additional donors would be necessary for multiple experiments as primary cell lines from humans can only divide a defined number of times. Finally, organ-on-a-chip (OoC) systems, predicated on the application of iPSCs and organoids, provide a unique platform to probe patient diversity. This diversity takes into account various factors like race, ethnicity, sex, age, and health or disease states as biological variables. Such systems also pave the way for conducting patient-specific studies concerning disease progression and treatment responses (Leung et al., 2022).

In rodents, cells for primary cultures can be derived from neonates or embryos. In both cases, acquiring male and female samples may be easier due to greater availability, but it is still time and labor intensive (Schiebinger et al., 2020). For embryonic cultures, cells can be derived

Table 1
Commonly used rodent models to study menopause, andropause and hormone therapy effects.

Model	Strengths	Weakness
Ovary intact 	• Provides tractable model of the irregular cycle of human perimenopause	• Only 30-40% of aging rodents transition to anestrus with consistent low levels of ovarian steroids • Requires a large aging colony
VCD (4-vinylcyclohexene diepoxide) 	• Allows dissociation between effects of age and ovarian hormone loss • Induces follicular atresia, mimicking human menopause • Effective in multiple strains	• Dose and duration dependent toxicity/carcinogenicity on lung and liver • Potential accumulation in the brain over period required to induce follicular atresia
Ovariectomized 	• Predictive model of oophorectomy in premenopausal women • Good for studying efficacy of hormone strategies	• No compensatory responses in brain metabolism as occurs during natural reproductive senescence • Hard to translate to humans, because humans taking hormone replacement therapy of dietary supplements generally have ovaries
Ovariectomized 	• Good translatability to men undergoing andropause syndrome (osteoporosis, sarcopenia, depression, cognitive decline, and anxiety, etc.)	

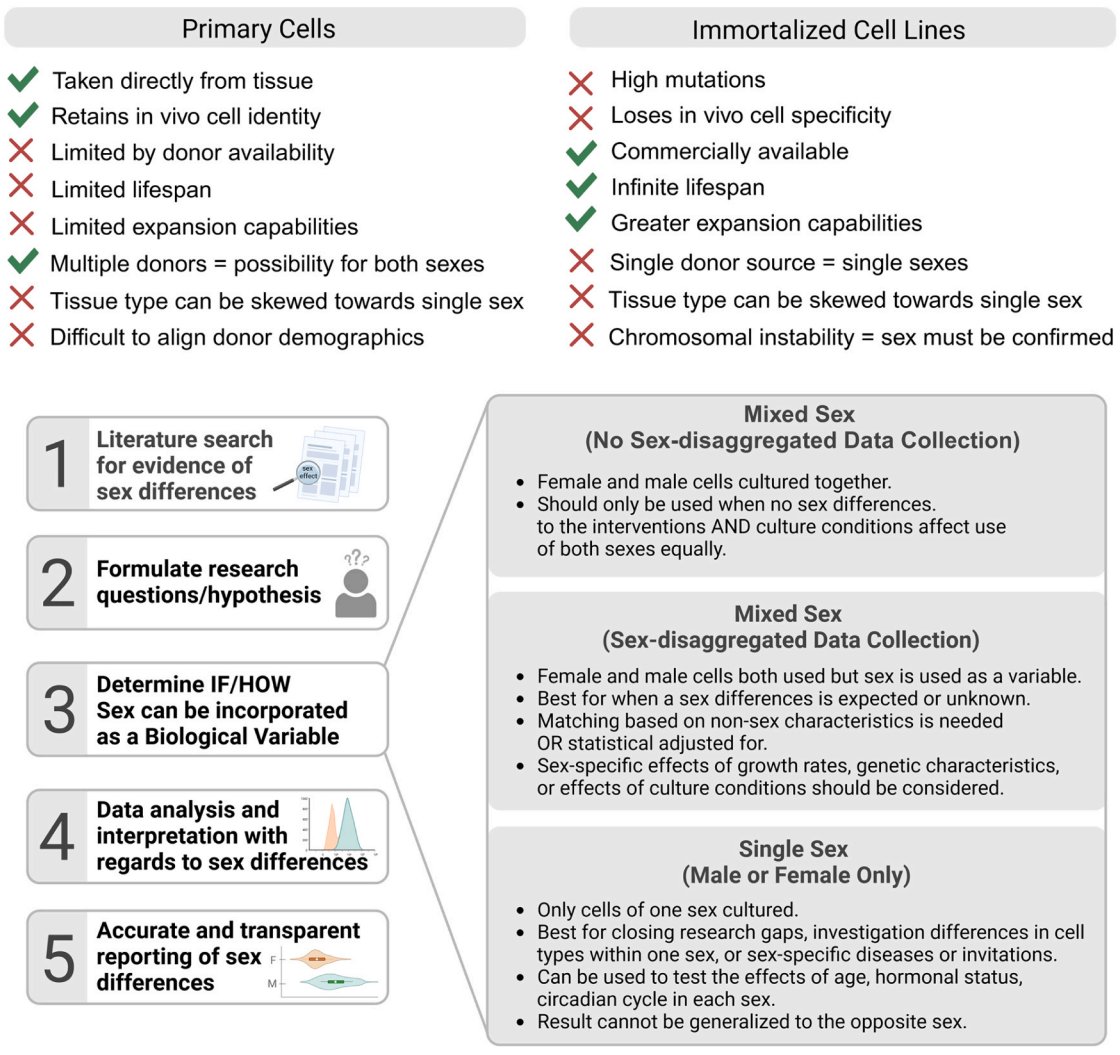
Source:(a) Adapted and modified from (Koebele and Bimonte-Nelson (2016).

from single pups and sexed after plating. But these cultures are typically less robust and have a limited number of cells compared with mixed-sex cultures, reducing the number of treatments and outcomes that can be evaluated. Neonatal cultures can be sexed and grouped by sex prior to plating.

There are several steps you should take to design an in vitro experiment that considers SABV (Table 2). First, a literature search should be performed with adequate search terms for "sex" and "gender" to fully assess previously documented sex differences in the research area (Step 1). This literature search should then be used as the basis for formulating your research questions and hypotheses (Step 2). Finally, you must determine if and how sex will be incorporated into the research (Step 3). While documented sex differences or a skew in disease prevalence to a single sex clearly provide a rationale for studying sex differences, the absence of sex differences does not justify the use of a single sex. Sex differences should always be investigated before they can be ruled out. There are 3 design options you can choose from when choosing which sex cells you will include in your study. 1) Mixed sex cultures without sex-disaggregated data collection refers to culturing cells of both sexes together in the same dish. This approach should only be used if it can be definitively stated that there are no known sex differences in response to the intervention and it is known that the culture conditions affect the cells of both sexes equally. Certain factors such as the cell media, growth factors, apoptotic agents and even some plastics used in culture dishes have been shown to exert estrogen-like actions (De Souza Santos et al., 2018). 2) Mixed sex cultures with sex-disaggregated data collection are ideal, especially if a sex difference is expected based on the literature search or sex differences are unknown. However, the tissues and cells should be matched according to non-sex characteristics that might influence the results, or the results should be adjusted statistically to account for these variables. 3) Single-sex cultures are less ideal because they do not allow both sexes to be directly compared in the same experiment. However, they may be used in comparative studies to, for example, fill gaps in the research, such as exploring an effect in females when there is already an established effect in males. In this case, a validation cohort should be used as with animal studies. Single sex studies can also be used to investigate female or male only interventions or diseases, investigate differences within cell types of one sex, or study how cells differ according to different factors.

In all cases, data should be interpreted cautiously. Care should be taken to avoid assuming that findings in one sex apply to the other, especially when single-sex cultures are used. Confounding variables related to the culture conditions or inherent differences in the cells based on sex should also be statistically factored. Finally, findings should be transparently reported and without over interpretation of the effects of the sex of the cells or tissue (De Souza Santos et al., 2018). The lack of

Table 2
Considerations and guidelines for incorporating SABV in in vitro experiments. The inherent advantages and challenges of primary cells and immortalized cell lines are described in detail. Three distinct options of how to incorporate SABV are explained.



sex differences should also be reported to guide future research (Schiebinger et al., 2020).

7. Inclusion of SABV in a single experiment: statistical design, analysis and reporting

The incorporation of SABV does not necessarily mean that sex differences need to be specifically investigated, nor that larger sample sizes need to be used (Beery, 2018). This is important as these are two of the most common arguments against including females into preclinical studies.

When designing a new study, the default option should always be to start with an exploratory study where each experimental group is composed of both males and females in a 1:1 ratio. Exploratory studies serve to specify hypotheses that may later be tested in confirmatory studies. As the term suggests, exploratory studies are designed to explore the effect of one or more factors (e.g., treatment, age, and sex) on one or more outcome variables. Because it is not meant to statistically test a hypothesis, a formal power analysis is not needed but data analysis should be limited to descriptive statistics. Thus, with half males and half females, one may explore (e.g., graphically, descriptive statistics, etc.) whether the data suggest generally a sex difference (i.e., a sex main effect or a significant sex co-variate) and/or a sex difference in the treatment effect (i.e., an interaction between a treatment and sex). If that is the case, a confirmatory study should be planned to formally test whether a sex difference is present. In this case, sex will become an independent variable (i.e., a fixed effect) and a formal power analysis (there are a number of publicly available programs for conducting power calculations (Percie du Sert et al., 2017; van Wilgenburg et al., 2003) should be conducted, ideally based on preliminary data or published effect sizes, to determine the adequate sample size. If no data are available, power calculations should be based on a best estimate of the minimum effect size that is considered biologically relevant.

Even if sex is included as an independent variable to formally test for a sex difference, in most cases it is not necessary to use twice as many animals compared to studies using only one sex. The reason for this is that factorial study designs (i.e., studies in which the effects of more than one variable, e.g., treatment and sex, are assessed) are statistically

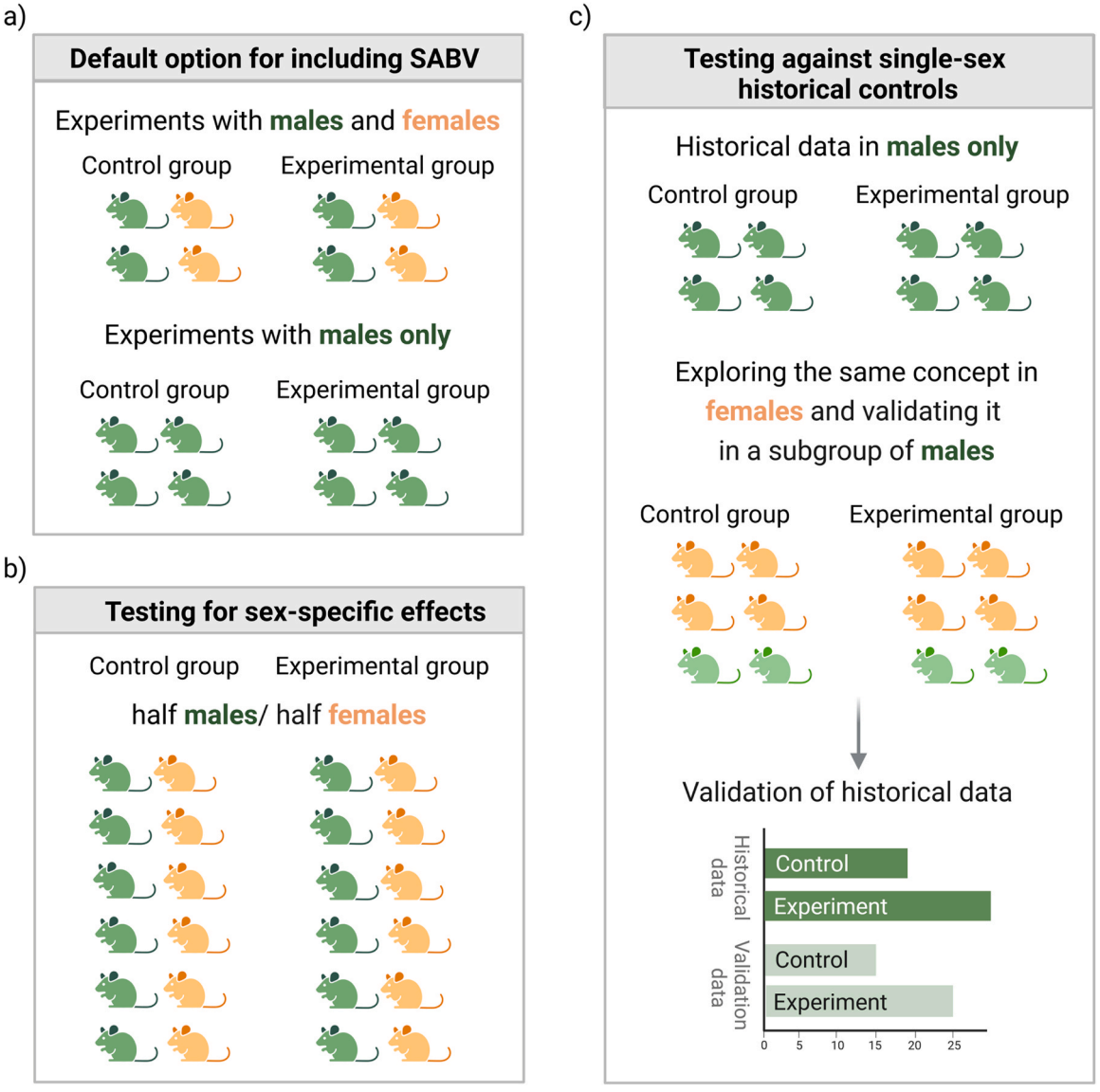


Fig. 3. Integration of SABV into study design. a) Inclusion of both sexes does not require a substantial increase in the sample size if females are added as a heterogenization factor only and included as a blocking factor during the analysis. b) If clear sex-specific effects are observed (or expected), a balanced factorial design can ensure that the sample size is only moderately increased. c) Validation of the historical data obtained from male only experiments by the inclusion of female cohorts that mimic the experimental conditions of the previous work in males, and by the inclusion of a "validation" subgroup of males.

more powerful. Nevertheless, if the sex difference is very small but the study will be powered to test it statistically (because the sex difference is considered biologically relevant despite being small), a much larger sample size may be needed.

If a confirmatory study is statistically powered to detect a relevant sex difference but no sex difference is observed in the results, then it can be reasonably concluded that sex does not seem to influence the outcome measure under these experimental conditions (Fig. 3a). However, such an outcome does not mean that one should revert to single-sex studies in future experiments. Instead, both males and females should be equally included and treated as a random effect (or blocking factor), similar to, e.g., cage. Including sex (and other factors that are not of primary interest to the experiment) helps to enhance the external validity of the results without reducing the statistical power of the study. Often, several such factors can be combined into nested (e.g., cage nested in age group) or crossed (e.g., sex and age group) random effects such that the statistical power of the test is actually increased because the random (i.e., unexplained) variation in the model is reduced.

While studies are ideally designed to include both sexes (Fig. 3b) (Becker et al., 2005; Percie du Sert et al., 2017), what should be done if a laboratory has an existing body of work on only males but now wants to expand its findings to females? There are some important guidelines for how to add females or males and relate it back to the existing work in a single sex (Fig. 3c). Because potential confounding factors might differ between independent experiments, it may be inappropriate to run an experiment on the missing sex and compare the results directly to the results of previous work on the other sex. This is due to the lack of statistical accountability of environmental factors that might differ between the two experiments. The better approach is to run a “validation” subgroup. For example, if you have historical data of an effect between control and experimental groups in males and now plan to conduct a similar exploration in females, the new experiment should include experimental and control groups of females that mimic the experimental conditions of the previous work in males plus a validation subgroup of males. Only then is it possible to determine whether the data from the validation group matches the historical data. If the two groups of males (the new subgroup and the historical group) are the same statistically, only then is it possible to compare the new work in females back to the previous work in males with confidence despite potential environmental differences.

Once data from both sexes are acquired, the most important final consideration is how to interpret and report the results in both sexes. As a general rule, all data analyses should be prespecified and data should always be reported in accordance with the ARRIVE 2.0 Guidelines (Percie du Sert et al., 2020). When appropriate, data should also be reported by 4 categories: male and female, control and experimental. This contrasts with a side-by-side comparison of control and experimental animals separately in males and females. When males and females are directly statistically compared, and a difference appears, this difference can be attributed to the sex of the animal. Another method to determine the presence of sex effects is to normalize all the data to one group (e.g., control males), which allows sex differences to be easily observed, particularly in the control groups.

When data must be analyzed separately for males and females because the experiment was first performed on one sex and then on the other, caution is required when reporting the results and it should be clearly stated that the two studies were performed at two different time points. This is because males and females cannot be directly compared using statistical analysis, and there is no longer a sex effect. Instead, it is preferable to use the following phrasing: "X effect is detected in one sex and not the other," or "X is a male-/female-specific effect." In other words, each sex should be discussed as if it were a different experiment.

Again, this is best done with a “validation” subgroup so previous results can be replicated to strengthen these comparisons (i.e., control for environmental changes). Even when no sex effect is found — whether statistically or even a bimodal distribution or trend — the data should be presented disaggregated by sex somewhere in the paper. This way data and outcomes can be best judged and used by the scientific community.

8. Investigating the biological source of sex-based differences

When conducting a study that specifically examines sex-based differences, it is important to understand the potential sources of the sex effect and how to characterize it. In terms of the source, sex effects primarily arise from two biological mechanisms that differ between males and females. 1) The first is sex hormones, either secreted in adulthood (Fig. 4, Section 1A) or as a consequence of developmental exposure (Fig. 4, Section 1B). 2) The second is the sex chromosome complement (Fig. 4, Section 2).

To determine which of these two biological sources is responsible for the observed effects, it is best to first focus on sex hormones released from the gonads during adulthood (Fig. 4, Section 1A) as this strategy is easier to employ and accounts for most of the observed sex differences in adult animals.

There are several strategies to design a study to examine whether gonadally-released hormones during adulthood are the source of the observed effects between males and females. The first strategy is to surgically remove the gonads of both the males and females in adulthood. If the effect persists when all gonadal hormones are removed, then the source is likely not gonadally-released hormones and developmental or chromosomal sources should be considered (Fig. 4, Section 1A, Strategy 1). The second strategy is to mimic the hormonal profile of the sex that is affecting the results, for example, males. This can be achieved by removing the gonads of the female animals and administering 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 (Fig. 4, Section 1A, Strategy 2). The third strategy focuses on the effects of hormones in a single sex, such as only males or only females. In this case, the gonads are removed and then a comparison of animals with or without hormone replacement is performed (Fig. 4, Section 1A, Strategy 3).

In all three strategies, however, the experiments should be delayed for a period of time after gonad removal to allow circulating steroids to clear from the bloodstream as well as from fat or other tissue deposits. Long-term gonadectomy can also downregulate steroid hormone receptors, changing the sensitivity of reintroduced steroids. Additionally, the chosen strategy should be based on a literature review and the desired outcome measures.

Alternatively, tracking hormone levels aids the determination of whether the observed sex effects are specific to a particular stage of the female estrous cycle or due to variations in male sex hormone levels. This is often one of the single biggest factors that deters many researchers from incorporating SABV. However, it has been shown that considering the estrous cycle may enhance the precision of neuroscience studies by revealing concealed sex differences and providing mechanistic insights into the identified sex differences across various neurobehavioral outcomes (Jaric et al., 2019a, 2019b; Chari et al., 2020; Schoepfer et al., 2020; Rocks et al., 2022; Saland et al., 2022; Duclot and Kabbaj, 2015; Dossat et al., 2018). 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. However, 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 all animals during a specific phase of the cycle or have a group of females at each cycle phase. 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

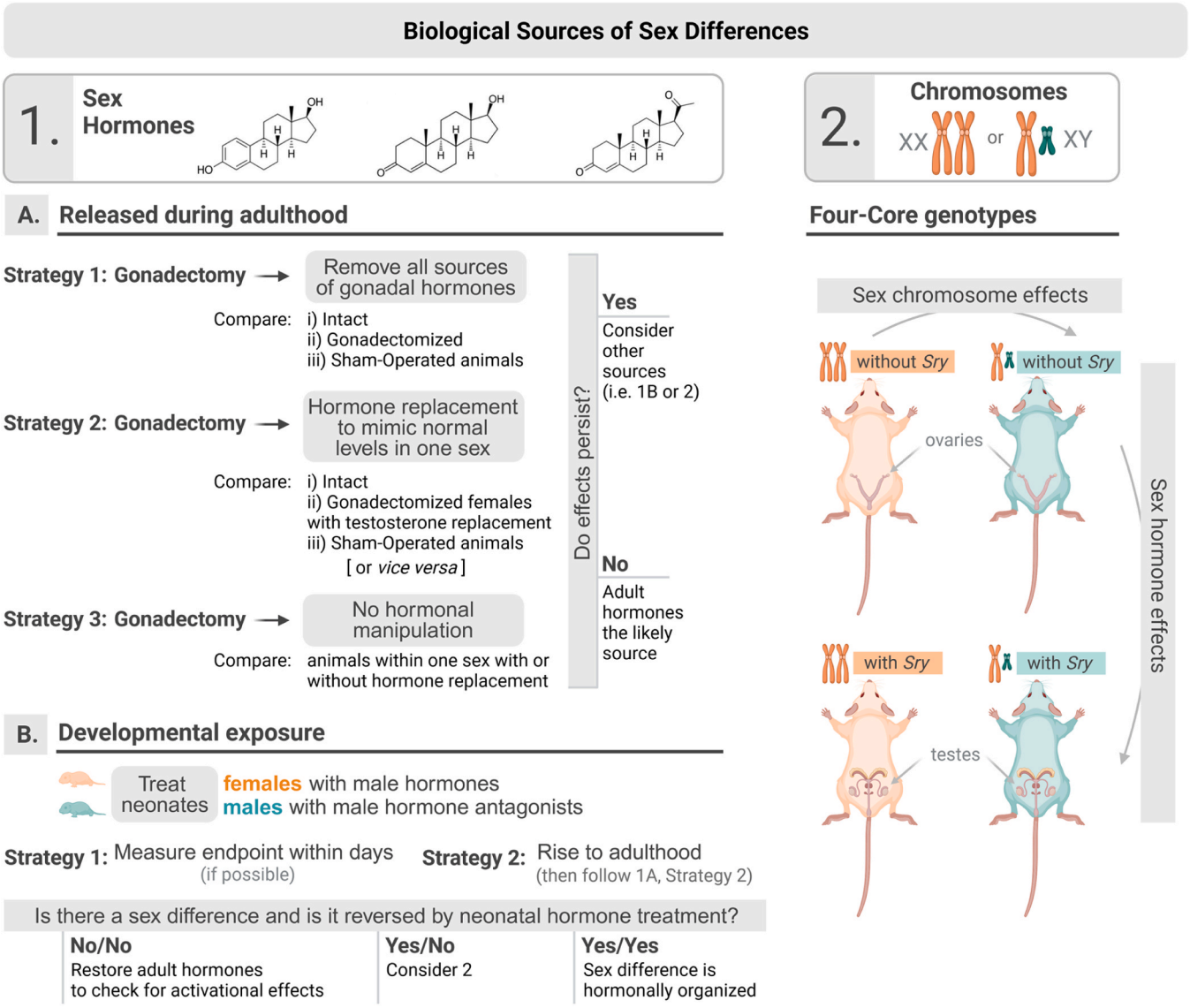


Fig. 4. (a) Biological sources of sex differences and/or differences in sex effects between males and females. (b) The potential sources of sex differences in experimental results are listed, along with recommended strategies for study design.

examination of data from an experiment conducted in females independent of the cycle stage.

If adult gonadally synthesized hormones are excluded as the source of the sex effect (Fig. 4. Section 1A), the next step is to determine whether the sources of these effects are developmental (i.e., exposure to gonadal hormones during specific developmental periods; Section 1B) or a sex chromosome effect (Section 2). These points have been reviewed elsewhere (see, for example, (Becker et al., 2005) for sex hormone developmental effects, and (Arnold and Chen, 2009) and (Büdefeld et al., 2008) for reviews of the Four Core Genotypes model). However, a few extra points should be noted. First, the developmental 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 (Becker et al., 2005).

For studies of chromosome-related sex differences, a relatively new genetic tool has emerged (Fig. 4). 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 of a male to an autosome. As a result, XX mice carrying ectopic *Sry* develop testes and XY mice devoid of *Sry* develop ovaries, although they lose germ cells and cease estrous cycling earlier in life (Arnold and Chen, 2009; Arnold, 2009; De Vries et al., 2002). This model allows for sex effects caused by chromosomal differences to be distinguished from those sourced from gonadally-produced sex hormones.

Correctly reporting sex effects is also important to provide guidance for both the design of subsequent experiments as well for the interpretation and reporting of current experimental findings. There are four operational categories of sex effects that should be used when reporting experimental results of sex differences studies (Fig. 5).

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 the other, many of which are associated with reproduction, such as maternal aggression, lordosis or male-specific courtship behaviors (Fig. 5a).

The second category, quantitative differences, is when an endpoint exists upon a continuum in both sexes. However, when compared

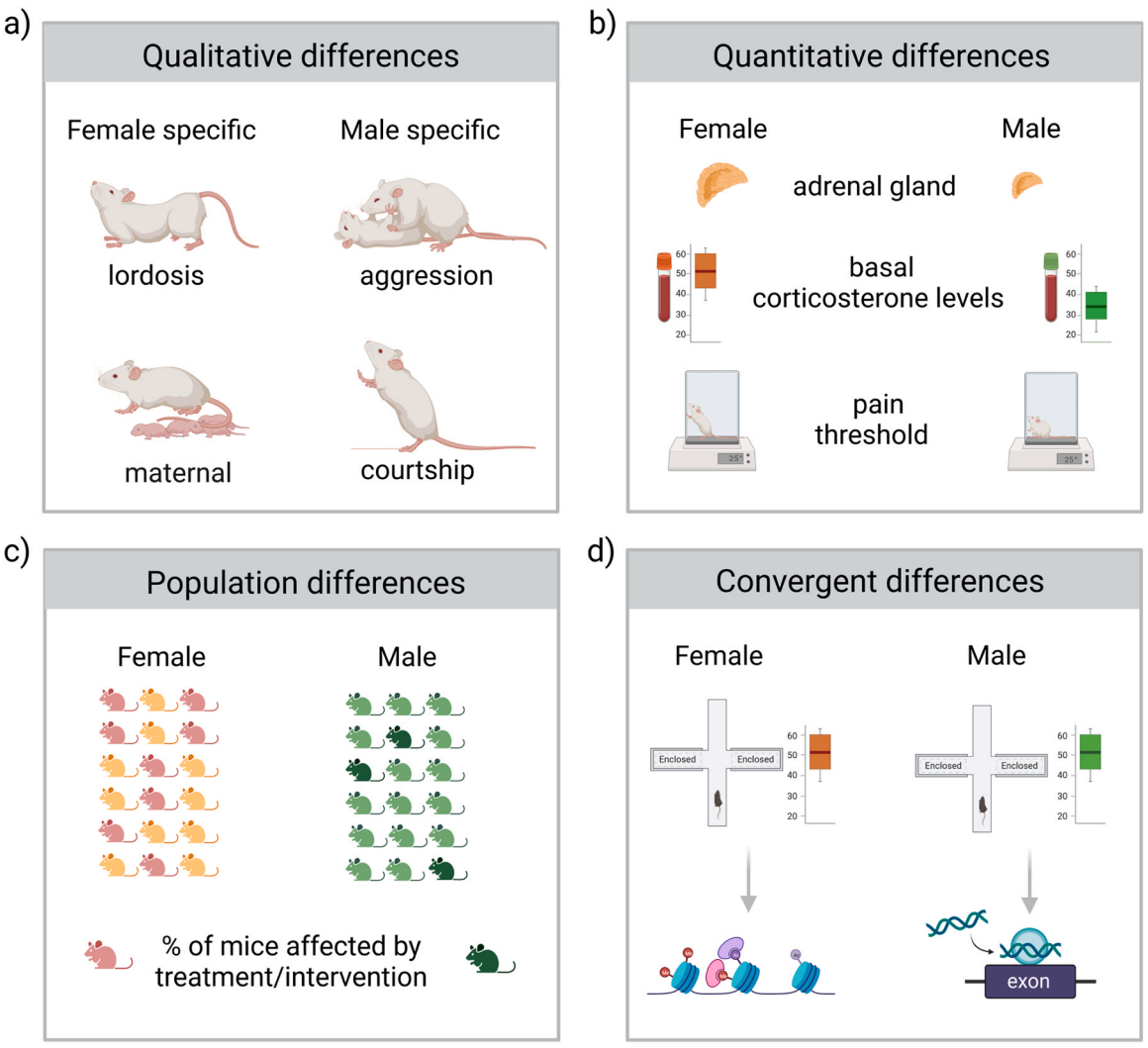


Fig. 5. Basic guidelines on how to evaluate sex effects through experimental designs and how to interpret and report experimental findings. Four operational categories of sex effects that should be used when reporting experimental results of sex differences studies: a) Qualitative differences are traits exhibited by males and females that do not look the same, i.e., maternal aggression, lordosis or male-specific courtship behaviors. b) Quantitative differences are found when an endpoint exists upon a continuum in both sexes, i.e., stress and anxiety responses, pain thresholds, social behavior, and learning and memory. c) Population differences are found when the incidence or distribution differs between males and females. Such differences may only emerge under specific conditions, such as after exposure to a stressor or pharmacological compound. d) Convergent sex differences refers to an endpoint that is similar in males and females but the molecular mechanisms are different.

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 (Fig. 5b).

The third 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 (50%) tend to choose cocaine over palatable pellets than males (16%), but the behaviors exhibited during cocaine taking do not differ between males and females (Perry et al., 2015, 2013). Sometimes, population differences only emerge under certain conditions, such as after exposure to a stressor, 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 (Fig. 5c).

The fourth and final category, convergent sex differences, refers to an endpoint that is the same in males and females but the underlying mechanisms are different (McCarthy et al., 2012; Bangasser and Cuarenta, 2021). 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 (Fig. 5d). For instance, estradiol triggers the potentiation of excitatory synaptic transmission in both male and female hippocampi. However, despite observing comparable increases in synaptic strength in males and females, the engagement and functions of cAMP-regulated protein kinase, internal calcium stores, and L-type calcium channels that regulate these processes differ between the sexes (Jain et al., 2019). In a study on sustained attention disrupted in depression, comparable deficits were observed in male and female rats exposed to a 6-day variable stress procedure. The stress in both sexes induced dendritic hypertrophy in cholinergic neurons, mediating

sustained attention. However, these effects are mediated through different sex-specific transcriptional regulations in the basal forebrain (Eck et al., 2020). This example effectively highlights the significance of comprehending convergent sex differences in the development of effective treatments for neuropsychiatric disorders.

It is important to mention the significance of genomics, epigenomics, and other high-throughput omics studies as valuable tools in preclinical and clinical neuroscience research. However, many genome-wide association studies (GWAS) overlook the biological variable of sex by excluding the X and Y chromosomes, hindering the investigation of sex differences in various diseases and traits (Wise et al., 2013; ANON, 2017; Gorlov and Amos, 2023). Recent studies have started to address this gap, providing a more comprehensive understanding of the genetic basis of psychiatric and neurological disorders (Silveira et al., 2023; Davis et al., 2021). This is particularly important in preclinical and clinical neuroscience research, as approximately 1500 genes on the X chromosome are expressed in the brain (Laumonnier et al., 2007), representing potential candidates for sex differences in neurological traits and disorders.

Although numerous studies highlight the importance of sex differences in gene expression and the epigenome in brain development (reviewed in (Gegenhuber and Tollkuhn, 2022) and Gegenhuber and Tollkuhn, 2019, 2020), the incorporation of sex as a variable is still inadequate in the "neuro-omics" field (Joel and McCarthy, 2017). However, several studies have investigated molecular mechanisms using high-throughput approaches and included SABV in their design. For example, Labonté and colleagues nicely demonstrated the importance and potential of the inclusion of SABV (Labonté et al., 2017). They investigated sex-specific transcriptional signatures in the brains of depressed men and women compared with healthy controls using a large cohort of human post-mortem brain samples, advanced bioinformatic tools, and a comparison between human and rodent data. This study analyzed six different brain regions and found different degrees of overlap in gene expression patterns between patients and controls. The study found that the amount of major depressive disorder-related transcriptional changes shared between men and women is limited and dependent on the observed brain region.

In addition, several rodent studies that included both sexes showed that male and female mice undergo different patterns of transcriptional regulation in response to stress. For instance, subchronic stress elicited a strong transcriptional response in males but not in females, suggesting that male rodents show an active resilience response not elicited in females (Hodes et al., 2015; Pfau et al., 2016). Another study demonstrated that acute stress induces a remarkable array of sex- and genotype-specific translational profiles of mRNA isolated from hippocampal CA3 pyramidal neurons (Marrocco et al., 2017). Interestingly, less than 5% of differentially expressed genes (DEGs) were shared between sexes, similar to findings in humans (Labonté et al., 2017; Breen et al., 2018; Propper and Brunyé, 2013). Using the same animal model, Caradonna and colleagues showed sex differences in response to pharmacological manipulation of the hypothalamic-pituitary-adrenal axis associated with differential methylation of the glucocorticoid receptor gene (Caradonna et al., 2022a). Recent work from the same group showed that a prior history of stress induces different degrees of epigenetic reorganization in the ventral hippocampus of male and female mice (Caradonna et al., 2022b). Additionally, different transcriptional signatures and differential patterns in signaling pathways relevant for hippocampal neuroplasticity differed between female and male rats exposed to chronic restraint stress (Olave et al., 2022).

The examples mentioned above nicely illustrate the crucial importance of including SABV in "neuro-omics" studies. Such studies can reveal sex-specific genes and transcriptional regulatory pathways, which can contribute to our understanding of the sex-specific nature of psychiatric disorders or may offer novel targets for therapies.

9. SABV and therapeutic drug development: from bench to bedside

It is widely acknowledged that precision medicine, or medical care optimized for a patient's unique characteristics, including their age, sex, ethnicity, and pharmacogenomics, is the future of clinical care. Yet, the inclusion of women in randomized controlled trials for novel treatments still lags behind that of men (Mauvais-Jarvis et al., 2020; Daitch et al., 2022). Moreover, the designs of numerous clinical trials lack optimality in detecting sex differences or sex-specific effects, which is of significant importance (Liu and Mager, 2016), with some fields making better strides than others. Considering that the drug development process is long and expensive, in the context of SABV, the limited inclusion and reporting of sex-specific effects in non-regulated preclinical research, as well as the lack of validated animal models for diseases with known mechanistic or phenotypic differences in males and females, further limits the realization of precision medicine. Depending on the therapeutic area, the average time to take a new drug target from discovery and preclinical evaluation through to FDA approval is 12 years (DiMasi et al., 2010), with an average estimated cost of $1–2.6 billion (DiMasi et al., 2016; Wouters et al., 2020) and only one compound in 5–10 thousand making it to FDA approval (Mohs and Greig, 2017). Thus, there is an immediate need to re-examine how and when to best integrate sex [as well as other key determinants of precision medicine] into the earliest stages of the drug development pathway to improve the efficacy of treatments in both men and women and avoid the dangers of a sex mismatch between preclinical research and clinical trials. It is important to highlight here that the translation of medical findings and information is a highly challenging task as it often concerns patients. Therefore, there can be a wide range of variation in both the disease and how patients respond to treatment that burdens the translatability of findings (Austin, 2021).

To achieve this goal, scientists should include SABV in all preclinical research to best inform the clinical research and development process at the earliest stages of drug development. In the context of in vitro research, the genetic sex [and age] of the cultured cells and cell lines should be noted and reported. Experiments should be conducted using both male and female cells whenever possible to identify sex differences at the earliest stage of drug development and potentially identify mechanistic insights. How the cells were maintained is also important to note, including the number of passages and the specific growth conditions. Both factors may lead to significant phenotypic differences in drug response. While limited in its translation to humans, it is also recommended, as a future research direction, that a single gonadal hormone, such as estrogen or testosterone, be added to cell cultures to examine the effect of the hormone on the outcome of interest.

As research projects are advanced to in vivo models, the importance of addressing sex in preclinical findings to human therapeutics increases. As a first step in this process, researchers should always conduct a careful literature search for high quality data upon which to design their experiments, assessing the quality of the information available on a particular test or model and prioritizing important methodological considerations (Sil et al., 2021). This can ensure that preclinical research is carried out to the highest standards and according to best practices (Percie du Sert et al., 2017; PREPARE Guidelines, 2023). The ARRIVE 2.0 guidelines describe the minimum reporting requirements for animal research (Percie du Sert et al., 2020) and include the transparent reporting of the sex [and age] of the animals (https://norecopa.no/prepare). Many diseases fluctuate in incidence, severity, and symptomatology across the lifespan, especially in women. Therefore, it is also important to consider incorporating diverse animal cohorts into drug development experiments, such as animals of different ages or reproductive statuses. When a significant sex effect on an outcome measure of interest does exist, properly pursuing the mechanism of that sex effect should be considered. Finally, a variety of other environmental factors related to housing and testing can also have an influence on observed sex

differences and should be clearly documented and accounted for statistically.

In addition to considering SABV earlier in the drug development process, sex-based pharmacology differences should also be considered in preclinical research. Most of the evidence of sex-based pharmacology differences is based on data collected during traditional pharmacokinetic (PK), pharmacodynamics (PD), and toxicology studies. However, some evidence of sex differences in PK, PD, and toxicology is available in animal models and is important to consider in all studies, regardless of whether the intended purpose of a research program is drug discovery or not.

The basics of PK are Absorption, Distribution, Metabolism, and Elimination - an acronym commonly referred to as ADME. Some sex-based differences have been observed across ADME factors in both humans and animals. Many of these findings are preliminary and not very robust, but the available evidence indicate sex differences in the secretion of gastric acid, transit times from the stomach to the intestine (Soldin and Mattison, 2009; Feldman et al., 1983; Caballeria et al., 1989; Bennett et al., 2000; Yonkers et al., 1992; Stephen et al., 1986; Hutson et al., 1989), body weight, intravascular volume, organ blood flow, muscle mass (Meibohm et al., 2002; Soldin et al., 2011; Gochfeld, 2017), renal blood flow, glomerular filtration, tubular secretion, and tubular reabsorption (Berg, 2006; Silvaggio and Mattison, 1994; Hytten and Chemberlain, 1980; Davison and Dunlop, 1980; Cerrutti et al., 2001), all of which are either lower or slower in women compared to men. However, it should be noted that there is no robust evidence regarding sex differences in PK or PD. Although during drug development, sex differences in regulated PK and toxicological studies are routinely evaluated, these comparisons are rarely included or covered in academic publications. Furthermore, there are also better established sex differences in the expression and activity of hepatic enzymes related to the metabolism of drugs, including the cytochrome P450 enzyme superfamily (Soldin et al., 2011; Gochfeld, 2017; Sramek et al., 2016a; Anderson, 2002; Kokras et al., 2011; Greenblatt and von Moltke, 2008; Hunt et al., 1992; Schmidt et al., 2001; Harris et al., 1995; Waxman and O'Connor, 2006). Preliminary sex-based differences have also been observed for multiple drugs including alcohol (Baraona et al., 2001; Frezza et al., 1990; Parlesak et al., 2002), sedatives, aspirin, and heparin (Cooper et al., 1984; Greenblatt et al., 1977; Aarons et al., 1989; Campbell et al., 1998; Trnavská and Trnavský, 1983).

PD is the relationship between the concentration of the drug at the site of action and the biochemical and physiological effects. PD is typically studied in the context of efficacy — the extent to which a given drug achieves its desired effect — and potency — the dose of drug required to achieve a desired effect. Efficacy is primarily measured by changes in functional outcomes. Some drug treatments may also lead to additional sex differences in functional outcome measures that should be considered. As a result, any outcome may show a sex difference that could reflect either a baseline sex difference and/or a sex difference in efficacy. Sex-based differences in therapeutic effects, attributed to PK/PD effects, have been observed in humans for beta-blockers (Luzier et al., 1999), analgesics (Berkley, 1997; Craft, 2003), antidepressants (Sramek et al., 2016b), antipsychotics (Yonkers et al., 1992; Smith, 2010; Melkersson et al., 2001; Seeman, 2019; Abel et al., 2010), and antimuscarinic therapies used for overactive bladder (Hartigan and Dmochowski, 2020). In addition, while they can influence some PK parameters, the influence of sex hormones, contraceptive use, and menopause on clinical efficacy is still poorly understood for most drugs (Meibohm et al., 2002; Harris et al., 1995; Damoiseaux et al., 2014). In rodents, sex differences in response to tricyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin-noradrenaline reuptake inhibitors have been observed (Kokras et al., 2015; Allen et al., 2012; Günther et al., 2011; Kokras et al., 2009; Caldarone et al., 2003; Kokras and Dalla, 2017). Of note, as most of these findings are exploratory, further hypothesis-testing or confirmatory studies are required.

Sex differences in PK and PD can be related to sex-based differences in toxicology and adverse drug reactions (Franconi and Campesi, 2014). In humans, women tend to have more adverse drug reactions than men and some studies have linked these effects with poor representation of females in preclinical studies (Gochfeld, 2017; Rodenburg et al., 2011). Some evidence exists that sex differences in PK contribute to sex differences in adverse drug reactions. A 2020 study examining 86 FDA-approved drugs found that most women had elevated blood concentrations and longer elimination times, and that these differences strongly predicted sex-specific adverse drug effects in women only (Zucker and Prendergast, 2020). However, for some adverse reactions, the correlation may not be so clear. For example, drug-acquired long QT syndrome is observed more often in women than men (Nicolson et al., 2010). Long QT syndrome is associated with an increased occurrence of ventricular tachyarrhythmias, which can lead to syncope, cardiac arrest, or sudden death (Gupta et al., 2007). These effects are thought to be linked to changes in sex hormones across the cycle as differences in cardiac ion channels are observed across the estrous cycle (Odening and Koren, 2014; Liu et al., 1999; Drici et al., 1996; Hara et al., 1998). Thus, observed effects might be missed in the absence of sex-stratified PK information (Hreiche et al., 2008). The effect of certain drugs on drug-acquired long QT is consistent across species. A 2015 literature review spanning 51 years of research determined that 91% of drugs that led to prolonged QT in non-rodent animal species also did so in humans; similarly 88% of drugs that did not prolong the QT in non-rodent animals models also showed no QT effect in humans (Vargas et al., 2015).

Because sex hormones have documented modulatory effects on many basic pharmacological parameters, as well as on many of the hormones and enzymes that influence these drug properties, the effect of sex hormones on PK, PD, and toxicology should be considered during the experimental design to avoid producing exposures that are either too low or too high to interpret a mechanistic hypothesis. This is particularly the case with drug dose selection, timing, and target engagement. So how should dose selection proceed? First, a thorough literature search should be conducted to look for existing evidence for a modulatory role of gonadal hormones by the drug of choice OR within the organ of interest. Then, a baseline for sex differences in the intended outcome measure, including variations across the estrous cycle, should be established. However, as mentioned previously, the lack of published data on sex differences in pharmacology does not suggest they do not exist. In contrast, as a good rule, it is best to always assume sex differences exist.

In the absence of comprehensive published data showing the use of the intended drug under similar biological and experimental parameters — such as the same strain, route of administration, and disease model — sex differences in basic pharmacology should be considered during drug dose selection. Ideally, this would be performed in conjunction with individuals with specialized knowledge of pharmacology. But some basic principles can be followed by all preclinical scientists. One of the simplest things to do is to establish a basic dose-response curve. Dose-response relationships are often used to examine the relationship between exposure to a substance in increasing doses (minimal 3 doses) and the effect on a parameter of choice, such as a desired behavioral effect or specific target receptor engagement. Dose-response relationships indicate two important facts about potential sex differences in the PD action of a drug. The first is differences in the potency of the drug between males and females. Potency is the amount of drug required to produce a particular effect. More specifically, it is the concentration or dose required to produce 50% of the maximal effect, or EC_{50}. The same drug administered to males and females may show differences in potency such that a higher amount is necessary in one sex to produce the same effect as that observed in the other.

The second concept is efficacy. The generation of a response from the drug-receptor complex is governed by a property described as efficacy (Louis et al., 2006). Two drugs may show different efficacies, or maximum responses, in males and females, leading to drastic misinterpretations of their effects on the outcome measure of interest. Sex

differences in drug efficacy may be related to sex differences in PD, such as the expression or binding affinity of the receptor in one sex versus the other. Therefore, sex differences in the mechanism of action of a drug are best studied in vitro.

Another consideration when administering a drug is ensuring it makes it to the desired target organ. One of the most challenging organs to reach is the brain due to the blood brain barrier. A 2016 review of preclinical studies of drugs targeting the brain found that nearly three quarters of the studies selected a dose without confirming that the selected drug dose was able to reach the site of action in the brain (Kleiman and Ehlers, 2016). Without measuring the concentrations in the brain, it will be impossible to know if the same dose of a drug achieves different concentrations in the brain of males versus females or has different time courses of action (Dalla et al., 2022). A recent review emphasized the significance of investigating sex-specific disparities in PK and PD of antimuscarinic medications designed for overactive bladder (Arrighi et al., 2008). Sex-related variation in neurotransmitter expression and muscarinic receptor subtypes could significantly contribute to differential responses to therapeutics. For example, adult men were found to express approximately three times the amount of M_2 mRNA at the bladder mucosa, compared to adult women, highlighting the necessity for sex-tailored adjustments in drug dosage in order to achieve optimal treatment efficacy (Hartigan and Dmochowski, 2020). Therefore, in certain cases, it might be necessary to administer a higher or lower drug dose to females than to males in order to attain equivalent target-organ concentrations and pharmacological response.

The concentration of the drug at the site of action may also be time dependent. In other words, a drug might show sex differences in the speed in which it is metabolized and thus brain concentrations might be initially similar between males and females but then show different time courses under which levels of the drug at the target decrease faster in one sex compared with the other. Ideally, this should be tested over at least 5–6 time points, including at the time point of the maximal concentration and during the washout phase, when no drug is in the system, to understand the time course of a potential drug's effect and if multiple doses are needed over time to maintain the effect (Aarons and Ogungbenro, 2010; Tuntland et al., 2014). There are several methods to assess drug distribution in the brain (Loryan et al., 2013; Bickel, 2005). Certain drug properties are also known to enhance crossing of the blood brain barrier (size of the molecule, whether it is hydrophilic, and its solubility) (Banks, 2009; Alavijeh et al., 2005; Pardridge, 2012). However, very little is known about how these properties differ across sexes or across the estrous cycle.

Drug dose selection and evaluation of target engagement are two areas of preclinical research that may show significant effects on the experimental outcomes and the interpretation of data. They are also largely underexplored areas in terms of potential sex differences. As more females are included in basic research, the potential effects of gonadal hormones on different pharmacology profiles will become clearer. Keeping these potential differences in mind during study design is important to ensure that conclusions about the effect of a drug on a particular outcome can be adequately made.

10. Conclusions

There is a growing consensus that the interpretation, validation, and generalizability of research findings is critically dependent on the consideration of key biological variables, including biological sex. The previously held notion of one patient one treatment is no longer realistic, and new therapeutics must be designed for a heterogeneous patient population. But out of this shift in thinking has emerged the need for more inclusive study populations across the research spectrum, including at the earliest stages of research.

The inclusion of SABV has the potential to enhance both preclinical and clinical research in four primary ways. First, at the experimental design stage, intentional study design at the outset to determine whether there are sex differences in a particular area of study will allow researchers to develop hypotheses and randomize and balance the sexes across experimental groups. Second, including both sexes in a study and analyzing the outcome measures separately for each sex allows for better interpretion of the treatment effects and helps to inform the design of additional tests and tools that consider fundamental differences between males and females. Third, in the analysis stage, disaggregating data by sex can reveal sex differences that are hidden when pooling data from males and females to establish whether there is a sex difference from a treatment (Clayton, 2018b). Fourth, at the reporting stage, improved reporting of the sex of the animals and cells used in research will inform others in their research and allow more researchers to pursue fruitful avenues of sex differences research. It is worth noting here that there have been relatively few high-quality studies, i.e., properly powered, randomized / blinded, with pre-specified hypotheses and predefined analysis plans, in published preclinical research on sex differences, setting an ideal starting point to urgently continue collecting information on sex differences and providing an opportunity to improve the rigor and translatability of preclinical research. As scientists, our thinking must be broadened to go beyond our past notions and embrace our ethical imperative to adopt these principles. We hope the guidelines described above will empower researchers at all career levels to do just that and include both males and females in all future experimental research.

CRediT authorship contribution statement

Christina Dalla: Conceptualization, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; **Ivana Jaric:** Investigation, Visualization, Writing – original draft, Writing – review & editing; **Pavlina Pavlidi:** Investigation, Visualization, Writing – original draft, Writing – review & editing; **Georgia Hodes**: Investigation, Writing – original draft, Writing – review & editing; **Nikolaos Kokras**: Investigation, Writing – original draft, Writing – review & editing; **Anton Bespalov**: Investigation, Writing – original draft, Writing – review & editing; **Martien Kas:** Investigation, Writing – original draft, Writing – review & editing; **Thomas Steckler:** Investigation, Writing – original draft, Writing – review & editing; **Mohammed Kabbaj**: Investigation, Writing – original draft, Writing – review & editing; **Hanno Wurbel:** Investigation, Writing – original draft, Writing – review & editing; **Jordan Marrocco**: Investigation, Writing – original draft, Writing – review & editing; **Jessica Tullkuhn**: Investigation, Writing – original draft, Writing – review & editing; **Rebecca Shansky**: Investigation, Writing – original draft, Writing – review & editing; **Jill Becker:** Investigation, Writing – original draft, Writing – review & editing; **Debra Bangasser:** Investigation, Writing – original draft, Writing – review & editing; **Margaret McCarthy**: Investigation, Writing – original draft, Writing – review & editing; **Chantelle Ferland-Beckham:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

CD and PP no conflicts. JM no conflicts. IJ no conflicts. MJK no conflicts. MMC no conflicts. HW no conflicts. RS no conflicts. GEH no conflicts. JBB no conflicts. JT no conflicts. CFB no conflicts. AB is an employee and shareholder of PAASP GmbH and a shareholder of PAASP US LLC.

Data availability

No data was used for the research described in the article.

Acknowledgments

This paper and the associated videos were made possible through a 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). CD, NK, and PP have received financial support from the Hellenic Foundation for Research and Innovation (HFRI-FM17-1676). IJ and HW are supported by the grant from The Swiss 3R Competence Centre (3RCC, grant number OC-2020-004). We would like to thank Dr. Mark Zervas for his careful revisions of the manuscript and Dr. Taryn Aubrecht for her work on the original video materials.

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