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SEX AND THE LAB: an alcohol-focused commentary on the NIH initiative to balance sex in cell and animal studies

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Abstract

Background—In May 2014, Dr. Francis Collins, the director of US National Institutes of Health, and Dr. Janine Clayton, the director of the US National Institutes of Health Office of Research on Women's Health (ORWH) published a commentary in the journal *Nature* announcing new policies to ensure that preclinical research funded by the NIH consider both males and females. While these policies are still developing, they have already generated great interest by the scientific community and triggered both criticism and applause. This review provides a description and interpretation of the NIH guidelines and it traces the history that led to their implementation. As expected, this NIH initiative generated some anxiety in the scientific community. The use of female animals in the investigation of basic mechanisms is perceived to increase variability in the results, and the use of both sexes has been claimed to slow the pace of scientific discoveries and to increase the cost at a time characterized by declining research support.

Purpose—This review discusses issues related to the study of sex as a biological variable in alcohol studies and provides examples of how researchers have successfully addressed some of them. A practical strategy is provided to include both sexes in biomedical research while maintaining control of the research direction.

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Conclusion—The inclusion of sex as an important biological variable in experimental design, analysis and reporting of preclinical alcohol research is likely to lead to a better understanding of alcohol pharmacology and the development of alcohol use disorder, may promote drug discovery for new pharmacotherapies by increasing scientific rigor, and may provide clinical benefit to women's health. This review aims to promote the understanding of the NIH sex as biological variable guidelines and to provide alcohol researchers with a theoretical and practical framework for working with both sexes in preclinical research.

Keywords

cell and animal studies; ethanol; gender; policy; preclinical research

1. The National Institute of Health policy on consideration of sex as a biological variable

In 2014, National Institute of Health (NIH) leaders raised concerns regarding an over-reliance on male animals in preclinical research—particularly for diseases occurring more frequently in women and for diseases that manifest differently in men and women (Clayton and Collins, 2014). They noted that such biases could mislead future clinical studies and ultimately, clinical practice and argued that NIH needed to promote balanced representation of both sexes in preclinical research. Plans for future policies drew feedback from the scientific community supporting the view that consideration of sex as a biological variable (SABV) could potentially influence the reproducibility, rigor, and generalizability of research findings in biomedical research. Perhaps not surprisingly, the scientific community favored giving scientists the discretion to decide when and how to address SABV, such that consideration of SABV would be evaluated on a case-by-case basis in grant applications and peer-reviewed publications (see http://orwh.od.nih.gov/about/director/pdf/RFIFinalReport20150520.pdf for full analysis of public comments).

NIH expects that SABV will be factored into research designs, analyses and reporting in vertebrate animal and human studies to the fullest extent possible. When proposing to study only one sex, investigators will be expected to justify their decision from the scientific literature, preliminary data, or other relevant considerations. Clearly, single sex studies are appropriate for the study of sex-specific conditions or phenomena such as maternal behavior or ovarian or prostate cancer. In addition, there are research topics—such as aggression in males—for which single sex studies can be scientifically appropriate. Resource scarcity and expense may also legitimately limit the ability to study both sexes, as in the case of non-human primate research.

The result is that exclusive use of male subjects as a default will no longer be acceptable in NIH-supported preclinical research. That said, NIH does not intend to require that *every* NIH-supported preclinical study include equivalent numbers of males and females in every experiment. Moreover, the anticipated policy also does not stipulate that sex differences be the focus of every study. Rather, NIH will require that investigators address the possibility that sex could influence the study's primary dependent variables in the study design and reporting. The NIH does not provide specific examples of when both sexes should be tested

and when a single sex is sufficient, as this could curtail the process of thinking through how SABV may affect the research hypothesis under consideration. Nevertheless, it is clear that the research topic will influence how SABV is likely to be considered in alcohol research. For example, in preclinical pharmacotherapy studies and other translational studies, it will be important to determine whether the experimental findings apply to both sexes. For other areas, simply including both sexes in the study will be sufficient to permit the discovery of any dramatic, unanticipated sex influences that could then be further pursued in studies adequately designed to characterize that apparent sex difference. At the same time, note that some sex differences that emerge may not be meaningful or interesting. For example, sex-dependent body-size differences could affect activity in behavioral testing apparatuses without influencing the main variables of interest; in such cases, it would be sufficient to simply note the observation. Of course, there are many areas where sex is already recognized as a crucial influence such that many alcohol researchers routinely study both sexes. These examples illustrate the need for a nuanced approach that takes into consideration the practices and priorities within the various research domains supported by NIH to ensure the appropriate consideration of SABV.

How will NIH's expectations be fulfilled? NIH will clarify and revise grant application instructions and review criteria to enhance reproducibility of research findings through increased scientific rigor and transparency. Consideration of sex and other relevant biological variables is among the new focuses of the revised review criterion instructions. Thus, grant applicants will be asked to explain how relevant biological variables, such as sex, are factored into research designs and analyses in the Research Strategies section. Consideration of SABV will be evaluated in the context of the overall research plan. In this way, scientists with detailed understanding of their respective fields will be in the best position to both formulate appropriate scenarios for accounting for SABV and to critically evaluate whether others are doing so sufficiently. The policies to be implemented in January 2016 are discussed further in the following NIH Guide Notices (http://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-102.html; http://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-103.html).

Understandably, introducing new variables into the peer-review mix can provoke anxiety. Yet, in light of the potentially important sex influences in the diverse landscape of biological and behavioral research supported by the NIH, relevant subject matter experts are best qualified to evaluate whether SABV considerations are appropriate in the context of overall research goals. To facilitate this approach, the NIH Office of Research on Women's Health (ORWH) is providing guidance to the scientific community (see http://orwh.od.nih.gov/sexinscience/index.asp) and the NIH Center for Scientific Review is providing training to scientific review officers and study section chairs. The 2015 Research Society on Alcoholism round table discussion (on which this critical review was based) also contributed to these goals as applied to alcohol-related research, as will future discussions on this topic.

2. Historical research perspective leading to the NIH policy

a. Historical perspective on clinical studies

The landmark clinical studies of the 1950–1980, such as the Physicians Health Study (1988b, 1988a, 1989), Multiple Risk Factor Intervention Trial (MRFIT) (1982), and the first 20 years of the Baltimore Longitudinal Study of Aging all excluded women. Ironically, the first-ever clinical evaluation of estrogen treatment for cardiovascular disease was in a study that only enrolled males (Canner et al., 1986). The reasons for excluding women were varied, ranging from evidence-based (such that at the time coronary disease was seen mostly in men), or the overcautious (as in women of reproductive age should be excluded due to unanticipated effects on future or current pregnancies), to the downright quirky (as in restroom facilities were not available for women on the premises).

Recognition of sex-dependent differences in drug responses led to NIH actively seeking to ensure that studies took into consideration sex and gender differences in design and analysis leading to the NIH Revitalization Act, signed into law in 1993. This bill required that women and minorities be included in appropriate numbers in NIH-supported research and that NIH clinical trials be designed to identify differences in research outcomes between women and men. This bill was motivated by the recognition that although NIH's internal guidelines urged that women be included in clinical trials, this policy was not adequately and systematically implemented during the grant review process. To address the disparity in sex representation in NIH funded research, the NIH ORWH was established. Over the span of the following two decades much progress has been made. Currently, approximately half of all participants in NIH-supported clinical research are female subjects. In 2003 (10 years following the NIH Revitalization Act), enrollment of women in Phase III clinical trials, excluding studies that are female only or male only, was more than 50% and more recently is reported to be 62.1% (FY2012).

However, there continues to be concern about the low inclusion of women in diseases that were traditionally thought to be male-centric, such as cardiovascular disease. Recent studies show that by age 40, cardiovascular disease prevalence is fairly equal among males and females and by 60, more women than men are affected (reviewed in (Kim and Menon, 2009). In contrast, an analysis of female enrollment in National Heart, Lung, and Blood Institute funded studies from 1996 to 2006 showed an average of 27% women enrollment (Kim et al., 2008). Why the discrepancy? Unfortunately, an overwhelming majority of drug trials for cardiovascular disease are performed by pharmaceutical industries, which is regulated ultimately by the Food and Drug Administration (FDA), where an old guideline specifically excluded women of reproductive age in such studies (Merkatz, 1998). A 2000 amendment to the Clinical Hold policy, however, now permits FDA to stop investigational new drugs studies for treatment of a serious or life-threatening disease if women or men are excluded due to reproductive potential (Clinical Hold for Products intended for life threatening conditions; 21 CFR 312.42(b)v). Nevertheless, poor inclusion of women in these studies is made worse by the fact that few studies use sex-based analyses (Geller et al., 2011), so that all data are analyzed together instead of being disaggregated for sex/gender.

Inclusion of female subjects in clinical research has led to better appreciation of the differences in responses to drugs between males and females. Sex differences may underlie adverse drug reactions affecting the heart, in particular cardiotoxicity and drug-induced long QT syndrome; the latter consists in a delayed repolarization of the heart after contraction and is associated with greater incidence of ventricular arrhythmias leading to cardiac arrest or sudden death (Hreiche et al., 2008). The role of sex hormones was suggested by the greater susceptibility to these adverse drug reactions during the ovulatory phase of the menstrual cycle (Rodriguez et al., 2001). Additional sex-related differences in responses to anesthetics and chemotherapeutic agents have also been identified. Female hormones have been reported to modulate opioid receptor density and dopaminergic function, with consequent impacts on respiratory depression and chronic pain (Nicolson et al., 2010). Sex hormones can modulate the γ-aminobutyric acid (GABA) receptor, a common site of anesthetic drugs, leading to sexually dimorphic effects of anesthetics (Frye and Duncan, 1994). Clinical studies show that differential preventive effects are achieved with low-dose aspirin in women and men (Adelman et al., 2011). Similarly, Zolpidem, a drug used to treat insomnia, requires different dosing in women and men (Greenblatt et al., 2014).

b. The need for the inclusion of Sex as Biological Variable in preclinical studies' guidelines

The recent (June 2015) announcement of the NIH Guide on the inclusion of SABV (see https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-102.html) in biomedical research comes more than 20 years after the NIH Revitalization Act of 1993. In view of the efforts already underway, is the new NIH guideline on SABV for basic science studies necessary? Data gathered by the NIH ORWH indicates that female inclusion lags in basic science studies even as it has improved in clinical studies. The upshot is that the inclusion of both sexes may occur for the first time when a drug or procedure goes to clinical trial.

Preclinical research studies continue to rely heavily on male animals and/or omit reporting of the sex of animal subjects. The problem with this is that preclinical or cell-based studies are meant to precede or inform clinical trial design. Without adequate understanding of sex-specific differences in disease processes or therapeutic responses, the generalizability of research findings is limited. Inclusion of both sexes in preclinical research is anticipated to improve data reproducibility and to identify potential differences in drug responses between males and females at earlier steps in translation.

Nearly all common diseases exhibit some degree of sex bias, whether male preponderant or female. In the case of neurologic diseases, multiple sclerosis is 3 times more likely to occur in females than males, while Parkinson's disease is 2 times more likely to occur in males as compared to females. While overt sex bias in disease incidence is well known, the extent of sex bias in basic biological processes is only now beginning to be appreciated. Microarray analysis of 23,574 mouse gene transcripts revealed that the extent of sexual dimorphism in gene expression was much greater than previously recognized. The majority of active genes were sexually dimorphic in at least three tissues: liver (87%), adipose (88%), and muscle (66%) (Yang et al., 2006). The link between adiposity and chronic disease such as hypertension, cancer and diabetes is well established in men, but this connection is less well understood in women. Furthermore, fat deposition in women differs from men in the

premenopausal years but resembles that of men in the postmenopausal years (Palmer and Clegg, 2015), revealing a complex age-by-sex variation for this tissue. Curiously, the proportion of sexually dimorphic active gene expression in the brain was low compared to other tissues (14.6%) (Yang et al., 2006), despite the sex bias sometimes seen in brain function, neuroanatomy and the incidence of neurologic diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and stroke.

As recently as 5 years ago, an analysis of 2000+ biomedical studies published in 2009 showed a male bias in 80% of the studies (Beery and Zucker, 2011). As expected there was variation based on disciplines so that the most male-biased fields were neuroscience (5.5:1, male: female) while a female bias occurred in reproduction-related journals. The ratio of male to female inclusion in studies was also greater for females in immunology-related journals; however this was offset by the fact that over 60% of the papers in this area simply did not report which sex was studied. At the present time the majority of journals do not insist on explicit information on which sex is used for cell or animal studies, resulting in a situation where single sex studies on males predominate. Even in dual sex studies, many studies are not disaggregated for sex, a problem endemic to both clinical and preclinical studies.

3. Myths and realities about the use of both sexes in preclinical research and suggested courses of action on how to balance sex in cell and animal studies

While the lack of equal usage of males and females in experimental studies is not disputed, the reasons for this are complicated. A recent paper (McCarthy, 2015) summarizes the many concerns biomedical researchers express regarding the use of both sexes, such as increasing the cost of the study, quadrupling animal numbers due to female estrous cycles necessitating a group for every phase of the cycle, or distracting from other important variables such as age. The issue of cost is certainly critical, but even without the inclusion of both sexes; investigators are making critical decisions about cost containment. Ultimately, these and related cost issues will and should drive the discussion for increased NIH budgets, both overall and at the level of the individual grant. For instance, the current modular grant may need to be revised to cope with increased experimental demands.

Another element of resistance to the incorporation of both sexes in preclinical studies is the notion that this somehow distracts from the main goal of the research program, making the research a "study of sex differences" as opposed to a "study of the disease." One problem with this mindset is that SABV is often a key factor in the manifestation or progression of a disease. Essentially, this resistance requires a shift in attitude from considering the use of both sexes as a burdensome addition to one where inclusion of both sexes is simply good science. Inclusion of both sexes should be treated within the purview of experimental design – an integral part of the research study, as necessary as a control group or a drug dosage group or an age group. Hence, at its root, investigators need to be convinced that the SABV guidelines are meant to increase rigor in science, to add robustness to experimental design and, ultimately, to better inform clinical trials that are based on these studies.

One commonly held misconception that has contributed to the exclusion of female animals in research studies is that results in females are much more variable, in part due to hormonal fluctuations across the estrous or menstrual cycle (Cahill, 2006, Prendergast et al., 2014). Because daily tracking of vaginal cytology across the 4–5 day estrous cycle in rodents was viewed as time consuming and expensive, particularly if females were tested at each of the four stages of the estrous cycle, females were often excluded from research studies. However, the results from two different meta-analyses, one of pain-related traits (Mogil and Chanda, 2005) and one of behavioral, morphological, physiological, and molecular traits (Prendergast et al., 2014), provide strong evidence that variability in females is not significantly greater than males for any endpoint that was examined. A recent preliminary analysis of published neuroscience literature suggests that female rats are also not inherently more variable than male rats on a range of neuroscience-related outcomes (Becker et al., 2015). An interesting finding from the Prendergast meta-analysis is that group housing compared to individual housing significantly increased variability in both male and female mice by 37%, suggesting that housing conditions (i.e., group size within a cage) contribute greater variation across studies than stage of estrous cycle (Prendergast et al., 2014).

Collectively, these results indicate that the initial examination of sex differences for a particular phenotype in neuroscience research does not require monitoring of the estrous cycle in female rodents. Strategies for assessing the role of estrous cycle stage have been published (McCarthy et al., 2012, Becker et al., 2005), but the initial recommendation is to measure adult females independent of estrous cycle stage. It also is important to search the existing literature – are there reports that phenotype female subjects to determine whether the genotype and species to be tested may exhibit estrous or menstrual cycle differences in the trait of interest? While the lack of published studies may just represent the paucity of data available on this topic, if published phenotyping of female subjects does not reveal estrous or menstrual cycle differences or such studies are not available, then a reasonable first approach is to initially ignore the estrous/menstrual cycle and determine whether there is a basic sex difference in one male group versus one female group (McCarthy et al., 2012).

We have outlined this practical approach in Figure 1, with the first step to obtain results from males and females. When the initial assessment of sex difference reveals that variability in females is greater than in males, or if existing data indicate that a particular trait of interest is known to vary as a function of the estrous or menstrual cycle, the investigator is faced with the decision of whether or not to mechanistically pursue these differences, which will likely require additional funding and will make sex differences a major area of research for the laboratory. It is important to emphasize that researchers retain the power to decide whether to investigate sex differences and/or estrous cycle-dependent changes in females, as indicated by the diamond shapes in the flow-chart shown in Figure 1. If investigators, after identifying a sexually dimorphic response, decide to continue their research in only one sex, *this decision should be rationalized in the context of their scientific findings*. For instance, if different behaviors are affected by alcohol in males and females, the decision to focus future studies only on one sex should be based on a clearly stated rationale about why the investigator is interested in that specific behavior; therefore, the choice is about the scientific question to be pursued, not about whether to use male or female animals. Indeed, this approach is more “scientific” and thoughtful than the more traditional approach to use male

animals as the “default” animal models. Furthermore, as sexually dimorphic effects are published, other laboratories have the opportunity to follow up on these newly identified, but not pursued, sex differences.

If the investigator decides to explore sex differences identified during her/his studies, comparison between one group of intact males and a minimum of two groups of intact females at known estrous cycle stages is a recommended option (Becker et al., 2005, Beery and Zucker, 2011, McCarthy et al., 2012). Available publications provide useful and detailed descriptions on the determination of estrous cycle phase in the rodent (Becker et al., 2005) and of the vaginal cytology of the rat and mouse estrous cycle (Cora et al., 2015). An alternate strategy would be to do vaginal smears to assess estrous cycle stage or to take a blood sample to asses estradiol levels on the day that a specific measure (behavioral, electrophysiological, etc) is taken or at the termination of the study when brains and other tissues are collected, and then use estrous cycle stage or estradiol levels as a covariate in the analysis.

When comparing males and females, it is important to consider the biology and age of the subject as well as the housing environment during development, time of day for testing, and the appropriate measurement of the trait of interest, because it is incorrect to assume that any observed sex difference will generalize to other ages or conditions (Becker et al., 2005). Once a sex difference has been identified, subsequent studies can be conducted to identify the factors that contribute to the trait difference in males and females (Becker et al., 2005, McCarthy et al., 2012). Considering that a majority of sex differences are identified in adulthood, these series of experiments would allow the investigator to determine whether the sex difference is determined by steroid hormones in adulthood, whether it is the consequence of developmental exposure to steroid hormones, or whether it is due to a direct sex chromosome effect. A straightforward way to begin is to determine if a sex difference is eliminated by gonadectomy and restored by steroid hormone replacement. Alternately, mechanistic studies that are focused on neurotransmitters or neuropeptides and downstream signaling molecules can be conducted to pursue the sex difference, but interpretation of the results will likely need to consider the modulatory influence of steroid hormones and actions at nuclear or membrane steroid receptors.

Another misconception is that studies utilizing primary cultures do not need to consider the sex of the animal. However, every neuron, glia, and other cell type carries the complement of male chromosomes (XY) or female chromosomes (XX), and there are hormone-independent mechanisms for sex differences that are under the control of the sex chromosome complement (Becker et al., 2005, Jazin and Cahill, 2010, McCarthy, 2015, McCarthy et al., 2012).

As a result, the NIH SABV guidelines should be interpreted as a recommendation that studies utilizing primary cultures should plate separately cells derived from male and female tissue. One approach is to plate cells from individual rodent pups; later, the sex of the individual pups used to prepare the cultures can be confirmed with PCR by genotyping for male-specific sex-determining region Y (*Sry*) (McClive and Sinclair, 2001). Another approach is to separate beforehand male and female pups or fetuses based on genital papilla

size/anogenital distance (Greenham and Greenham, 1977, McCarthy et al., 2012). Veterinarians attending animal facilities at any given institution can be asked to train laboratory personnel on this simple task. The reliability of this method is very high in adequately trained people; this method can be validated by PCR analysis of Sry.

A possible course of action for *in vitro* studies is to test the initial hypothesis in sex-specific cultures; if different effects are found, the investigator should make the decision of whether to carry out more in-depth mechanistic studies in cultures derived from one sex or whether to study both sexes and unravel the sex difference mechanism. This decision should be based on scientific findings, questions, and interests. If no sex differences are found, the investigator can easily justify the use of mixed-sex cultures in future studies.

The importance of examining sex differences with primary cultures is highlighted by the recent findings that female (but not male) mice exhibit astrocyte reactivity following chronic ethanol exposure and withdrawal *in vivo* and in *in vitro* sex-specific primary astrocyte cultures (Wilhelm et al., 2015). Thus, use of sex-specific primary cultures has the potential to increase the translational value of *in vitro* findings and to facilitate the comparison with *in vivo* work.

4. Examples of sex differences in alcohol research

There are many examples of sex differences in clinical and preclinical alcohol research as alcohol exposure throughout life has been shown to have sexually dimorphic effects. Discussion of all these studies is beyond the focus of this article, but excellent reviews have been published on sex differences on the developing brain in prenatal (Weinberg et al., 2008) and adolescent (Kuhn, 2015, Spear, 2015) models of ethanol exposure as well as on every stage of alcohol addiction in adult animals (Becker and Koob, 2016). While we refer readers to those more comprehensive reviews on the topic of sex differences in alcohol responses, we describe here one mechanistic example of how alcohol may affect differently males and females at the molecular level with the goal to emphasize that the assumption that research carried out in one sex can be generalized to both sexes is unwarranted.

Research in rodent models document that females consume higher doses of ethanol than males in a variety of ethanol access conditions (Cozzoli et al., 2014b, Finn et al., 2004, Juarez and Barrios de Tomasi, 1999, Middaugh and Kelley, 1999, Middaugh et al., 1999, Sinnott et al., 2002, Yoneyama et al., 2008, Lancaster and Spiegel, 1992), but this is partially due to an effect of testosterone to reduce intake (Vetter-O'Hagen et al., 2011) and there is minimal evidence for an influence of estrous cycle phase on ethanol intake or self-administration. In particular, lower ethanol consumption was observed during proestrus in early work in female rats (Forger and Morin, 1982), but this finding was not replicated in later studies in freely cycling female rats (Ford et al., 2002, Roberts et al., 1998, van Haaren and Anderson, 1994) and was only observed in females whose cycles had been synchronized (in this case, the lower ethanol self-administration was observed primarily in estrus and proestrus) (Roberts et al., 1998) or when microanalysis of ethanol intake patterns were examined (with higher bout frequency but lower bout size in proestrus) (Ford et al., 2002). Consequently, sex differences in ethanol consumption are typically examined without

monitoring estrous cycle stage, with similar variance in results for both male and female rodents.

Data from the Finn laboratory illustrate a potential mechanism underlying sex differences in the regulation of ethanol drinking involving differences in metabotropic glutamate receptor 5 (mGlu5) signaling. This study examined the biological implication of sexually divergent changes in the effect of repeated binge drinking on signaling molecules downstream of mGlu5 in adult male and female C57BL/6J mice (Figure 2A). Initial studies determined that the mGlu5 antagonist MTEP [3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine] was equally effective to decrease binge ethanol intake in male and female mice (Figure 2B), but that later ethanol intake after a period of abstinence was increased in females and unaltered in males (Cozzoli et al., 2014a). Thus, it is possible that male and female mice have similar sensitivity to mGlu5 antagonists at the receptor level while the signaling downstream of mGlu5 might differ between the sexes. Based on evidence that glutamatergic signaling molecules in the nucleus accumbens are sensitive to the neuroadaptive properties of ethanol (Ary et al., 2012, Besheer et al., 2010, Cozzoli et al., 2012, Cozzoli et al., 2009, Goulding et al., 2011, Marty and Spigelman, 2012, Neasta et al., 2010, Neasta et al., 2011), the next studies determined whether there were sex differences in the effect of repeated binge ethanol consumption on protein levels of signaling molecules in the phosphoinositide 3-kinase (PI3K) signaling cascade (Figure 2A). Notably, 24-hour withdrawal from repeated binge ethanol consumption significantly altered the phosphorylation of PI3K, mammalian target of rapamycin (mTOR), 4E-binding protein 1 and p70 ribosomal protein S6 kinase in the accumbens of male but not female mice (Cozzoli et al., 2016). To study the functional ramifications of the observed sex-specific alteration, mTOR was targeted because repeated binge drinking produced a divergent response in protein levels. Bilateral infusion of the mTOR inhibitor rapamycin into the accumbens established that mTOR inhibition did not alter binge ethanol intake in females, whereas it produced a dose-dependent and selective reduction in binge ethanol intake in males (Figure 2C) (Cozzoli et al., 2016). Together, the results highlight that mTOR signaling in the accumbens is necessary to maintain binge ethanol consumption only in male mice. Because mTOR complex 1 is proposed to be a common point of neuronal signaling that is important for drug-induced plasticity (Neasta et al., 2014), the resistance of female mice to the ability of rapamycin to decrease binge drinking emphasizes the sex specificity of potential pharmacotherapies for alcohol use disorders.

4. Balancing sex in animal and cell studies in drug development

The percentage of drugs that fail clinical trials is extremely high, approaching 90%, according to a recent study (Hay et al., 2014). As a logical approach aimed at reducing the enormous cost associated with failed clinical trials, the NIH intends to increase the rigor and reproducibility of preclinical studies by introducing the SABV guidelines and related policies. Rodent and non-rodent mammalian models are used to delineate the pharmacokinetic profile and general safety of potential drugs, as well as to identify toxicity patterns of drugs during drug development. Animal models are used to determine the drug's half-life, which depends on rate of absorption, volume of distribution, metabolism, and excretion properties – all of which can be influenced by the sex of the animal. Evidence that sex differences in these parameters exist in animal models suggests that accounting for

SABV in physiological responses will lead to better experimental study design. Particularly in translational studies leading to clinical trials, important factors that contribute to variability in pharmacokinetics include sex-dependent differences in body weight, plasma volume, gastric emptying time, plasma protein levels, enzymatic activity, drug transporter function, and excretion activity. Several of these differences are present in rodent models. For example, gastric alcohol dehydrogenase activity is higher in male than in female rats (Aasmoe and Aarbakke, 1999), which would impact blood ethanol concentrations after oral administration. Moreover, cytochrome P450 levels (i.e., CYP3A9 and CYP4Fs) show higher mRNA and protein expression in livers of females versus male rats (Aasmoe and Aarbakke, 1999, Kalsotra et al., 2002), which may also impact ethanol metabolism. Other factors involved in drug metabolism are less well understood from the perspective of sex differences. Para-aminohippuric acid (PAH), a reference substance used to estimate renal organic anion transport, has a lower rate of clearance and excretion rate in female rats compared to male rats (Cerrutti et al., 2002b, Cerrutti et al., 2002a), which would be relevant in establishing dosing and frequency of drugs using this mechanism of excretion.

Drug development for alcohol use disorder is relatively unexplored compared to other diseases such as cancer, cardiovascular and psychiatric disorders (Litten et al., 2012). One barrier is the lack of information of how SABV may impact the molecular target(s) by which alcohol exerts its pharmacologic activity. However, in addition to efficacy, one needs to consider sex differences in toxicology when developing a new pharmacotherapy for alcohol use disorder. For example, the half-life of perfluorooctanoic acid, which is excreted in the kidney by an organic anion transporter, is 70 times longer in male than in female rats and this effect is modified by changes in sex hormone levels (Vahter et al., 2007). Another example of a classically direct sex hormone effect on P450 is CYP2J5 in the mouse kidney, which is up-regulated by testosterone and down-regulated by estrogen (Vahter et al., 2007). Changes in clearance and metabolism will alter the response between the sexes, particularly in the exaggerated doses given in toxicological studies.

5. Concluding Remarks

Starting on January 2016, investigators are required by NIH to consider SABV in research designs, analyses, and reporting in preclinical studies or to provide a solid rationale (based on scientific considerations or other relevant factors) supporting the decision to study only one sex. The inclusion of sex in the biological variables that are accounted for in preclinical studies is expected to increase rigor and reproducibility in the preclinical findings and to facilitate the successful translation of these findings into the clinic. Based on the sex differences already known in alcohol consumption, metabolism and pharmacology, it is likely that inclusion of SABV in preclinical experimental design can help to advance pharmacotherapeutic development for alcohol use disorder. Many concerns associated with the use of both sexes in animal research involve resource management. Figure 1 depicts a suggested flowchart that may be useful when designing experiments in accordance with the SABV guidelines and intends to emphasize how researchers retain the power to decide whether to investigate sex differences and/or estrous cycle-dependent changes in females.

The SABV guidelines can be interpreted as intending to challenge researchers to rethink the "default" models mostly used in preclinical research, which are male animals and mixed-sex primary cultures, and to prevent unwarranted generalizations of results obtained in only one sex. In this context, it is apparent how these guidelines have the potential to increase the rigor of preclinical results that may lead to an increased number of drugs going successfully through clinical trials.

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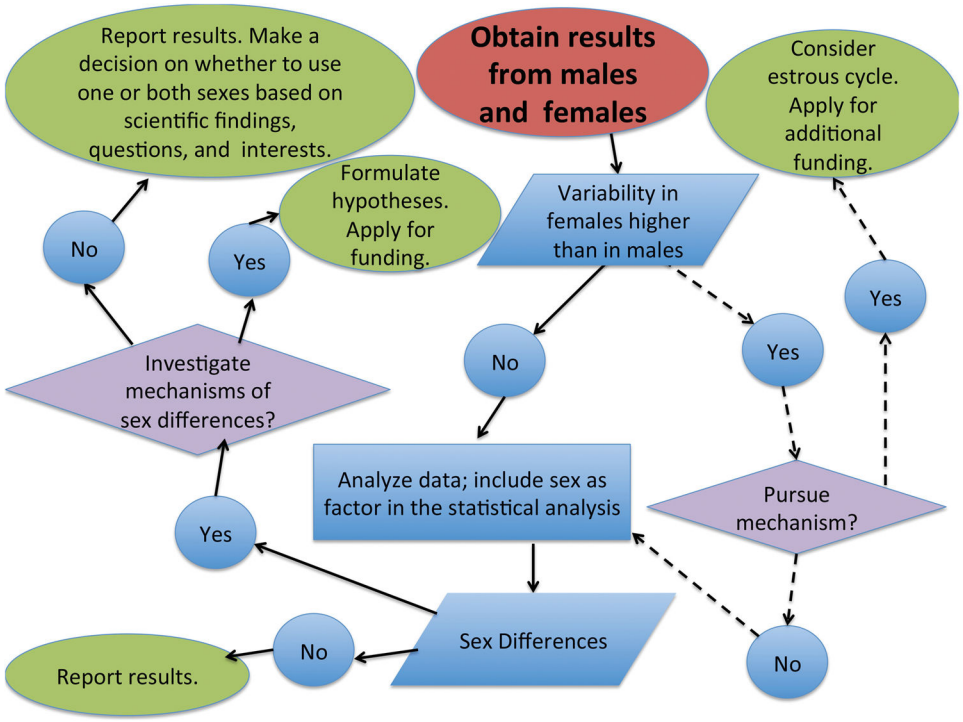

Figure 1.

Flow-chart of a potential course of action when designing experiments that use animals of both sexes. A major misconception that has contributed to the exclusion of female animals in research studies is that results in females are much more variable and that tracking of vaginal cytology across the estrous cycle is necessary to reduce this variability. Results from meta-analyses indicated that variability in females on most biological measurements is not significantly greater than males (Becker et al., 2015; Mogil and Chanda, 2005; Prendergast et al., 2014). Researchers that work with both sexes advise to initially ignore the estrous/menstrual cycle and to first determine whether there is a basic sex difference in one male group versus one female group (McCarthy et al., 2012). This flow-chart shows how research can be developed after collecting data from males and females on any given end-point of interest. Ovals represent the start (red) or the end (green) points of the chart; the parallelograms represent outputs; the rectangle represents a process (action); and the diamonds indicate decisions to be made. After obtaining results from males and females (in which estrous cycle has not been monitored), the results are analyzed to determine whether females are more variable than males. More likely, females and males will present similar variability; at this point, data will be analyzed to identify whether there is a sex difference in the response. If no sex differences are found, data are reported. If sex differences are identified, the investigator is presented with the decision (left diamond) of whether to pursue them, in which case she/he will formulate new hypotheses and apply for new funding, or whether to simply report them and pursue other research. In the less-likely event that data from females present a much higher variability than data from males, the investigator is presented with the decision (right diamond) of whether to pursue the biological mechanism behind this variability, consider the estrous cycle and hormonal fluctuations and apply for new funding for this research, or whether to not pursue this finding and analyze and report

the data obtained from males and females as described before. This figure intends to emphasize how the researcher retains the power to decide whether to investigate sex differences and/or estrous cycle-dependent changes in females. After analyzing the results for sex differences and reporting them, the investigator has the choice of using one or both sexes for future studies by providing a convincing rationale based on scientific findings, questions, and interests. In this contest, the NIH SABV guidelines discourage the use of a given sex as the “default” model to be used in research.

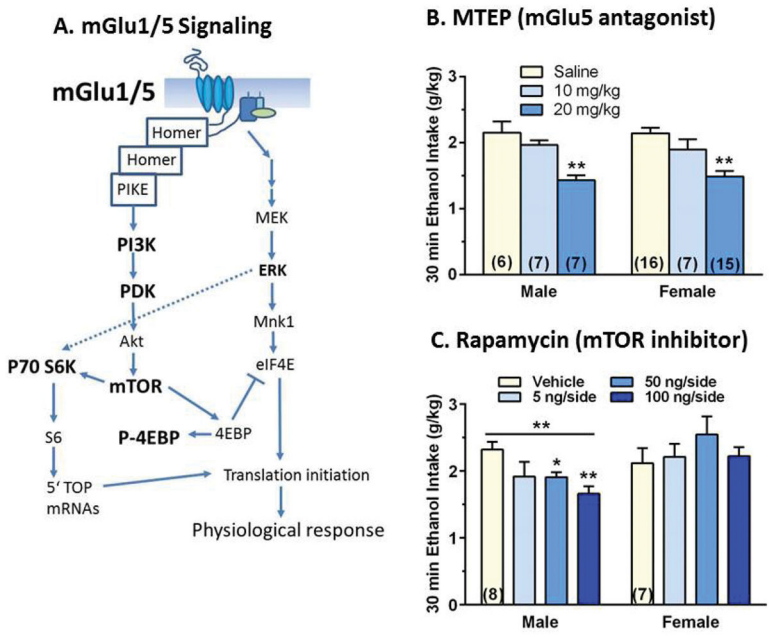

Figure 2.

Pharmacological manipulation of binge drinking reveals that male and female mice have similar sensitivity to a metabotropic glutamate receptor 5 (mGlu5) antagonist at the receptor level (B) but different sensitivity to rapamycin (C) an inhibitor of mTOR, consistent with the sexually divergent changes in phosphoinositide 3-kinase (PI3K) and downstream signaling molecules following repeated binge drinking (A). **Panel A:** Depicted is a simplified diagram of mGlu1/5 intracellular signaling. Downstream signaling molecules that differ by sex and influence alcohol binge drinking are highlighted in bold font. Notably, 24-hour withdrawal from repeated binge drinking significantly altered the phosphorylation of PI3K, mTOR, 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (P70 S6K) and tended to alter phosphoinositide-dependent protein kinase 1 (PDK) in the nucleus accumbens of male but not female mice (results in Cozzoli et al., 2016). **Panel B:** Binge ethanol consumption, averaged across seven 30-min sessions, following intraperitoneal injection of saline or different doses of MTEP. The efficacy of MTEP to decrease binge drinking was similar in the male and female mice, as the 20 mg/kg MTEP dose significantly decreased binge ethanol intake by 33% or 31% in the male and female mice, respectively. Values are the mean ± SEM for the number of animals on the figure. **p<0.01 versus respective saline group; adapted from (Cozzoli et al., 2014a). **Panel C:** Intra-accumbens rapamycin dose-dependently decreased binge ethanol intake during a 30-min binge session in male but not female mice, with a 28% decrease in binge ethanol intake following the 100 ng/side dose in male mice. Mice received bilateral infusions of vehicle or rapamycin prior to a binge ethanol session in a within-subjects design. Values are the mean ± SEM for the number of animals on the figure. *p<0.05, **p<0.01 versus vehicle infusion; line represents significant ANOVA; adapted from (Cozzoli et al., 2016).