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PERSPECTIVE

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Considering sex as a biological variable will require a global shift in science culture

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For over half a century, male rodents have been the default model organism in preclinical neuroscience research, a convention that has likely contributed to higher rates of misdiagnosis and adverse side effects from drug treatment in women. Studying both sexes could help to rectify these public health problems, but incentive structures in publishing and career advancement deter many researchers from doing so. Moreover, funding agency directives to include male and female animals and human participants in grant proposals lack mechanisms to hold recipients accountable. In this Perspective, we highlight areas of behavioral, cellular and systems neuroscience in which fundamental sex differences have been identified, demonstrating that truly rigorous science must include males and females. We call for a cultural and structural change in how we conduct research and evaluate scientific progress, realigning our professional reward systems and experimental standards to produce a more equitable, representative and therefore translational body of knowledge.

The last decade has seen increased public awareness that women are vastly more likely than men to be misdiagnosed in a wide array of medical conditions. One of the most well-known examples is cardiac arrest: men and women experience distinct sets of symptoms, but the focus on men's symptoms as 'textbook' diagnostic criteria has led to delayed treatment for women, with sometimes fatal consequences[1]. This flawed view—that the way a disease presents in men is the standard by which all cases are determined—is pervasive across many areas of public health, including brain health. Afflictions from stroke[2] to attention deficit/hyperactivity disorder (ADHD)[3] are vastly under- or misdiagnosed in women and girls, usually because their symptom profiles lack one of the male-derived criteria. And like heart attack misdiagnoses, these failures to accurately identify psychiatric and neurological conditions in women have had devastating and long-lasting after-effects, including permanent disability, depression and suicide. Compounding the problem is evidence that, even when accurately diagnosed, women experience a greater frequency and number of negative side effects from pharmacological treatments than men do[4]—again, as a result of men's outcomes being the default measuring stick.

Our incomplete understanding of the etiology, symptomatology and treatment of mental and neurological disease in women is due in large part to the neglect of female subjects in preclinical neuroscience research. A now-landmark 2011 evaluation of biomedical publications found that neuroscience studies used male animals six times more often than they used females[5]. A more recent analysis of papers published in 2017 sadly suggests that this imbalance has only barely begun to improve[6,7], despite the more widespread recognition of the disparities in women's health mentioned above. Following similar policies by the Canadian Institutes of Health Research and the European Commission, the US National Institutes of Health (NIH) introduced the Considering Sex as a Biological Variable (SABV) mandate in 2016, as part of a broader initiative to improve the rigor and reproducibility of research funded by the NIH. The policy states that grant applications must include both male and female subjects and/or cells in experimental design and analysis, with primary objectives of broadening the general knowledge base and delivering a more refined understanding of how, and in whom, basic science findings will best translate into clinical applications[8]. If any of these funding agencies hope to improve personalized medicine through the research they support, then understanding the influence of biological sex on the data we collect is a necessary and fundamental step toward achieving this goal.

We are currently four years into the implementation of NIH's SABV policy, or about three years into active funding of NIH grants that 'adhered' to the mandate in their proposals. It is clearly too early to determine whether SABV has been a sweeping success, but it is not too early to ask whether we are taking actions to ensure that it will be. Early surveys of NIH study section members suggest that reviewers generally support the initiative and agree that the number of proposals that satisfactorily address SABV is increasing[9]. This is a promising start, but much more is required to guarantee that the initiative goes beyond lip service paid by grant writers. In this Perspective, we discuss several areas of basic neuroscience in which careful consideration of SABV has led to critical discoveries of the ways in which fundamental neurobiological processes—from cell signaling to complex decision-making—differ in males and females. These findings highlight the broad need to study the brain in both sexes and call for large-scale, systemic change in the way neuroscience research is conducted. As we argue later in this piece, effecting these changes requires active participation, not just from grant reviewers, but also from good-faith efforts by funded researchers and commitments from both journals and funding agencies to hold them accountable.

Sex-dependent behavioral strategies

Behavioral neuroscientists quantify select aspects of an animal's physical activity to make inferences about its cognitive or psychological state. Standard behavioral paradigms and their associated metrics were developed to facilitate interpretation and allow cross-lab comparisons, but what has rarely been asked is whether the metrics themselves mean the same thing in both sexes. Most common behavioral tests were validated decades ago, when the exclusive use of male rodents was standard practice[10]. In the rare instances where

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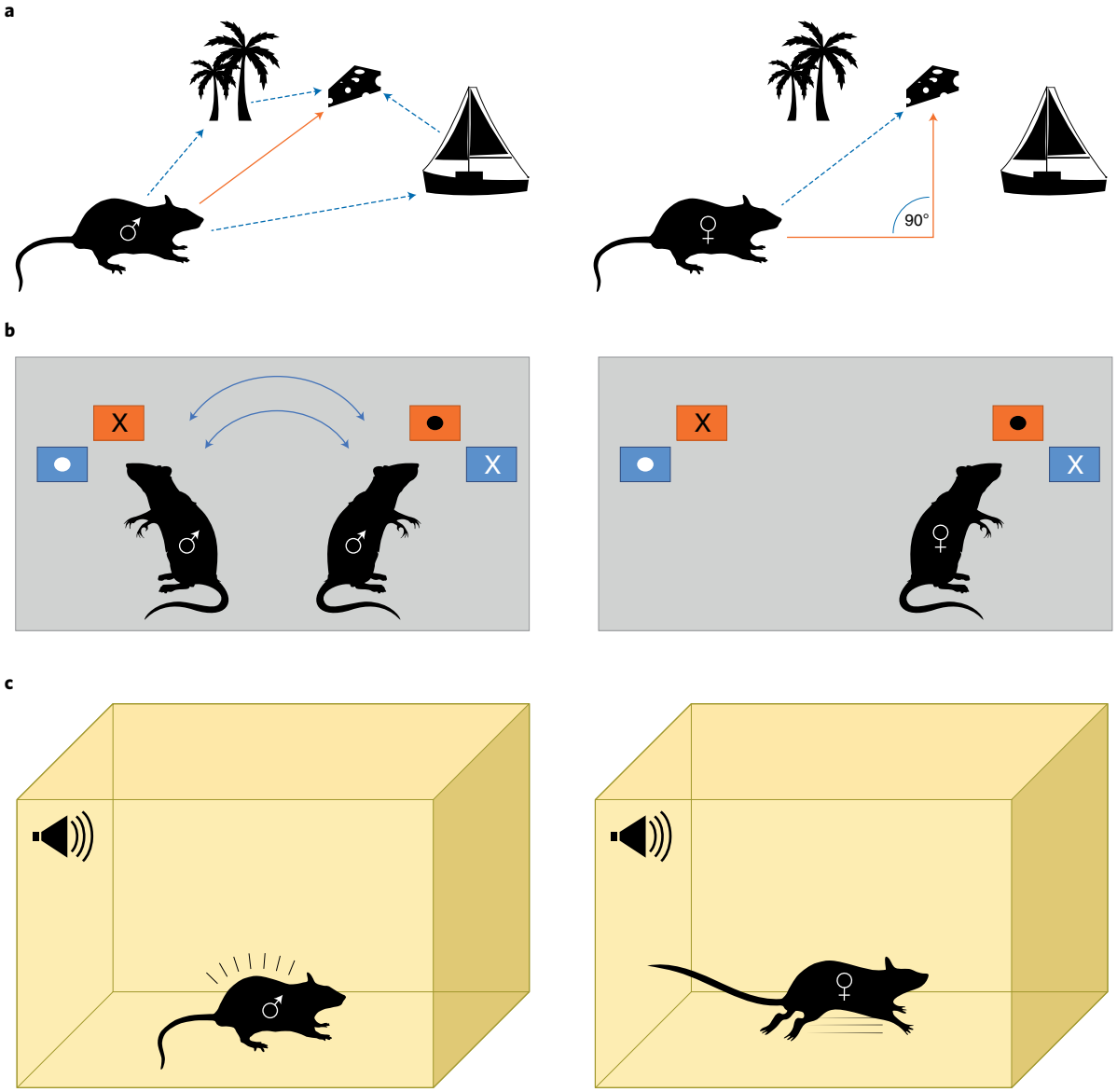

Fig. 1 | Sex-dependent behavioral strategies. a, Males and females use discrete strategies in spatial navigation tasks. While males navigate to a target by using nearby landmarks, females are more likely to use a strategy based on their own position in space. **b**, In a complex reward learning task, males' choices from trial to trial appear more random, as they sample all dimensions of the task. In contrast, females restrict the space in which they make their choices, allowing them to learn the task rules systematically. **c**, In classical Pavlovian fear conditioning paradigms, males predominantly express fear learning with a freezing response, while females are more likely to also exhibit an escape-like darting response.

experimenters included female subjects, assessments were based on the same metrics, asking simply whether the magnitude of a given outcome was different in males and females (that is, quantitatively different), not whether females might be exhibiting different outcomes altogether (that is, qualitative differences).

In several behavioral paradigms, what appear to be quantitative sex differences in learning, decision-making or emotion may actually reflect qualitatively different, sex-dependent strategies[11]. For example, several decades of research in spatial navigation paradigms—traditionally considered to be hippocampus dependent—have shown that, although both sexes ultimately perform the task at comparable levels, males generally learn to navigate more quickly than females[12,13]. However, later research showed that females switch in an estrous-dependent manner to a striatal-based strategy (Fig. 1a)[14]. This idiothetic (self-based) strategy can result in females traveling farther and thus having longer latencies to complete the task, a quantitative difference that was originally interpreted as females being spatially impaired in comparison to males[13]. Rather, females were using a qualitatively different strategy, one that, although more circuitous, minimized exposure to predators and other dangers. Subsequent studies further showed that sex differences in time to complete a water maze task were completely eliminated if the animals had prior exposure to the maze[15]. This example illustrates the need to reevaluate the biases inherent in our experimental designs regarding the 'right' way to solve problems and ask whether what appear to be errors in fact simply reflect the selection of a different strategy[16].

Sex differences in behavioral strategies can also be observed in non-spatial learning tasks. An excellent example comes from a recent paper by Chen et al.[17], who trained male and female mice on a multidimensional reward task. An initial assessment of learning speed suggested that the females had an advantage, but a subsequent

computational analysis revealed that the 'slower learning' in males was due to their making more random choices from trial to trial, indicating that they might be gathering information about multiple dimensions, perhaps building a more complex representation of the task rules. In contrast, the females restricted themselves to a single dimension, thus allowing them to learn one rule at a time (Fig. 1b). By the end of the experiment, males and females were performing equally, but it was clear that the paths each sex took to achieve that level of success were distinct. Moreover, the authors found that neural activity patterns predicted the female-biased strategy only in females; in other words, even if an individual male happened to use this strategy, it was through the engagement of a unique set of circuits. Once again, we see here that understanding the neurobiology of a fundamental process like reward learning requires the consideration of an animal's sex to fully interpret experimental outcomes.

One final example of sex-dependent behavioral strategies comes from the field of conditioned fear learning. The vast majority of published fear conditioning work has been conducted in male rodents[18], and measuring fear has traditionally been limited to a single behavior: freezing. The amount of time an animal spends in a freezing posture is traditionally interpreted to reflect both the degree of fear the animal is experiencing and the strength of the associative memory it formed during the learning stage[19]. But this focus on freezing behavior means that an animal exhibiting a non-freezing fear behavior (such as flight or active vigilance) is essentially interpreted as either not being afraid or not having learned. In rats, females are more likely than males to exhibit these alternate fear responses[20] and are therefore more subject to 'misdiagnosis' in terms of learning ability or emotional magnitude when evaluated on freezing alone (Fig. 1c). This finding has been corroborated across multiple paradigms, including a risky foraging task[21] and a cue discrimination task[22], making it clear that assessing freezing alone is an inadequate approach to reliably study aversive learning in females. Thus, rather than relying on a single, one-dimensional behavior, comprehensive multidimensional analyses are clearly needed if we are to precisely interpret the psychological state of our subjects. Fortunately, recent years have seen a burst of new machine learning-based programs that conduct automated complex behavioral tracking, such as DeepLabCut[23] and MoSeq[24]. These tools will likely reveal nuanced sex differences in a variety of behavioral assays, offering key insights into how to best study behavior in both males and females.

These studies demonstrate that making the SABV initiative successful will require going beyond the simple assessment of females in behavioral paradigms designed for males. If our goal is to accurately understand the neurobiological basis of learning or emotional processes in both sexes, we must be open to the idea that discrete sets of behavioral parameters might best convey these constructs in each. These early days of SABV are critical—without thoughtful examination of our datasets and videos to identify both quantitative and qualitative multidimensional measures, we risk misinterpreting sex differences in experimental outcomes as differences in ability. Such misrepresentations not only can be dangerous if the findings make their way into the public eye, but can also lead other scientists astray in investigating underlying mechanisms.

Sex-dependent structural and synaptic plasticity

Past arguments for the exclusion of female animals in neuroscience research were based, in part, on assumptions that 'fundamental' biological processes such as neural transmission must be the same across the sexes, and therefore whatever was discovered in males would surely generalize to females[25]. Neuroscientists reasoned that, if male and female brains were the same, there was no need to bother studying both (more on this and other SABV myths in Box 1). We now know that this is not always true and that, even when there appears to be congruence between the sexes at one level, sex-dependent mechanisms upstream may mediate these common outcomes. In a seminal paper from 2004, Geert De Vries proposed that in such cases some sex differences may essentially serve as compensation for other sex differences, to allow brain functions in males and females to ultimately realign[26].

A clear example of these 'latent sex differences' can be found in electrophysiological inquiries into rapid estradiol signaling in the hippocampus. Despite a threefold quantitative sex difference (higher in females) in circulating serum estradiol concentrations, males and females exhibit similar estradiol levels within the hippocampus itself[27]. Oberlander and Woolley[28] found that application of estradiol to an ex vivo hippocampal slice preparation resulted in potentiated glutamate transmission in recordings from either males or females. On the surface, this outcome suggests that there are no sex differences in estradiol-mediated synaptic transmission.

Box 1 | SABV myths

Many of the reasons biomedical scientists cite for choosing not to use female animals in their research are based on false assumptions, poor logic or long-standing myths that are not grounded in fact. Here we debunk a few.

- "**Female data will be more variable than data from males.**" This myth has been resoundingly disproved by several comprehensive meta-analyses in both rats and mice across neuroscience subfields[87,88]. If anything, these studies found that male data are often more variable than those from females.
- **"Using females means we need to know the estrous cycle phase or remove the ovaries."** Ovarian hormones are unquestionably powerful neuromodulators, but, as we have argued previously, gonadal hormones are not a uniquely 'female problem' for neuroscientists[85]. Examining the influence of the estrous cycle on a particular experimental question is always an option, but is not required for research in females, just as assessing testosterone levels (which can vary up to tenfold across a cohort[89]) is not standard practice for experiments in males.
- **"We tried using both sexes but did not find any differences, so we went back to using just males."** If there are truly no sex differences, then continuing with mixed cohorts is the right course of action for several reasons. It will satisfy SABV, produce datasets that represent both sexes, thereby improving translatability as well as reproducibility and rigor, and, importantly, allow the discovery of potential points of divergence as the research progresses (for example, sex-dependent mechanisms underlying a common outcome). Reverting back to a single sex betrays an assumption that continuing to collect data from both sexes will be 'messier' or that you believe you might in fact discover a sex difference, despite your claim to the contrary. This mentality thus represents a flawed and biased logic that works to defeat the goals of SABV, again centering males as the foundation of neurobiological knowledge.
- **"We started this work in males, so it makes sense to keep going in males. We will follow up with females when this project is finished."** Be honest, when is a project ever truly finished? There is always another level of 'mechanistic insight' one can claim to need. Playing catch-up can be daunting, but it is better to do as much work in both sexes at the same time, rather than a streamlined follow-up study in females years after the original male work was published. This latter approach risks framing the female work as a lower-impact 'replication study' instead of equally valuable to scientific knowledge.

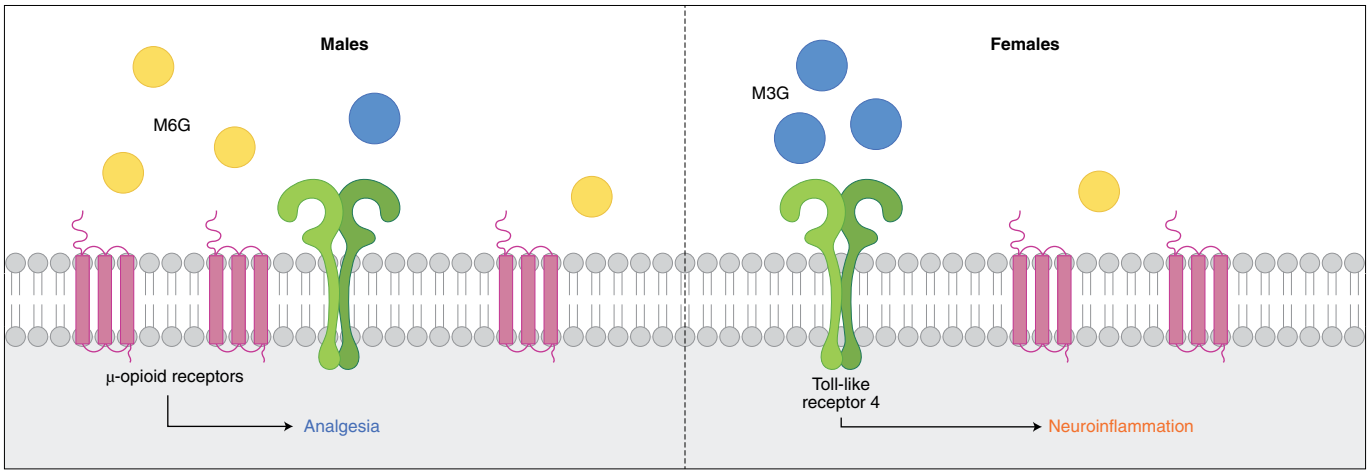

Fig. 2 | A mechanism for sex differences in morphine efficacy. Morphine is metabolized into morphine-6-glucuronide (M6G), which binds to MORs and elicits an analgesic response, and morphine-3-glucuronide (M3G), which binds to TLR4 receptors and elicits a neuroinflammatory response. Morphine may be less effective in females, not only because females have lower expression of MORs in the vlPAG as compared to males, but also because morphine metabolism in females results in disproportionately high levels of M3G versus M6G.

However, a careful dissection of the pre- and postsynaptic components of this observation revealed that estradiol acts through entirely distinct signaling mechanisms in males and females to achieve the same effect. In males, estradiol action occurred through presynaptic estrogen receptor α (ERα) and postsynaptic ERβ. In contrast, presynaptic ERβ and postsynaptic G-protein-coupled ER-1 mediated these effects in females. Follow-up studies by the same research group further identified sex-specific roles for protein kinase A and multiple calcium sources in long-term potentiation induction[29]—a critical component of synaptic plasticity, especially as it relates to learning and memory. This body of work illustrates how the observation that 'X causes Y' in both sexes does not necessarily mean that the path from X to Y is the same. Given the influence that mechanistic studies such as these can have on understanding of memory-related disorders such as Alzheimer's disease or post-traumatic stress disorder (PTSD), it is clear that key points of divergence and convergence between the sexes must be identified to develop more effective therapeutics.

Brain function is inextricably linked to brain structure, and, although male and female brains may be nearly indistinguishable when observed by the naked eye, a recent large-scale analysis identified sex differences in volume in discrete subregions that are conserved from rodents to humans[30]. In addition to these regional sex differences, the structural plasticity that individual neurons undergo in response to experiences has also turned out to be surprisingly sex specific[31,32]. This phenomenon is perhaps best catalogued in rodent studies of how chronic stress exposure affects dendritic and spine morphology in areas like the hippocampus, prefrontal cortex (PFC) and amygdala[32]. Early work in male rats demonstrated that repeated restraint stress elicits apical dendritic atrophy and spine elimination in both hippocampal and prefrontal pyramidal cells[33–35]. In contrast, the same treatment induces dendritic and spine growth in the basolateral amygdala[36,37]. In light of evidence that stress-related illnesses like PTSD or depression can be characterized by a hyperactive amygdala and hypoactive PFC and hippocampus[38], the directionality of these findings makes intuitive sense. However, when the same experiments are conducted in female rats, markedly different effects have been observed. In the hippocampus, chronic stress elicits only a slight change in basal branch number[39], while PFC dendrites and spine density increase in stressed females[40,41]. In the amygdala, chronic stress induces dendrite and spine loss in females[42], the polar opposite of observations in males.

Reconciling these divergent findings between the sexes with epidemiological data that women are twice as likely as men to develop stress-related disorders[43,44] challenges our biases about what is 'good' or 'bad' for the brain. Do the structural alterations we observe in these key brain regions reflect a disease state or healthy adaptation? Are the opposing stress effects in males and females functionally meaningful in understanding sex-specific disease risk factors and biomarkers? We will only find the answers to these questions by conducting rigorous parallel investigations in both sexes. Importantly, even when preclinical results do not directly translate to humans along a biological sex divide, gaining a deeper understanding of the brain's mechanistic diversity increases the likelihood of treating people of all sexes and genders. But we note here that, had translational work in this area progressed exclusively in males, the search for treatments that reverse the impact of stress on neural structure in males could have inadvertently resulted in selecting for those that exacerbate the effects in females. Once again, we see the potential for dangerous and unwanted outcomes in females when males are seen as the default.

Sex-dependent pain pathways

A perfect example of a problematic sex bias in preclinical research is in the field of pain. Chronic pain disproportionately affects women, including migraine, rheumatoid arthritis, fibromyalgia and irritable bowel syndrome[45–47]. But despite this imbalance in clinical populations, preclinical investigations into the biological underpinnings of pain have been overwhelmingly conducted in males[48,49]. Even in clinical studies, which commonly recruit both men and women as participants, sex is rarely included as a factor for analysis[5,48,50].

Opioids remain the 'gold standard' for pain management[51,52], and research over the last three decades suggests that opioid potency is greater in males[53–59]. The midbrain periaqueductal gray (PAG), a critical hub for both endogenous and exogenous pain modulation[60,61], has been implicated as a primary contributing factor. The cells of the PAG—in particular, the ventrolateral PAG (vlPAG)—contain dense populations of μ-opioid receptors (MORs), the preferred receptor for morphine, and vlPAG administration of morphine produces long-lasting analgesia in males. In contrast, intra-PAG administration of morphine in females is remarkably ineffective in modulating acute or chronic pain[61–63], likely owing to comparatively lower levels of MOR protein and binding[61], as well as lower MOR binding efficiency and G protein activation[64,65]. Sex differences have also been reported in both the structure and function of the PAG

Box 2 | A systematic approach to SABV

Although the SABV mandate does not require grant applicants to power their experiments to detect sex-dependent effects, it is clear from the examples discussed in the text that in some cases this is the scientifically sound thing to do. How can you know what the right way to proceed is? Here are some considerations.

- At the bare minimum, adhering to SABV means using experimental cohorts that include both males and females in every experiment, without necessarily analyzing data by sex[8]. Such an approach will ensure that, at the very least, the data we put out into the world will represent both sexes. We strongly recommend that, even if data are not analyzed by sex, visual representations of the data disaggregate by sex (for example, distinguish individual data points) so that readers may discern for themselves whether sex may factor into experimental outcomes. This approach is detailed further in an excellent recent Perspective at *Nature*[90].
- If your data suggest there might be a sex difference (even if you are underpowered to test this statistically), it is worth taking the time to establish what kind of sex difference you may be observing (that is, quantitative or qualitative). This will probably involve increasing your sample sizes and then conducting a formal test for sex differences. Determining whether your effects reflect quantitative or qualitative sex differences, sexual divergence or convergence, or sexual dimorphism is critical, and several excellent resources discuss these types in more detail than we have space for here[91,92].
- Once you have conclusively defined your sex differences, what next? There are many directions you can take to probe the biological underpinnings of these sex differences. Again, we point you to a few superb resources that provide useful decision trees, flow charts, and analysis strategies for designing rigorous experiments that consider sex as a biological variable[92–95].

and its descending projections to the rostral ventromedial medulla (RVM) and dorsal horn of the spinal cord[66–70], which would also contribute to the differences in opioid modulation of pain. More recent studies have further identified a sex-specific role for PAG microglia in morphine efficacy. In females, PAG microglia are more likely to be in a 'reactive' baseline state than they are in males, and morphine action at the innate pattern receptor Toll-like receptor 4 (TLR4) preferentially initiates a neuroinflammatory response (indicated by increased levels of proinflammatory cytokines) within the PAG in females that directly opposes the analgesic effects of morphine[59,71–75] (Fig. 2). Interestingly, the story is quite different within the spinal cord, where microglial TLR4 is critical for pain signaling in males but not in females[49,76,77], highlighting the importance of site specificity when considering the impact of sex on a biological variable of interest.

The necessity of sex-specific research on pain and pain management is clear. Although the pain field has historically been plagued by the same male-skewed bias as much of neuroscience research[78], an encouraging new analysis of literature trends suggests that this may be one area of research in which the SABV initiative has been effective at increasing the use of female subjects[49]. We look forward to future reports that these changes are taking place across neuroscience subdisciplines, but in most cases the needle has not yet moved[6], and therefore more action is needed.

Moving forward—who is responsible?

Neuroscientists who received NIH funding in the last three years presumably addressed SABV in their proposals to the satisfaction of the study section. But whether they have done so in their actual research remains to be seen. NIH grants are nonbinding, meaning that awardees are not required to conduct the exact experiments they propose. Moreover, there is no explicit language from NIH stating that SABV adherence will be enforced once the funds are awarded. Without accountability measures in place, no one is prevented from exclusively using male subjects in research funded under SABV policies. And who would not be tempted to do so? Studying both sexes—even in an underpowered 50/50 design, as is recommended when starting out—carries the implicit 'risk' of identifying sex differences, which may require designing future mechanistic studies of males and females in parallel, rather than continuing with mixed cohorts. By sticking with males only, a lab insulates itself against this potential discovery, allowing them to more quickly advance their work beyond the scope of the proposal and setting them up for future funding opportunities.

The issue is compounded by the current publishing culture. Publishing trends—especially in high-impact journals like this one—have seen a rapid rise in the number of data figures included in each paper, as demands for 'mechanistic insight' escalate and criteria for what constitutes a 'complete story' multiply[79–81]. This shift is at odds with the goals of SABV, because neuroscientists who spend X dollars on experiments in males are thus motivated to allocate their next X dollars to further fleshing out the circuitry and signaling processes that underlie the results of the first set of experiments, rather than being motivated to use those funds to determine whether their findings hold true in females (see Boxes 1 and 2 for why the 'males first' approach is problematic even for those who do intend to study females eventually). When a high-impact paper can be a postdoc's ticket to a prestigious faculty job[82], the inclination for labs to take the former approach over the latter in the name of advancing their trainees' careers is understandable. However, it is no longer defensible.

The use of both sexes in basic neuroscience research is an essential step in rectifying sex- and gender-based health disparities, including life-threatening misdiagnoses in women. But as long as the incentive structure in scientific publishing prioritizes extended research of a phenomenon in males over careful dissection of that phenomenon in males and females, SABV initiatives everywhere will fail. Ensuring they do not will require a cultural shift in what impactful, high-profile science looks like. A truly complete story must be one in which we know the story's 'ending' in both sexes. What we consider 'rigorous' must be a body of work that includes males and females in all experiments (with the obvious exception of those that can only be done in one sex, such as pregnancy-related studies). SABV policies are part of broader initiatives to improve rigor, reproducibility and inclusivity in publicly funded research, and therefore the use of both sexes should be standard in assessing high-impact work that is supported by those funds.

This cultural shift will not come naturally—or voluntarily—to many. A 2017 evaluation of SABV efforts by the European Commission reported that, despite requests, applications considering SABV rose by only 3% (from 16% to 19% of all applications) over two years[83], spurring the implementation of more explicit requirements for upcoming Horizon Europe funding opportunities[84]. Additionally, a recent survey found that male NIH study section members (who make up two-thirds of panel membership) were less likely than their female counterparts to believe that SABV is an important policy[9]. This suggests that not only are men more likely to give non-SABV-adherent proposals better scores, but they may also be less likely to comply with SABV in their own research. Some voiced concerns that SABV would slow scientific progress. These opinions are in line with long-standing problematic views that using male subjects allows researchers to discover hard scientific truths, while study of female subjects can only reveal phenomena that are relevant to the 'niche' topic of women's health[85]. Indeed, how can we

conduct rigorous research in both sexes while maintaining the pace of scientific progress? The two goals are only at odds if it is considered 'progress' to answer a biomedical question in a way that ignores over half the population.

The mission statements of large-scale science funding agencies generally boil down to "uncovering new knowledge that will lead to better health for everyone," and the agencies are therefore obligated to enforce the policies they have put in place to help accomplish this mission. Currently, the success of the SABV initiative depends entirely on good-faith efforts by researchers who, as outlined above, have clear incentives not to make that effort. This is not enough. Researchers should be held accountable by making documentation of SABV compliance mandatory in yearly progress reports and by using compliance as a contingency for grant renewals (both noncompetitive and competitive). In addition, we scientists must also hold each other accountable through manuscript peer review—by prioritizing papers that use both sexes, requiring subject sex to be reported in manuscript titles[86] and fighting against the 'follow-up in females' approach (Box 1). Finally, journal editors—who arguably have the greatest power to shape the culture of scientific research practices—should also hold authors to these standards, especially at high-profile journals. In 2016, the European Association of Science Editors put forth the Sex and Gender Equity in Research (SAGER) guidelines, which detail expectations and best practices for reporting sex and gender in scientific publications. These guidelines should be widely implemented across publishing platforms.

The SABV initiative will succeed in broadening basic science knowledge and improving public health when a critical mass of scientists around the globe feels compelled to apply the policy guidelines to their research programs. There are many outstanding resources available to assist with experimental and statistical designs that consider sex as a biological variable (discussed in Box 2), and we encourage researchers new to studying both sexes to take full advantage of these tools. But until the current incentive structure of academic science is at least partially dismantled and rebuilt to value these outcomes, the day that we can call SABV policies a success will not arrive. The scientific community must come together to perform a conscious, intentional realignment of our reward systems and standards for scientific rigor that counters implicit biases against the utility of female research subjects[77] and recognizes our responsibility to the public as taxpayer-funded investigators. The result will be a new definition of 'progress' that is more equitable, more translational and more beneficial to society as a whole.

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Competing interests

The authors declare no competing interests.

Additional information

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