Trends in Neurosciences

CellPress OPEN ACCESS

Opinion

Hidden variables in stress neurobiology research

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Among the central goals of stress neurobiology research is to understand the mechanisms by which stressors change neural circuit function to precipitate or exacerbate psychiatric symptoms. Yet despite decades of effort, psychiatric medications that target the biological substrates of the stress response are largely lacking. We propose that the clinical advancement of stress response-based therapeutics for psychiatric disorders may be hindered by 'hidden variables' in stress research, including considerations of behavioral study design (stressors and outcome measures), individual variability, sex differences, and the interaction of the body's stress hormone system with endogenous circadian and ultradian rhythms. We highlight key issues and suggest ways forward in stress neurobiology research that may improve the ability to assess stress mechanisms and translate preclinical findings.

Highlights

Mapping stress-induced behavioral adaptations onto specific cognitive domains and neural circuits is essential for facilitating the translation of preclinical stress findings.

'Hidden variables' can thwart mapping efforts, hindering the development of stress response-based therapeutics for psychiatric disorders.

Inclusion of sex in stress neurobiology research is important, but sex must be interpreted as just one biological variable among many that may influence an individual's stress response.

Stress hormone secretion is dynamically regulated by circadian and ultradian mechanisms. A key hidden variable of stress research is the extent to which stressors disrupt normal biological rhythms to produce their observed effects.

Hidden variables can influence stress-related behavioral and physiological outcomes

Numerous psychiatric disorders are precipitated and exacerbated by stress, including major depressive disorder (MDD), substance use disorders (SUDs), and post-traumatic stress disorder (PTSD). Therefore, understanding how stress responses can become dysregulated or maladaptive is central to advancing psychiatry. Hormones involved in responding to stress are fundamental to normal brain function. Cortisol – corticosterone in rodents – (CORT) is the body's major 'stress' hormone; it circulates throughout the body even under non-stressful conditions, following regular circadian and ultradian rhythms that help regulate gene transcription and adaptive behavior [1–4]. Feedback through the hypothalamic–pituitary–adrenal (HPA) axis works to keep stress hormones in check, ebbing and flowing throughout the day and in line with the needs of the organism (Figure 1). Spikes in CORT during acute events (such as predator attack) are adaptive, they support life-preserving responses to danger, but dysregulated CORT rhythms under conditions of chronic stress put organisms at risk for maladaptive behavioral changes associated with psychiatric disorders.

Understanding the stress response system, the types of stressors that lead to its dysfunction in psychiatric disorders, and the consequences of its dysfunction for neural circuits are important goals. In the service of these goals, stress neurobiology researchers commonly use readouts of stress hormone physiology and behavior as outcome measures of stress reactivity, and attempt to identify mechanisms underlying changes in both readouts to guide translational efforts. However, 'hidden variables' in study design can influence measures of stress reactivity and, thus, our understanding of the neurobiological mechanisms of stress-induced behavioral deficits. In this opinion article we discuss how 'hidden variables' may impact interpretation of results from stress neurobiology studies. We then delineate paths forward to understand the influence of hidden variables on stress-induced changes in physiology and behavior.

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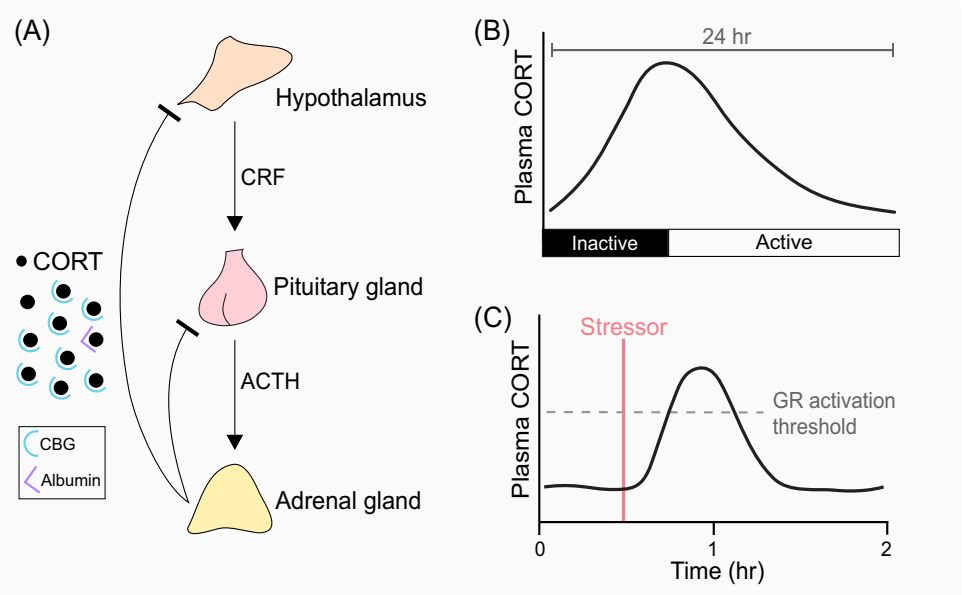

Figure 1. Hypothalamic–pituitary–adrenal (HPA) axis function. (A) Schematic illustration of the HPA axis, its hormonal mediators, and relevant blood proteins. Cortisol/corticosterone (CORT) regulates the activity of the HPA axis by binding to glucocorticoid receptors (GRs) in the hypothalamus and pituitary gland, a process termed 'negative feedback'. (B) Relative pattern of plasma CORT levels across the 24-h light/dark cycle in rodents and humans. (C) Relative plasma CORT levels after an acute stressor. When plasma CORT levels are sufficiently high, they activate GRs throughout the body and brain to enact transient physiological responses that allow an organism to cope with an acute stressor. Abbreviations: ACTH, adrenocorticotropic hormone; CBG, corticosteroid-binding globulin; CRF, corticotropin-releasing factor.

Dissecting stress-induced behavioral changes across cognitive domains

A fundamental goal of animal research on the neurobiology of stress is to determine how stress causes behavioral impairments relevant to a given psychiatric disorder. However, it can be problematic to characterize stress-induced changes in specific behavioral assays as 'illness-like' (e.g., 'depression-like' or 'anxiety-like' behaviors). By reducing interpretations of stress-induced behaviors to the likeness of a human phenotype, researchers lose clarity on the neurobiological processes changed by stress because of the need to fit a specific illness narrative. In fact, many human psychiatric disorders are highly comorbid, perhaps in part because stress causes changes across a wide variety of cognitive domains and associated neural circuits. Linking neural circuit function to behavior without trying to fit specific illness criteria is among the goals of the National Institutes of Health (NIH) Research Domain Criteria (RDoC) framework[i].

With regard to stress models, there is a large variation in the types of stressors used, the duration of stress exposure, and the endpoints one may measure [5]. Regardless of the stress model being used, when choosing and conducting a behavioral test to measure the outcome of a stress manipulation, it is important to recognize that a finding of little or no effect of a stress-related intervention in one behavioral test does not rule out effects on other behavioral tests. Many behavioral tests are not cross-predictive for each other [6–8]. For example, in male C57BL/6J mice, chronic social defeat stress (CSDS)-induced social avoidance behavior is not predictive of increased anxiety-related behaviors in the elevated plus and open field tests [6]. As another example, in female C57BL/6J mice, chronic CORT treatment does not have effects in the light–dark test, elevated plus maze, or novelty-suppressed feeding test [9], but it impairs operant reward-seeking for sucrose pellets [10].

When an effect of stress on a particular behavioral test is observed, it is also important to carefully interpret the meaning of the performance change. What cognitive domain is affected? Interpretations accepted as standards may not always be correct. For example, researchers commonly report that rodents scoring poorly in the sucrose preference test are experiencing a loss of pleasure (anhedonia). However, these rodents might also be displaying novelty-suppressed feeding (hyponeophagia), or could be unable to learn and remember the value and location of the sweet solution, or could be lacking motivation to pursue sucrose [11,12]. Careful analysis and proper controls can be applied to distinguish these possibilities [12], but attention to the problem is necessary. In many cases, careful dissection of behavior can be aided by advances in behavioral tracking (e.g., DeepLabCut [13]) that allow the dissection of behavioral microstructures.

Similar problems arise when interpreting operant behavior, where deficits can arise for multiple reasons. For example, a common operant test of motivation, the progressive ratio task, requires mice to progressively increase the number of times they perform an action to receive a reward, until they reach a 'breakpoint' where they are unwilling to work any harder. However, the progressive ratio task conflates work and time: performing numerous repetitions of an action, in addition to involving more work, requires more time than a single action. Differences in temporal discounting for rewards could therefore explain differences between groups on the progressive ratio task. To remove this temporal discounting confound, a task can be used where a single lever press leads to food reward, but the force required to press the lever progressively increases. On this task, obese mice exert more force (i.e., work harder) for food than lean mice [14].

Finally, similar behavioral outcomes amongst groups or individuals do not guarantee similar underlying mechanisms. For example, although chronic CORT treatment impairs operant responding for rewards in both male and female C57BL/6J mice, different impairments in dopamine system function are observed in the two sexes [10]. Another example of the discrepancy between mechanism and behavior arises from two studies examining the effects of optogenetic stimulation of dopamine neurons following two different stress paradigms: CSDS and chronic mild stress (CMS). One study found that optogenetically inducing phasic bursting of dopamine neurons relieves behavioral despair and anhedonia after CMS [15], while the other study found that this optogenetic stimulation worsens social deficits and anhedonia following a subthreshold CSDS paradigm [16]. These two studies may appear at odds with each other. They emphasize that finding consistent explanations of circuit changes in response to stress may be elusive, and that the many manipulations that can be characterized as 'stressful' are not necessarily equivalent manipulations. These studies also underscore the need for a stronger conceptual framework for explaining how stress-induced impairments in multiple psychological processes – likely governed by separate neural circuits – lead to convergent and divergent behavioral outcomes [12,17]. Careful dissection of behavior is imperative for isolating the psychological and neurobiological processes that are altered by stress.

Handling individual variability in behavior

Humans are not diagnosed with psychiatric disorders based on the stressors they have endured, but based on the symptoms they develop. Different stressors can lead to the same diagnosis, and the same stressor can cause different symptoms in different people (Figure 2). In the context of animal studies, therefore, the expectation that all or even most subjects in a stress treatment group will follow a specific illness-like symptom course may be misplaced. Just as it is reasonable to expect humans to develop different psychiatric symptoms in response to stress, it is reasonable to expect individual variability in animal subjects' stress responses. In both humans and rodents (even genetically inbred rodents), there is high inter-individual variability in behavior, both at a baseline and in response to stress [18–22].

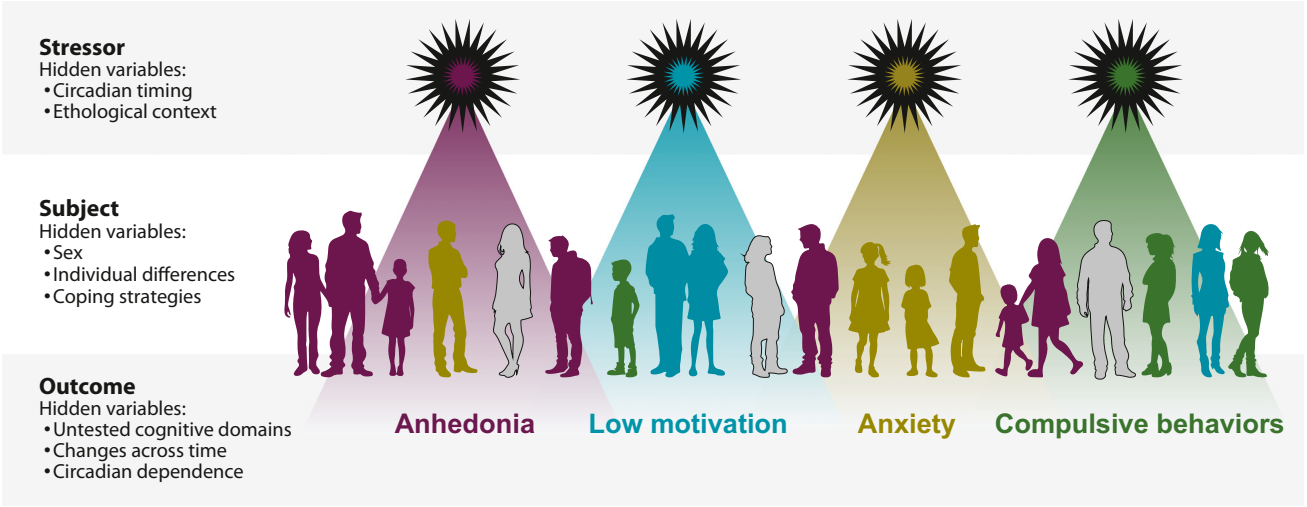

Figure 2. Hidden variables at three levels of experimental design can influence apparent results in stress neurobiology studies. The graphic depicts individual differences in response to different types of stressors, which, on average, lead to particular phenotypes (e.g., anhedonia, low motivation, anxiety, compulsive behaviors). Stressors may induce variable outcomes depending on individual differences amongst subjects and experimental choices about outcome measures. For example, the hypothetical stressor symbolized by the color purple generally leads to anhedonia (purple silhouettes), but some individuals are unaffected by this stressor (gray silhouette), while others are affected but show symptoms in a different domain (chartreuse silhouette, anxiety). To the left, we list 'hidden variables' to consider with regard to stressors, subjects, and outcome measures that may influence the apparent mapping between stressors and changes in behavior and neural function. Top: stressors used across studies vary in duration, severity, and other parameters. In addition, there are often overlooked or 'hidden' variables that may require consideration. These include, for instance, the interactions of stressors with circadian timing and ethological context (e.g., in animal studies, if a social stressor is applied, is it within the realm of social experiences the animal might encounter in natural contexts?). Middle: subject-level variability in the responses to stressors can be notable. Factors influencing variability can include sex (whose effects may be dependent or independent of hormones), individual differences (which may interact with sex, but can also encompass other differences in genetic background and life experiences), and baseline traits that can influence stress-coping strategies during stressor exposure. Bottom: the choice of outcome measures is another key factor in experimental design that can influence apparent outcomes. Hidden variables include untested cognitive domains (where apparently 'resilient' individuals could show symptoms), changes in outcomes across time (e.g., such that an individual might appear 'susceptible' at an early time point and 'resilient' later as they recover), and circadian dependence of behaviors (e.g., stress might evoke changes in the circadian rhythms of certain behaviors, rather than in their overall performance levels, and deficits might only be observed during particular times of day).

Individual variability in vulnerability to stress has been an important focal point in stress research. Many researchers have turned to categorizing rodents into susceptible or resilient groups. On the one hand, categorization of subjects based on a behavioral phenotype is advantageous because it ensures that individual differences in response to stress are not washed out in a group average. On the other hand, binarization into susceptible and resilient groups does not fully consider each individual animal's response to stress. Particularly if only one behavioral test is used for categorization, an apparently 'resilient' individual could be susceptible in a different cognitive domain than what has been tested and used for categorization (Figure 2). For example, as discussed above, male mice experiencing CSDS may be resilient on a social avoidance assay, but still display changes on the elevated plus and open field tests [6]. Therefore, researchers should be careful to limit interpretations of resilience to the specific test being used, and not to assume they are modeling global resilience to stress-induced deficits in all cognitive domains.

The term 'resilience' is another potential source of complexity, in terms of how the temporal dynamics of recovery are interpreted and analyzed [23]. Resilience is often defined by performance in a behavioral task at a single time point after stressor exposure [6,7]. However, this approach does not account for behavioral adaptations that may occur longitudinally during stressor exposure. Did the 'resilient' subjects experience stress-induced disruptions in normal functioning but recover through some coping mechanism? Were they entirely impervious to the manipulation? To clarify the meaning of 'resilience', it is imperative to understand the time courses and mechanisms

underlying behavioral adaptations to stressor exposure (see Outstanding questions). One interesting recent effort to look at time courses of resilience took advantage of advances in machine-learning-assisted behavioral quantification to determine which C57BL/6J mice would be susceptible to changes in social interaction following CSDS, based on their behavior during CSDS [20]. While susceptible male mice were submissive during attacks by an aggressor, resilient mice fought back. Interestingly, different behaviors were associated with resilience in female mice. In females, resilience was predicted by 'close vigilance' of the aggressor, but not by fighting. By analyzing behavior during an ongoing stressor, researchers delineated immediate coping behaviors that reflected resilience in a later social interaction test. However, it remains unclear whether behaviors during CSDS predict resilience in other cognitive domains, such as reward processing. Perhaps there are cognitive domains where alternative coping strategies during CSDS would appear more advantageous than fighting back. An important goal going forward would be to better understand how various coping strategies and other trait characteristics of individuals interact with stressors to produce specific domains of resilience and susceptibility. While researchers cannot test all cognitive domains in all experiments, they must be cautious not to overgeneralize behavioral outcomes on a single test to multiple cognitive domains. To better define 'resilience trajectories' and areas for targeted translational intervention, it would be useful to assess behavioral responses to stressors longitudinally, in a manner that encompasses pre-stress states, immediate responses to ongoing stressors, and post-stress coping and recovery periods [8,20,22,24,25].

Sex as a biological variable

Recognition of the importance of sex as a biological variable in clinical and preclinical research has been growing. The argument for sex inclusion in stress research is especially strong because stress is a well-known risk factor for a broad range of neuropsychiatric disorders with documented sex differences in diagnoses. For example, women are more likely than men to be diagnosed with MDD [26], while men have earlier ages of onset for schizophrenia and more prominent negative symptoms than women [27]. Understanding sex differences in stress biology could offer insights into the underlying mechanisms for disparate sex ratios in neuropsychiatric disorders that are precipitated or worsened by stress.

In preclinical research, female animals have been historically excluded due to beliefs about behavioral variability induced by hormonal cycling. Although female sex hormones can matter for behavior and physiology, sex and hormones can independently influence outcomes, and males, like females, have fluctuating or variable levels of sex hormones [28–30]. Numerous studies have pointed to crosstalk between the gonadotropic and HPA axes, suggesting that gonadal hormones regulate HPA axis function, and thus stress responsivity, in all animals regardless of sex [31,32]. Unless the influence of estrous cycle hormones on behavior is specifically under study, it is not necessary to treat estrous as a hidden variable to track, above and beyond other variables. In stress research, there is a trade-off between female estrous tracking to gain information about hormone status and the stress caused by vaginal swabbing to gather such information. In the meantime, it should be recognized that female variability on common behavioral tasks is often lower than male variability [21,29,33–36]. Therefore, including female animals in preclinical research (without estrous tracking) does not make studies inherently less reliable. On the contrary, understanding when and how sex differences occur is likely to lead to robustness in research findings, enhancing translation of basic biology into medical innovation. An example is evident in the development of corticotropin-releasing factor type 1 (CRF-1) receptor antagonists as potential antidepressants. Most preclinical studies on antidepressant efficacy of CRF-1 receptor antagonists used exclusively male subjects. In clinical studies, CRF-1 receptor antagonists were only reported as effective in trials that recruited exclusively males [37]. The inclusion of female-

only or mixed-sex preclinical studies (with analysis stratified by sex) could have clarified the sex-specific antidepressant potential of CRF-1 receptor antagonists prior to clinical trials, avoiding the treatment of human subjects with therapies unlikely to produce benefit.

In designing studies in stress research to address sex as a biological variable, one must consider whether the behavioral paradigms being used can be applied across the sexes. For example, CSDS is a popular adult stress paradigm used in male mice, but adapting this stress paradigm to females, which do not naturally display the same type of territorial aggression, is difficult [38]. Some stress paradigms, such as chronic variable stress, can be used in both sexes, but different durations are required to elicit similar behavioral outcomes in males and females [22]. Whenever male and female subjects are treated differently in a study design, it becomes difficult to interpret sex differences in outcomes unless careful mechanistic follow-ups are conducted. Further, treating male and female subjects differently from study outset lends itself to thinking of sex as a dichotomy. However, it is ideal to understand sex differences in behavior not as sexual dichotomies (which are uncommon), but as overlapping spectrums of individual variability that are skewed (on average) by sex. Regardless of whether or not sex differences in behavior occur in response to a particular manipulation, mechanistic follow-up studies in both sexes are important, as males and females may not use the same mechanisms for behavioral adaptation to stress [10,39].

Different treatments for male and female animals may occur even when they are not intentionally built into the study design. For example, when male and female pups are subjected to early life stress, it is unclear whether male and female pups get equivalent maternal care. In the limited bedding and nesting paradigm for early life stress, which causes fragmented maternal care [40,41], several studies have observed stronger effects on female animals [42–44]. Does this sex difference occur because male and female pups respond differently to fragmented maternal care? Or could it occur because dams with poor resources allocate their care differently to their male and female offspring? These two different reasons for the observed sex differences would have different biological implications. Dissecting the reasons that sex differences occur thus becomes critical.

Finally, although sex-inclusive animal studies are important for translation, sex differences in animal models should be interpreted with caution. Sex is only one variable that influences behavioral outcomes, and it can have complex interactions with other hidden variables (see Outstanding questions). Studies of genetically inbred mouse lines isolate the influence of chromosomal sex, but they do not allow for dissection of complex genomic interactions. When genetically diverse animals are surveyed on common behavioral tasks, enormous behavioral variability can be observed [45–48]. Is the effect of sex consistently in one direction when other sources of genetic diversity are evaluated? The answer to this question has clear importance for translation. In animal studies, while it may not be necessary or feasible to examine the effects of sex on large numbers of genetically diverse subjects in every study, researchers can be more careful when interpreting sex differences from inbred strains and assessing whether the specific observed sex differences will be applicable in more genetically diverse populations.

Circadian and ultradian rhythm disturbances as crucial hidden variables

Circadian rhythm disturbances, including changes in patterns of eating and sleeping, are key symptoms of numerous stress-related psychiatric disorders, including MDD and SUDs[ii] [49,50]. Furthermore, stress hormones are regulated on a circadian schedule. CORT peaks at wake time (beginning of the light cycle for humans, beginning of the dark cycle for rodents) and declines throughout the wake period (Figure 1B). Treatments with exogenous CORT or chronic

stressors can interfere with the circadian regulation of CORT release [51–54], as can other experimental manipulations such as high-fat diet and food restriction [55–57].

On top of circadian rhythms, ultradian rhythms cause regular pulses of CORT throughout the day and can be a crucial hidden variable regulating the enduring impact of stress [58]. Genetic variability can result in different circadian and ultradian rhythms of CORT release among different strains of rodents, which may relate to stress susceptibility [59]. Humans with stress-related psychiatric disorders can also have disrupted CORT rhythms [60–63]. Understanding how stress manipulations and other environmental factors interfere with rhythms of stress hormone release may inform interpretations of behavioral changes and offer insight for translation. Recent studies have reported that stressors applied at different times across the circadian cycle have different effects on behavior, hormonal responses to stress, and molecular circadian rhythms in tissues throughout the body [51,64–66]. The importance of stressor timing has significant implications for the translatability of preclinical findings.

Due to endogenous circadian rhythms, outcomes of stressors (including behaviors, circuit activity, and molecular changes) must likewise be interpreted with regard to time of day [67–71]. Inherent to circadian rhythms are peaks and nadirs in the normal ranges of outcome measures across the light/dark cycle, leading to potential ceiling and floor effects, respectively. It may be harder to observe inhibitory effects of stress on behavior and biological systems during the nadir of their expression (when floor effects are likely), and vice versa for stimulatory effects of stress. Going forward, it will be essential to monitor outcomes across time (see Outstanding questions). Efforts to do so will be aided by the advent of high-throughput home-cage monitoring devices [67,70,72]. Combining multiple, compatible experimental approaches – such as home-cage operant devices, wireless electroencephalographic systems, and long-term video monitoring – may allow researchers to capture the full circadian behavioral repertoire of individual animals before, during, and after exposure to stressors. One exciting possibility is that circadian monitoring in stress studies will help to identify shared biological mechanisms that contribute to both the affective symptoms and the circadian disruptions observed in psychiatric disorders [73–75].

Concluding remarks

Stress neurobiology research is essential for the advancement of psychiatric medicine. To best ensure the translatability of basic stress studies, it is necessary to design experiments that elucidate the specific cognitive domain(s) affected by a given stressor. Rigorous experimental design and careful analysis of behavioral outcomes will aid in understanding how stress affects specific cognitive processes, thus facilitating the development of therapeutics for specific stress-induced behavioral impairments. Exciting technological developments in behavioral tracking and machine learning will aid in these efforts by allowing researchers access to data on the microstructures of behavior, and by allowing the analysis of large datasets that can appropriately account for individual and sex-based variability in performance.

When considering experimental design, it is essential to account for sex as a biological variable in all aspects of stress neurobiology research, from behavioral to mechanistic experiments. Sex inclusion is crucial for understanding mechanisms underlying stress-induced behavioral changes in different sexes, and for appropriately designing translational approaches that will benefit all patients. In human populations, it is important to recognize gender as a variable and to avoid binary conceptualizations of sex that do not account for the full range of human experience.

Furthermore, whenever possible, it is advantageous to assess physiology and behavior longitudinally: before, during, and after exposure to stressors. A great advantage of animal research is that

Outstanding questions

Which tasks are best suited to examine the cognitive domains most relevant for psychiatry? How can tasks be designed to delineate between cognitive domains? Can a battery of tasks be used to resolve overlapping domains? Can tasks be designed to track outcomes over time, thus revealing important temporal components of stress responsivity?

What is the best way to account for individual variability in stress reactivity? Are there consistent aspects of neural circuit function that predict different components of stress susceptibility and resilience across cognitive domains?

How does sex skew distributions of individual variability? What other genetic or environmental variables interact with sex to influence outcomes?

How do times of stress delivery and behavioral testing affect our ability to detect stress-induced phenotypes that vary with circadian or ultradian rhythms? Can approaches be designed to continuously monitor stress-induced changes in behavior and physiology across time, accounting for the effects of these rhythms?

prospective studies are much easier to accomplish than in human research. Longitudinal, prospective studies in stress neurobiology will help identify the molecular and circuit mechanisms of stress vulnerability, resilience, and resistance to stress-induced changes in different cognitive domains, which can be targeted for intervention.

Finally, future studies need to consider how stress interacts with endogenous circadian and ultradian rhythms. Because of the intimate relationship between stress hormone signaling and these biological rhythms, understanding the molecular mechanisms of stress-induced changes in circadian and ultradian rhythms may be crucial for illuminating the mechanisms of susceptibility to psychiatric disorders.

By considering the 'hidden variables' discussed herein, preclinical researchers can conduct studies that dissect the mechanisms by which stress precipitates and exacerbates behavioral symptoms associated with psychiatric disorders, pinpointing new translational targets involved in the stress response.

Acknowledgments

We thank members of the Lerner laboratory for helpful feedback and discussions. This work was supported by an NIH New Innovator Award (DP2 MH122401) to T.N.L., and an National Institute of Neurological Disorders and Stroke (NINDS) DSPAN Award (F99 NS130873-01) to A.L.H.

Declaration of interests

The authors declare no competing interests.

Resources

[i]www.nimh.nih.gov/research/research-funded-by-nimh/rdoc

[ii]https://doi.org/10.1176/appi.books.9780890425787

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