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# Modeling heritability of temperamental differences, stress reactivity, and risk for anxiety and depression: Relevance to research domain criteria (RDoC)

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## Abstract

Animal models provide important tools to study biological and environmental factors that shape brain function and behavior. These models can be effectively leveraged by drawing on concepts from the National Institute of Mental Health Research Domain Criteria (RDoC) Initiative, which aims to delineate molecular pathways and neural circuits that underpin behavioral anomalies that transcend psychiatric conditions. To study factors that contribute to individual differences in emotionality and stress reactivity, our laboratory utilized Sprague–Dawley rats that were selectively bred for differences in novelty exploration. Selective breeding for low versus high locomotor response to novelty produced rat lines that differ in behavioral domains relevant to anxiety and depression, particularly the RDoC Negative Valence domains, including acute threat, potential threat, and loss. Bred Low Novelty Responder (LR) rats, relative to their High Responder (HR) counterparts, display high levels of behavioral inhibition, conditioned and unconditioned fear, avoidance, passive stress coping, anhedonia, and psychomotor retardation. The HR/LR traits are heritable, emerge in the first weeks of life, and appear to be driven by alterations in the developing amygdala and hippocampus. Epigenomic and transcriptomic profiling in the developing and adult HR/LR brain suggest that DNA methylation and microRNAs, as well as differences in monoaminergic transmission (dopamine and serotonin in particular), contribute to their distinct behavioral phenotypes. This work exemplifies ways that animal models such as the HR/LR rats can be effectively used to study neural and molecular factors driving emotional behavior, which may pave the way toward improved understanding the neurobiological mechanisms involved in emotional disorders.

**Abbreviations:** cDRD, Caudal aspect of the dorsal raphe nucleus, dorsal part; CORT, corticosterone; DAT, Dopamine transporter; DNMT, DNA methyltransferase; EPM, Elevated Plus Maze; EZH2, Enhancer of zeste homolog 2; FST, Forced Swim Test; H3K27-me3, Trimethylation of histone H3 at lysine 27; HR, High novelty responder rat; LR, Low novelty responder rat; miRNA, microRNA; NIMH, National Institute of Mental Health; NPU, No-shock, predictable-shock, unpredictable-shock threat task; OFT, Open Field Test; P, Postnatal day; PTSD, Post-traumatic stress disorder; RDoC, Research Domain Criteria; SSRI, Selective serotonin reuptake inhibitor; VTA, Ventral tegmental area.

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**KEYWORDS**
amygdala, anhedonia, hippocampus, negative valence, passive coping, threat

## 1 | INTRODUCTION

Nearly half of all Americans will meet criteria for a major psychiatric disorder at some point in their lives (Andrade et al., 2003; Kessler et al., 2007). Major depressive disorder and anxiety disorders are highly comorbid (Keller et al., 2005; Kessler et al., 2008; Middeldorp et al., 2005) and among the most prevalent and debilitating psychiatric illnesses, with a lifetime prevalence of 13.2% (Hasin et al., 2018) and 33.7% (Kessler et al., 2012), respectively. In addition to the pain and suffering that patients and their families face, these illnesses pose an enormous financial burden, costing over $210 billion in the USA annually (Greenberg et al., 2015). Despite the magnitude of this problem, the etiology and pathophysiology of mood and anxiety disorders remain poorly understood.

Animal models play a critical role in revealing neurobiological mechanisms of emotional behavior. These models provide important tools for developing theoretical frameworks to understand how biological and environmental factors contribute to emotional dysfunction, which could ultimately be applied to developing novel therapeutics. A longstanding challenge in the field of translational neuroscience has been that animal models cannot recapitulate the complex array of symptoms and pathophysiological changes associated with human psychiatric illness. Traditionally, animal models relevant to psychiatric disorders have been expected to meet several validation criteria, namely face validity, predictive validity, and construct validity (for an excellent in-depth review see (Nestler & Hyman, 2010)). For models relevant to depression, some of these validation criteria (such as face validity) may be satisfied when animals exhibit behavioral anomalies within domains pertinent to the human condition, such as anhedonia or behavioral despair. Predictive validity (also known as pharmacological validity) refers to when a model animal responds to a treatment in a way that predicts similar effects in humans. The concept of predictive validity is quite challenging in psychiatry as there is limited understanding of how drugs such as antidepressants impact a wide range of behavioral domains and whether mechanisms that lead to behavior changes in experimental animals are similar to or different from those that lead to improvement in patients suffering with depression. The notion of construct (or etiologic) validity relates to whether an underlying disease processes that leads to human disease in humans can be replicated in model animals. This concept is fraught in animal models relevant to emotional disorders given the still limited understanding of genes that influence predisposition to illnesses like depression (Nestler & Hyman, 2010). This perspective began to shift in the advent of the National Institute of Mental Health (NIMH) Research Domain Criteria (RDoC) Initiative. RDoC provides a framework for conceptualizing how abnormalities within specific molecular pathways and neural circuits underpin emotional behaviors and give rise to symptoms that transcend multiple psychiatric conditions (Insel et al., 2010). By employing an RDoC framework, preclinical animal models can be leveraged to study neural and molecular mechanisms that drive specific behavioral abnormalities, such as anhedonia, that are relevant not only for depression, but for other psychiatric disorders (e.g., schizophrenia) as well.

In order to study neurobiological factors that contribute to individual differences in emotional behavior, our laboratory has spent the last several years working with selectively bred lines of Sprague–Dawley rats that were bred based on differences in novelty-induced exploratory behavior (Clinton et al., 2011; Cohen et al., 2015; McCoy et al., 2016; Stead et al., 2006). As detailed below, selective breeding for high versus low locomotor response to novelty produced two phenotypically distinct lines of rats that differ in several behavioral domains relevant to anxiety and depression. This review article begins by summarizing human studies showing the link between individual differences in emotionality and risk for depression and anxiety. We then describe parallel findings in the selectively bred high/low novelty responding rats, with particular focus on RDoC negative valence domains (e.g., acute threat, potential threat, and loss) commonly associated with depression and anxiety disorders. Lastly, we describe neural circuits and molecular mechanisms that contribute to emotional behavior differences in the bred high/low novelty responder rats, including inborn differences in epigenetic regulation in the developing brain. Such findings may provide insight into neuropathological changes involved in the etiology of psychiatric disorders like depression and anxiety. Furthermore, the High Responder/Low Responder model provides a powerful pre-clinical model to elucidate the neurobiology of RDoC transdiagnostic emotional domains adversely impacted by mood disorders and related conditions.

## 2 | INDIVIDUAL DIFFERENCES IN HUMAN TEMPERAMENT AND PROCLIVITY TO DEPRESSION AND ANXIETY

The pathogenesis of stress-related mood disorders, such as depression and anxiety, is influenced by myriad genetic,

biological, and environmental factors. Inborn differences in personality and emotional reactivity strongly shape how individuals respond to stress and increase vulnerability to depression and anxiety. Studies in children describe how certain temperaments predict emotional dysfunction later in life (Biederman et al., 1990, 2001; Kagan et al., 1987; Rosenbaum et al., 2000). For instance, toddlers that show high levels of behavioral inhibition (acting restrained, wary, and fearful in novel situations) face increased risk for anxiety disorders (Biederman et al., 1990, 2001; Gladstone & Parker, 2006; Hayward et al., 1998; Muris et al., 1999, 2001; Schwartz et al., 1999) and depression (Caspi et al., 1996; Muris et al., 1999, 2001) later in childhood, adolescence, and/or adulthood. By contrast, toddlers that act impulsively are at greater risk of later substance abuse and antisocial behavior (Eigsti et al., 2006). Behavioral inhibition during early childhood is also associated with enhanced cortisol secretion and stress reactivity (Hastings et al., 2008; Mackrell et al., 2014), which may relate to hypothalamic–pituitary–adrenal (HPA) axis dysregulation that can occur in adult depressed patients (Vreeburg et al., 2009). Clinical studies have identified alterations in and complex interactions among many behaviors, including response to novelty, as a reliable predictor of vulnerability for depression or anxiety (Duclot & Kabbaj, 2013; Josefsson et al., 2011; Vreeburg et al., 2009; Wu et al., 2012). Thus, temperamental differences represent a viable manner to identify individuals at-risk for developing stress-related mood disorders.

Early evaluations of genetic and environmental influences in family, adoption, and twin studies show that additive genetic effects contribute 31%–42% of liability for heritability of major depressive disorder (Ono et al., 2002; Sullivan et al., 2000). Indeed, parental depression is one of the greatest predictors of a child's risk for developing depression (Klein et al., 2002; Mackrell et al., 2014), with a vast literature implicating both maternal and paternal depression in offspring developing internalizing psychopathology (Cote et al., 2018; Kane & Garber, 2004; Mackrell et al., 2014; Pilowsky et al., 2008, 2014; Wickramaratne et al., 2011). One study of USA mothers and infants demonstrated that maternal depression or anxiety during the third trimester of pregnancy was associated with greater negative behavioral reactivity to novelty in the infant at 4 months of age (Davis et al., 2004). Such altered responses to novelty can, in turn, contribute to higher levels of behavioral inhibition in children and predict later behavioral issues such as adolescent social anxiety (Davis et al., 2004; Kagan & Snidman, 1999; Schwartz et al., 1999).

It has been suggested that inheritance of specific traits from parents, rather than of depression itself, is what contributes to heightened risk for psychopathology in vulnerable children (Manki et al., 1996; Ono et al., 2002). A study of German mothers and infants, for example, found that personality characteristics like agreeableness in mothers predicted higher cry scores in a behavioral battery designed to assess distressed response to novelty in 4-month-old infants (Mohler et al., 2006). Thus, heritability of traits such as response to novelty could be used to predict vulnerability to stress-related disorders. However, the onset of major depression and anxiety is influenced by both genetic predisposition and environmental factors (Sullivan et al., 2000). Triggering events during childhood, adolescence, or adulthood, including exposure to psychosocial stress or trauma, can interface with inborn trait heritability to give rise to depression or anxiety-related disorders in susceptible individuals.

Altogether, the present body of work describes a clear relationship between temperamental differences in children and heightened emotional dysregulation and risk for developing mood disorders later in life. However, the neurobiological mechanisms underlying this relationship remain unclear. Observations of behavioral inhibition or response to novelty may represent a unique area of study for exploring how inborn differences in temperament and emotional reactivity influence susceptibility for stress-related mood disorders. Understanding how heritable and environmental factors interact to contribute to the onset of affective dysfunction in the developing brain is crucial to help pave the way for generating therapeutic interventions.

## 3 | MODELING INDIVIDUAL DIFFERENCES IN EMOTIONALITY IN RODENTS: HIGH RESPONDER/ LOW RESPONDER (HR/LR) RATS

While data from humans demonstrate the importance of temperament in shaping risk for psychiatric disorders, human studies are inherently limited in scope, in part due to the long time course of human neurodevelopment and limited ability to study brain tissue directly. Rodent studies permit observation and measurement across the lifespan and grant access to brain tissue for genetic, molecular, and circuit-based analyses. Furthermore, animal models also permit the ability to directly assess how various environmental factors, such as stress exposure, influence these measures across the developmental trajectory.

Rats, like all organisms, display an array of behavioral responses when placed in a novel situation. Some rats show extensive exploratory behavior (High Responders, HRs), characteristic of “behavioral disinhibition,” while others display much lower levels of exploratory behavior (Low Responders, LRs), characteristic of “behavioral overinhibition.” Thirty years ago, Piazza et al. reported that locomotor response to novelty predicted a rat's propensity to psychostimulant self-administration, which made HR/LR rats an attractive model for studying the neurobiology of addiction (Piazza et al., 1989). Later work showed that novelty-induced

locomotion also predicted differences in anxiety-like behavior and neuroendocrine stress reactivity (Kabbaj et al., 2000). These and other studies demonstrated that LR rats naturally exhibit exaggerated anxiety-like behavior compared to HRs (Kabbaj et al., 2000; White et al., 2007) and act passively under stressful conditions (Clinton et al., 2014; Cohen et al., 2015; Garcia-Fuster et al., 2011; Stedenfeld et al., 2011; Turner et al., 2011). LRs also exhibit a blunted corticosterone (CORT) stress response to novelty (Kabbaj et al., 2000; Marquez et al., 2006) and distinct patterns of neuronal activation in response to novelty stress (Kabbaj & Akil, 2001) compared to HRs. Taken together, these data suggested that LR and HR animals exhibit fundamental differences in emotional reactivity, leading them to interact differently with the environment. These distinct behavioral characteristics are highly reminiscent of the child temperament differences described by Kagan and colleagues, which showed that highly behaviorally inhibited children were more likely to develop anxiety and depression later in life (Kagan & Snidman, 1999). Based on these similarities, we became interested in using the HR/LR model to study the underlying neurobiology of this phenomenon.

### 3.1 | Selective breeding for the HR/LR traits

To explore genetic and early-life environmental factors that influence the HR/LR phenotypes, rats were selectively bred to enrich for the LR or HR behavioral traits (Stead et al., 2006). The founding population of the original HR and LR lines was comprised of 60 male and 60 female Sprague–Dawley rats. Each rat was screened for locomotor response to novelty in a novel test cage (43 × 21.5 × 24.5 cm) equipped with photobeams to monitor horizontal and rearing movements for 60 min. Each animal's locomotor score was determined by summing the horizontal and rearing movements, and the top and bottom 20% scoring males and females (categorized as HR and LR, respectively) were chosen for mating. At each subsequent breeding generation, we screened male and female rats from the HR/LR lines in the same locomotor test screen, choosing the best male and female representatives from each of our 12 HR and 12 LR families for the next breeding round. Additional details for the selective breeding paradigm can be found in the original publication describing and characterizing the lines (Stead et al., 2006). The bred HR/LR lines were initially created in the laboratory of Dr. Huda Akil in 2004 where they have been maintained for more than 50 generations. Our laboratory regenerated the bred lines in 2011 using a similar selective breeding strategy, which created HR and LR lines that exhibited phenotypic differences on par with those observed in the original bred animals.

Over the last 15 years, the bred HR/LR lines have been subjected to an extensive behavioral characterization (outlined below and also described in (Flagel et al., 2014)). These studies illustrated that selective breeding for one trait (locomotor response to a novel environment) produced two lines of rats that exhibit distinct behavior across numerous measures, including anxiety-like behaviors, stress coping style, social interaction, cognition, and behavioral response to psychostimulants, which can be mapped to RDoC domains including Negative Valence, Positive Valence, Social Processes, and Arousal and Regulatory Processes. The characterization of the bred HR/LR animals included comparing them to commercially purchased outbred Sprague–Dawley rats (Stead et al., 2006) as well as a cross-bred F1 line of rats generated by breeding between HR and LR animals (Figure 1). Studies with the cross-bred line showed that male and female cross-bred F1 progeny display intermediate behavioral phenotypes relative to the bred HR/LR extremes (Flagel et al., 2014). For instance, relative to bred HR/LR male and female offspring, the F1 animals exhibit intermediate levels of locomotor response to novelty (Figure 1a), anxiety-like behavior in the Elevated Plus Maze (EPM; Figure 1b), and passive stress-coping behavior in the Forced Swim Test (FST; Figure 1c). Comparisons of the bred HR/LR lines to commercially purchased rats yielded similar results (Stead et al., 2006).

The sections below summarize a series of behavioral findings in the bred HR/LR animals, focusing on behavioral traits that are relevant to mood disorders, with particular attention to how these behavioral domains are described in the context of the RDoC.

### 3.2 | Behavioral Characterization in the HR/LR lines—focus on the RDoC Negative Valence Systems Domain

In 2008, the NIMH Strategic Plan articulated the goal of developing novel approaches for the classification of mental illnesses (https://www.nimh.nih.gov/about/strategic-planning-reports/highlights/highlight-what-is-rdoc.shtml). In support of this effort, the RDoC Initiative was introduced to provide a transdiagnostic research framework meant to facilitate integration of genetic, molecular, cellular, circuit, and behavioral data to provide theoretical and neurobiological underpinnings for altered human behavior. This approach was in contrast to widely used diagnostic criteria employed in clinical practice (summarized in the Diagnostic and Statistical Manual of Mental Disorders [DSM-5] and the International Classification of Diseases [ICD-11]) that define psychiatric disorders based on symptom clusters (American Psychiatric Association, 2013).

The RDoC framework focuses on observable neurobehavioral systems that regulate core psychological functions including motivation, cognition, and social behavior. At present it is composed of six principle domains: (1) Negative

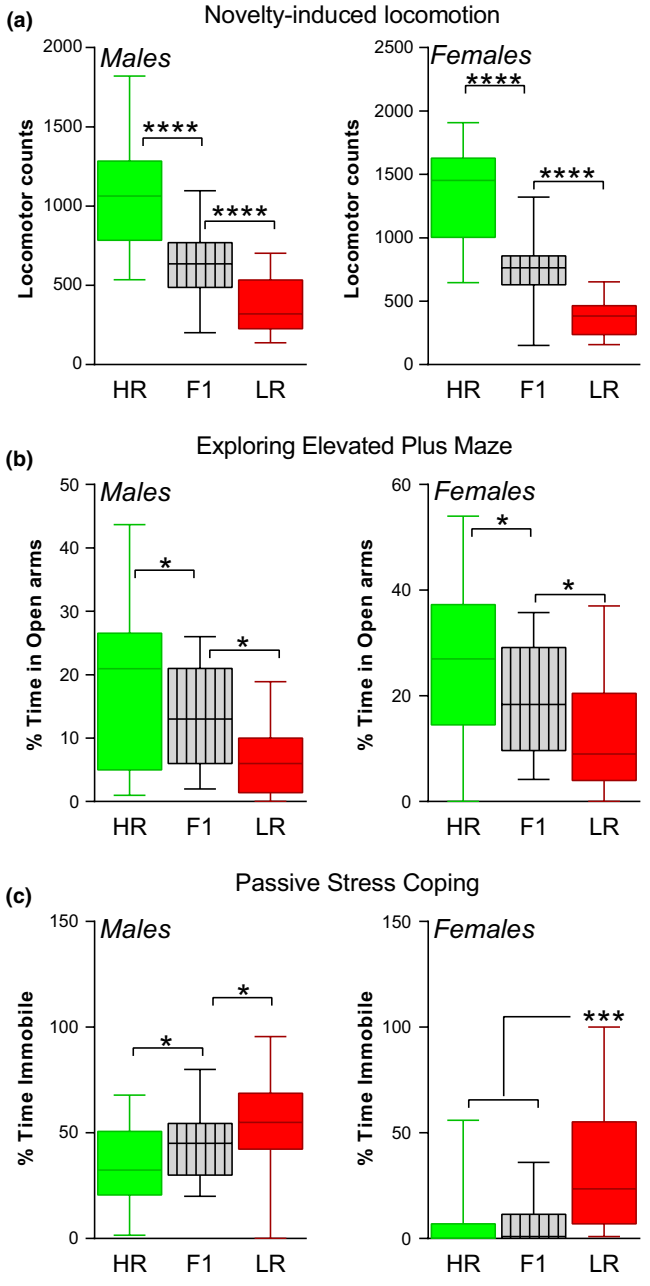


**FIGURE 1** Cross breeding the High Novelty Responder (HR) and Low Responder (LR) lines produces rats with an intermediate behavioral phenotype. F1 progeny are created by mating an HR male/LR female or LR male/HR female. Adult male and female cross-bred F1 offspring were compared to bred HR/LR males and females in a locomotor response to novelty task (a); the Elevated Plus Maze (b); and the Forced Swim Test (c). In the 1-hr novelty-induced locomotor task, F1 cross-bred animals displayed an intermediate level of activity that was significantly lower than the highly active bred HR offspring and higher than LRs (a; **** $p$ <0.0001). In the Elevated Plus Maze, HR rats spent the most time in the anxiogenic open arms relative to LR and F1 groups; LRs showed the least exploration of the open arms and the F1 animals displayed an intermediate level of activity that was significantly different than HR and LR groups (b; * $p$ <0.05). In the Forced Swim Test, LR rats exhibit high levels of passive stress coping (immobility) relative to both HR and F1 animals. Male F1 rats showed an intermediate level of immobility relative to HR and LR animals (c; *$p$ <0.05; ***$p$ <0.001). Data are means ± *SEM*; groups were compared by one-way ANOVA followed by Fisher's LSD post hoc as needed; group sizes were $n$ = 16–17 for HR males/females, $n$ = 33–34 for F1 males/females, and $n$ = 16–18 for LR males/females

Valence Systems, (2) Positive Valence Systems, (3) Cognitive Systems, (4) Social Processes, (5) Arousal and Regulatory Systems, and (6) Sensorimotor Systems (Cuthbert, 2014). These domains are further subdivided into constructs and sub-constructs that can be interrogated using multiple levels of analysis spanning from genes and molecules to behavior and self-reports (Cuthbert, 2014; Insel et al., 2010). These units of analysis constitute putative biological underpinnings of specific functional domains. Importantly, RDoC is continuously evolving, with NIMH holding a number of workshops in the years following its initial introduction to add additional elements to the RDoC matrix. For example, the Sensorimotor Systems domain was added fairly recently (Garvey & Cuthbert, 2017). Likewise, it is notable that each domain is not completely dissociable from the other (e.g., social processes involves elements related to cognition and motivation), and that some behavioral assays (particularly those used in rodents) may tap into multiple domains simultaneously.

The RDoC concept is rooted in the notion that specific behaviors may be characteristic of multiple distinct clinical manifestations or psychiatric disorders. With that in mind, RDoC posits that neurobiological mechanisms driving such behaviors would be conserved among different disorders. For example, psychosis (i.e., delusions and hallucinations) is a core symptom of primary psychotic disorders including schizophrenia or schizophreniform disorder, but psychotic symptoms may also emerge as part of depressive disorders and bipolar disorders (American Psychiatric Association, 2013). The RDoC framework attempts to overcome such challenges created by the clustering clinical symptoms to promote discovery of the neurobiological underpinnings of defined behavioral phenotypes.

Alterations in nearly every RDoC domain and subdomain have been documented in human patients suffering from depression and/or anxiety disorders (Gibb et al., 2016; Holroyd & Umemoto, 2016; Woody & Gibb, 2015). Likewise, the selectively bred HR/LR rat lines are characterized by behavioral differences that fall into multiple RDoC domains, including Positive Valence Systems (for review, see (Flagel et al., 2014; Stedenfeld et al., 2011); Social Processes (Clinton, Kerman, et al., 2011; Davis et al., 2008; Kerman et al., 2011), and Arousal and Regulatory Systems (Kerman, Clinton, et al., 2012). Figure 2 provides a broad overview of emotional behavior and neurobiological differences found in the bred HR/LR

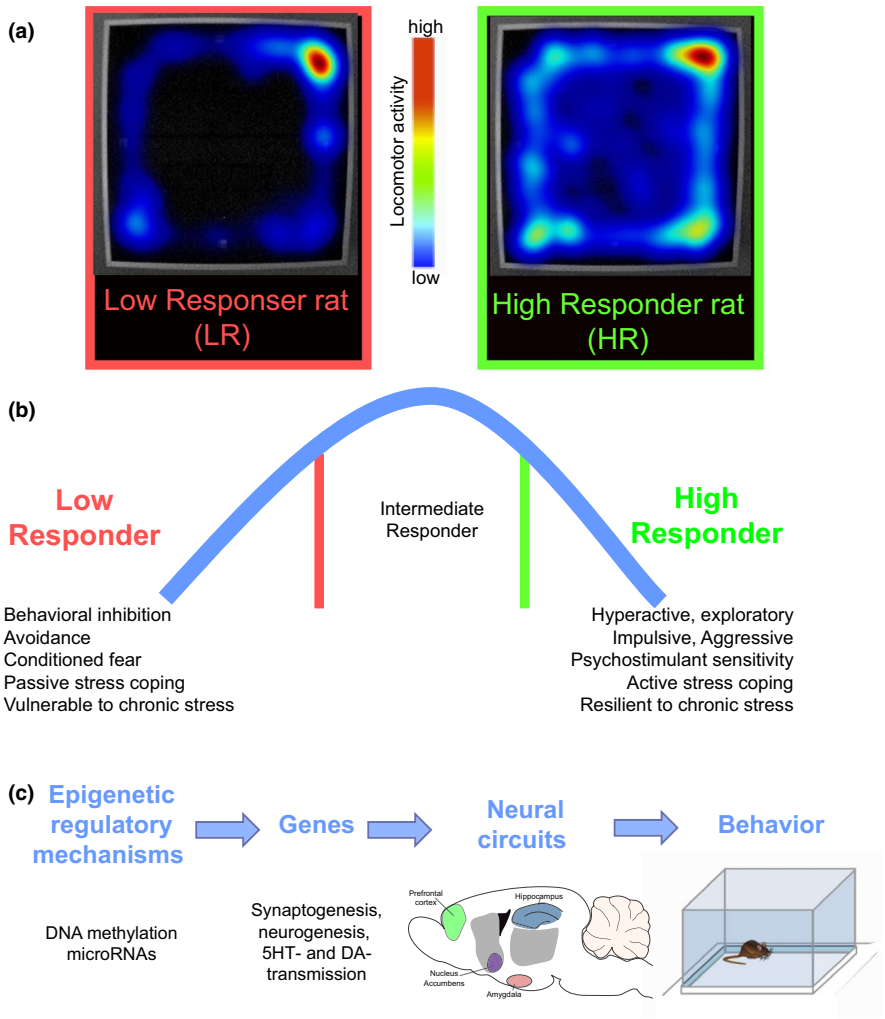


**FIGURE 2** Overview of emotional behavioral and neurobiological differences in the selectively bred Low Novelty Responder (LR) and High Novelty Responder (HR) rats. Heatmaps illustrate typical LR and HR locomotor response to novelty in the 5-min Open Field test (a). The U-shaped curve represents a typical range of locomotor response to novelty, highlighting LR rats in the bottom third of responders, HRs in the top third, and Intermediate Responder rats falling midway between these extremes. Extensive behavioral characterization of the LR/HR lines demonstrates that low versus high locomotor response to novelty predicts behavioral differences across several behavioral domains relevant to depression, with LR rats exhibiting extreme behavioral inhibition, avoidance, fear, passive stress coping, and vulnerability to chronic stress (b). Epigenomic and transcriptomic profiling in multiple limbic brain regions revealed a range of molecular differences in the LR versus HR brain, with the most dramatic alterations occurring in the hippocampus and amygdala (c)

model. For the purpose of this review article, we will focus on HR/LR behavioral differences pertinent to the Negative Valence Systems Domain.

The NIMH convened a workshop of psychiatry and behavioral neuroscience experts to define the Negative Valence Systems domain constructs (https://www.nimh.nih.gov/research/research-funded-by-nimh/rdoc/negative-valence-systems-workshop-proceedings.shtml) in March 2011. Preliminary discussions focused on the constructs of fear, aggression, and distress, which were further refined to include Acute Threat (fear), Potential Threat (anxiety), Sustained Threat, Frustrative Nonreward, and Loss. In the following sections, we highlight each of the Negative Valence Systems domain constructs with the goals of: (1) describing each construct; (2) discussing how these constructs are impacted in depression and anxiety disorders and how the constructs are often examined in rodent behavioral tests; and (3) reviewing data collected in the bred HR/LR animals that recapitulates observed changes in these constructs with respect to clinical depression and anxiety (summarized in Table 1). Subsequent sections of this article explore novel insights provided by using the HR/LR animal model, which are amenable to translational investigations further characterizing neurobiological mechanisms underlying behaviors encompassed within the Negative Valence Systems domain to provide a useful preclinical tool for identifying novel circuits and molecules that underpin dysregulation within the transdiagnostic RDoC Negative Valence Constructs.

**TABLE 1** Summary of behavioral differences in selectively bred High Novelty Responder (HR) versus bred Low Novelty Responder (LR) rats within the context of three Research Criteria Domain (RDoC) Negative Valence domains

| RDoC Negative Valence Constructs | Behaviors | Tests | Phenotype difference | Reference(s) |
|---|---|---|---|---|
| Acute Threat | Conditioned fear (freezing) | Contextual fear conditioning | LR > HR | (Prater et al., 2017; Widman et al., 2019) |
| | Unconditioned fear (freezing) | Defensive Burying Test | LR > HR | (Cohen, Jackson, et al., 2017) |
| | Avoidance | Active Avoidance Task | LR > HR | (Widman et al., 2019) |
| | Passive stress coping | Forced Swim Test | LR > HR | (Clinton et al., 2014; Cohen et al., 2015; Garcia-Fuster et al., 2011; Stedenfeld et al., 2011; Turner et al., 2011) |
| Potential Threat | Exploring new environment | Open-field test, | LR < HR | (Clinton et al., 2008, 2014; Clinton, Stead, et al., 2011; Cohen et al., 2015; Davis et al., 2008; Garcia-Fuster et al., 2011; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011) (Cohen et al., 2015) (Stedenfeld et al., 2011) |
| | Social approach | Elevated plus maze, Light-dark box | LR < HR | |
| | Increased conflict detection | Social interaction Test | LR > HR | |
| | | Novelty-suppressed feeding | | |
| Loss | Anhedonia | Sucrose Preference Test | LR > HR | (Stedenfeld et al., 2011) |
| | Decreased libido | Male sexual behavior | LR > HR | (Cummings et al., 2013) |
| | Psychomotor retardation | Homecage activity | LR > HR | (Kerman, Clinton, et al., 2012) |

## 3.3 | Acute Threat (fear)

The *Acute Threat (fear)* construct is defined as engagement of neurobiological systems to promote defensive behaviors that protect an organism from perceived danger (NIMH, 2011). Clinical observations suggest that individuals diagnosed with a variety of psychiatric ailments, including depression (Hamilton et al., 2012; Miller et al., 2015), bipolar disorder (Phillips & Vieta, 2007), and anxiety (Etkin & Wager, 2007), are more sensitive to negative, potentially threatening stimuli, indicating dysregulation in the Acute Threat construct (Nusslock et al., 2015). Importantly, functional imaging studies have documented shared patterns of dysregulated neuronal activation across a set of common limbic and cortical brain regions in these disorders, including the amygdala and the dorsolateral prefrontal, insular, and anterior cingulate cortices (Etkin & Wager, 2007; Hamilton et al., 2012; Miller et al., 2015; Phillips & Vieta, 2007). These observations are consistent with the theoretical underpinnings of RDoC as they represent a shared neuronal substrate engaged by threatening stimuli across distinct clinical entities.

Fear can be an adaptive response to a situation when an organism is faced with an environment that threatens its survival. Fear can be triggered by a range of stimuli such as the prospect of falling from a great height, exposure to a loud noise or predator scent, or fear of abandonment. Inborn fear is deeply ingrained and evoked even without prior exposure to a fearful stimulus, although it can also be a learned response where an animal associates a previously innocuous stimulus with potential harm (LeDoux, 2003). While multiple approaches have been developed to assess fear in rodents, we will focus on the defensive burying test (Treit et al., 1981) and the active avoidance task (Servatius et al., 2008) as paradigms for responses to an inherently aversive stimulus. We will also discuss contextual and cued fear conditioning as models of learned fear (Phillips & LeDoux, 1992, 1994).

The defensive burying test measures stress coping style where an animal may actively cope with stress by shoveling bedding material onto a noxious stimulus (i.e., a wall-mounted shock probe) or passively cope by avoiding the noxious stimulus and freezing (De Boer & Koolhaas, 2003). We have observed dramatic HR/LR behavioral differences in the defensive burying task, with LRs manifesting high levels of passive coping in the task while HRs display active coping (Cohen, Ata, et al., 2017). Following shock exposure, immobility in LRs increased to nearly 200% of baseline levels, with no change in the HRs' freezing behavior. However, HR rats spent nearly ten times as much time burying the probe following shock exposure (with no HR/LR differences at baseline). Both HR and LR rats greatly decreased their time spent near the probe following shock exposure, so that they both spent virtually no time near the probe (Cohen, Ata, et al., 2017). These observations are consistent with the

notion of LRs being reactive/passive copers and HRs being (pro)active copers.

Related to the notion of LR rats being reactive/passive stress copers relative to proactive coping HRs is their disparate behavioral responses in the Forced Swim Test (FST). In this test, LR rats exhibit a high level of immobility (passive coping) compared to HRs that display high levels of swimming, climbing, and escape behaviors (active coping) (Clinton et al., 2014; Cohen et al., 2015; Garcia-Fuster et al., 2011; Stedenfeld et al., 2011; Turner et al., 2011). The FST is not explicitly addressed by RDoC at present. It seems plausible that stress coping strategy might be appropriately categorized under the "Acute Threat" construct. This is considered more fully in the "Other Considerations" section below.

The active avoidance test is a fear-motivated associative avoidance task where animals learn to associate the occurrence of an aversive stimulus (footshock) with an auditory cue (Servatius et al., 2008). They are then presented with an auditory cue in advance of footshock exposure with the opportunity to avoid or discontinue the shock by pressing a lever. Lever pressing within a short period after the auditory cue to avoid footshock (shock avoidance) represents an active coping strategy because the animal anticipates shock presentation and chooses to turn it off in advance (Servatius et al., 2008). If the animal does not press the lever, the footshock will begin. Lever pressing after commencement of the footshock (escape behavior) represents a more passive coping strategy. We found that the bred HRs and LRs adopted different strategies in the active avoidance task, with HRs adopting an active coping strategy (lever pressing during the tone to avoid shock) while LRs adopted a passive coping strategy (waiting until the shock to lever press) (Widman et al., 2019).

Contextual and cue-induced fear conditioning represent two models of conditioned fear learning. On the first day of contextual fear conditioning, an animal is placed into a test chamber and exposed to multiple inescapable footshocks over a brief period, followed by a second day in which the animal is placed into the same cage for a brief period without footshock. Extensive evidence indicates that acquisition of contextual fear conditioning is a hippocampus-dependent process (Lubin et al., 2008; Lubin & Sweatt, 2007; Phillips & LeDoux, 1992, 1994, 1995). During cue-induced fear conditioning, footshock exposure on the first day is paired with an auditory stimulus. On the subsequent day, the auditory stimulus is presented in the absence of footshock (Phillips & LeDoux, 1992). Freezing behavior is measured on the second day of each of these tests and interpreted as an index of "fear memory."

The bred HR/LR rats have been evaluated both in contextual fear conditioning (Widman et al., 2019) as well as auditory cue-induced fear conditioning (Prater et al., 2017). These studies showed that bred LR rats exhibit markedly greater cue- and context-dependent fear conditioning compared to HRs. It is important to keep in mind the large baseline HR/LR differences in locomotor behavior (Stead et al., 2006), which may have contributed to the differences in freezing. However, no baseline differences in grooming behavior were detected between HRs and LRs, suggested that freezing differences were not solely related to general differences in motor activity. Taken together these observations suggest that as compared to HRs, LR rats have a greater propensity toward forming "fear memory."

Another important factor to consider here is whether there is evidence of the bred HR/LR rats showing general differences in associative learning. Previous studies have demonstrated that the bred HR and LR rats exhibit similar rates of learning on measures of classical conditioning and in operant learning (Flagel et al., 2010). For example, when the bred HR/LR rats are exposed to a Pavlovian conditioning paradigm where the conditioned stimulus (illuminated lever) is paired with food reward, bred HR rats display a "sign-tracking" response (approach the lever when presented), whereas bred LR rats develop a goal-tracking response (approach the food receptacle rather than the lever) (Flagel et al., 2010).

## 3.4 | Potential Threat ("anxiety")

RDoC differentiates the *Potential Threat (anxiety)* construct from the *Acute Threat (fear)* construct by stating that Potential Harm leads to "activation of a brain system in which harm may potentially occur but is distant, ambiguous, or low/uncertain in probability" (NIMH, 2011). The current RDoC matrix lists a single paradigm under this construct—the no-shock, predictable-shock, and unpredictable-shock (NPU) threat task. The NPU task is utilized in clinical research that uses fear-potentiated startle to study fear and anxiety in humans (Schmitz & Grillon, 2012). It consists of delivering shocks in completely predictable manner for a part of the test, as well as in a completely unpredictable manner (Schmitz & Grillon, 2012). Human research utilizing this test has shown that individuals diagnosed with Post-Traumatic Stress Disorder (PTSD) and panic tend to have greater startle responses during the unpredictable shock portion of this test (Grillon, 2008; Grillon et al., 2009). It would be interesting to examine potential HR/LR differences in fear-potentiated startle paradigms, both in the predictable and the unpredictable manners, but, at the time of this writing, there are no such reports.

Several rodent tests of unconditioned anxiety, such as the Open Field Test (OFT), the EPM, and Light-Dark Box, could also fit within the *Potential Harm* construct. Each of these tests involve placing an animal in a novel "approach-avoidance" conflict situation where one area is relatively safe while another area is perceived as less safe. (For example, light, open areas may increase the risk or

probability for predation.) The OFT measures exploratory behavior in a circular or square arena. Rodents find bright open spaces to be aversive and prefer to stay close to the walls. Differences in total exploration, time spent in the anxiogenic center of the maze, latency to enter the center, and anxiety-associated behaviors such as defecation and self-grooming are used to measure an animal's level of anxiety in the Open Field. The EPM consists of four elevated arms arranged in a cross, with two opposite arms enclosed by tall vertical walls while the other two arms are open. The amount of time spent in and number of entries into the safer enclosed arms versus the open arms, as well as latency to enter the open arms, are measures of anxiety-like behavior. The Light-Dark Box is a shuttle box divided into two equal-sized compartments, with one side painted white and brightly illuminated and the other compartment painted black and dimly lit. Again, rodents prefer dark areas to bright ones, and less time spent in the light box and increased latency to initially enter the light compartment are considered signs of anxiety-like behavior.

Rodent avoidance behavior of the center of the Open Field, open arms of the EPM, or brightly illuminated chamber of the Light-Dark box test is thought to be similar to the avoidance behavior observed in humans and in anxiety disorders. This is due in part to the fact that clinically prescribed anxiolytic drugs improve rodent performance on these tasks. For example, treating rats with benzodiazepines prior to testing increases their exploration in the EPM (Simpson et al., 2012), Light-Dark Box (Bourin & Hascoet, 2003), and Open Field (Prut & Belzung, 2003). Additional technical considerations for measuring anxiety in rodents, as well as a detailed discussion of considerations important to the interpretation of the above-mentioned tests, are reviewed elsewhere (Bouwknecht & Paylor, 2008).

The bred HR/LR rats consistently show differences in anxiety-like behavior in the OFT, EPM, and Light Dark Box (Clinton et al., 2008, 2014; Clinton, Stead, et al., 2011; Cohen et al., 2015; Davis et al., 2008; Garcia-Fuster et al., 2011; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011). Bred LR rats spend less time exploring anxiogenic portions of a test apparatus compared to HRs and show increased latency to initially explore the potentially threatening region of the apparatus. In addition, the bred LR rats (compared to HRs) exhibit far greater stress-induced defecation, a physiological indicator of emotional distress (Archer, 1973; Walsh & Cummins, 1976). These findings are consistent with those from other rodent models relevant to anxiety and depression, such as the Maudsley Reactive rat (Berrettini et al., 1994; Commissaris et al., 1990, 1996); Roman High Avoidance Rat (Ferre et al., 1995); and the Wistar Kyoto rat (Courvoisier et al., 1996; Delini-Stula & Hunn, 1985; Nam et al., 2014; Pare, 1993). Another important consideration for these apparent anxiety-related behavior differences is whether they relate to baseline locomotor differences between the lines. Because locomotor activity is a key aspect of the most common rodent tests of anxiety (e.g., the Open Field test, EPM, and Light-Dark box), it is challenging to dissociate general motoric activity from other emotionally relevant behaviors. First, we have found that bred HR and LR rats display similar baseline locomotor activity in their homecage during the light phase of the circadian cycle, which is the time of day when all other behavioral tests are conducted. Second, one study found that treating LR rats with the benzodiapepine chlordiazepoxide effectively reduced anxiety-like behavior (increased time spent in the center of the open field) without changing novelty-induced locomotion (Stead et al., 2006).

## 3.5 | Loss

The RDoC construct of *Loss* is defined as a state of deprivation of a meaningful object, situation, or relationship (e.g., loss of a loved one, a relationship, status, shelter, or loss of behavioral control). Specific behaviors associated with the Loss construct fall into multiple categories, including (1) emotional aspects (rumination, social withdrawal, sadness, amotivation, and anhedonia); (2) cognitive aspects (e.g., deficits in executive function); and (3) physical or physiological aspects (e.g., psychomotor retardation, loss of drive for sleep, and loss of appetite) (NIMH, 2011). Each of these aspects feature prominently in symptomatology of major depression (Woody & Gibb, 2015).

In spite of the wide range of behaviors associated with the Loss construct, at present, only a single paradigm is listed in the RDoC matrix—"sadness eliciting film clips", which is not applicable to rodents. Nevertheless, several relevant behaviors noted in this domain, including social withdrawal, anhedonia, executive function, psychomotor activity, sleep, and appetite, can be readily measured in rodent models. In terms of hedonic drive, LR rats show a relative lack of hedonic drive relative than HRs, showing diminished behavioral response to psychostimulants (Clinton et al., 2012; Cummings et al., 2011; Davis et al., 2008; Flagel et al., 2010, 2016), anhedonia in the sucrose preference test (Stedenfeld et al., 2011), and diminished sexual behavior (Cummings et al., 2013) compared to HRs. LR rats, too, exhibit a greater level of psychomotor retardation compared to HRs, evident both in diminished behavioral response to novelty (Clinton et al., 2008, 2014; Clinton, Stead, et al., 2011; Cohen et al., 2015; Davis et al., 2008; Garcia-Fuster et al., 2011; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011) as well as reduced locomotor activity in their homecage environment during the dark phase of the circadian cycle (Kerman, Clinton, et al., 2012).

## 3.6 | Sustained Threat

RDoC defines the "*Sustained Threat"* construct as an aversive emotional, cognitive, physiological, and behavioral state caused by prolonged (weeks to months) exposure to threatening conditions (NIMH, 2011). Several behaviors categorized under the "Sustained Threat" construct overlap with behaviors noted under other Negative Valance constructs, such as (1) avoidance (noted under "Acute Threat" construct); (2) anxious arousal or attentional bias to threat (relevant to "Potential Harm" construct); and (3) anhedonia or decreased libido (noted under "Loss" construct). Notably, the Negative Valence Systems workshop proceedings mention uncertainty about whether "Sustained Threat" should indeed be considered a separate construct. The group provisionally decided to include Sustained Threat as a distinct Construct but suggest that the topic will be revisited in the future.

Given the ambiguity of the "Sustained Threat" construct, we did not include it in the Table 1 summary of HR/LR differences as multiple relevant behaviors are already noted under the constructs of Acute Threat, Potential Threat, and Loss. One perhaps important detail to note is that the HR/LR behavioral differences described up to this point occur at baseline in HR and LR rats. An important component of the "Sustained Threat" construct relates to the prolonged nature of the threat exposure. We have conducted a series of studies in HR/LR rats that were exposed to a range of chronic stressors, including prenatal stress (Clinton et al., 2008), maternal separation stress (Clinton et al., 2014), and chronic mild stress during adolescence (Rana et al., 2016) or adulthood (Stedenfeld et al., 2011). Each chronic stressor evokes somewhat different effects on behavior and physiology (i.e., HPA-axis reactivity) depending on the timing of the stress exposure period and/or the timing of behavioral and physiology assessment. By and large, though, we found that the bred LR rats were routinely adversely influenced by chronic stress exposure, whereas HRs were consistently resilient.

## 3.7 | Frustrative Nonreward

The last Negative Valence System construct, *"Frustrative Nonreward"*, is defined as aggressive responses elicited by withdrawal of a reward or thwarted attempt of obtaining a reward after repeated efforts (NIMH, 2011). Within the discussion of aggressive behavior, the workshop participants discussed three broad forms of aggression: (1) frustrative nonreward, (2) offensive aggression, and (3) defensive aggression. Defensive aggression was considered a response to real or perceived threat, which would more appropriately fit under the Acute Threat construct. HR/LR rats do display differences in defensive aggression such that HR rats exhibit a relatively high level of attack behavior in a Resident Intruder Test compared to LRs (Kerman et al., 2011). Offensive aggression relates to competition for resources, such as improved social status or access to food, a mate, etc., which falls under the Social Processes Domain of RDoC. No studies to date have evaluated offensive aggression or frustrative nonreward in the bred HR/LR rats.

## 3.8 | Other Considerations

The FST has been a traditional test of rodent "depressive-relevant" behavior for more than 40 years, although this test was curiously absent from Negative Valence System RDoC discussions. Admittedly, there is a great deal of consternation in the field of psychiatric neuroscience as to the appropriate interpretation of FST behavior. The standard format of the test is for rodents to be immersed in an inescapable cylinder of water (~25°C) for a 15-min pretest. Twenty-four hours later, the animal undergoes a 5-min test in which time spent floating/immobile is scored as the primary measure, with some also scoring time spent swimming or climbing and latency to first immobile epoch. Higher immobility time was originally believed to be indicative of greater "depression-like" behavior (sometimes referred to as behavioral despair or helplessness), in part because this measure was found to be sensitive to antidepressant treatment. Despite the widespread use of the FST, the test has undergone intense scrutiny in recent years, noting its lack of face, construct, and predictive validity (for excellent reviews see de Kloet & Molendijk, 2016; Molendijk & de Kloet, 2019). Perhaps some of these points contributed to the exclusion of FST in the RDoC framework. Here, we will discuss our reasoning behind suggesting that FST behavior be included in the Acute Threat construct of the RDoC Negative Valence System domain.

One hallmark of testing procedures under the Acute Threat construct is the presence of an imminent danger to the organism. The section on acute threat also notes that these tests often utilize an escapable or an inescapable stressor in which animals may adopt a proactive (active) or reactive (passive) coping strategy. The FST meets this primary requirement as animals are forced to tread water or sink. Researchers then measure the behavioral response adopted by the rodent—active coping responses (such as swimming, climbing, and diving) and passive coping responses (floating)—when presented with the imminent threat of drowning. In this framework, the switch from active to passive coping is the adaptation of a behavior designed to conserve energy until an escape route presents itself.

We would argue that distinctive HR and LR behavioral responses in the FST, with HRs showing high levels of climbing and swimming while LRs exhibit high levels of immobility, reflect overall differences in HR versus LR stress coping style. This is evident in other behavioral tests relevant to the

Acute Threat construct, including the Defensive Burying test (Cohen, Ata, et al., 2017) and the Resident Intruder test (Kerman et al., 2011), where LRs likewise exhibit passive stress coping responses.

Apart from specific behavioral tests used to evaluate the bred HR/LR rats is to consider how this model compares to other work that has used rats selectively-bred for other behaviors. Over the years, a number of laboratories embarked on selective breeding paradigms to develop lines of rats that exhibit differences in several behaviors relevant to anxiety and depression, including passive stress coping (Weiss et al., 1998) and anxiety-like behavior (Commissaris et al., 1986; Landgraf & Wigger, 2002; Steimer & Driscoll, 2003)). For example, the Swim High-Active versus Swim Low-Active lines were selected based on FST performance (Weiss et al., 1998). Maudsley High- versus Low-Reactive rats were bred according to differences in high versus low defecation when placed in a novel open field (Commissaris et al., 1986). Another group developed the High Anxiety- and Low Anxiety-Bred lines based on performance in the Elevated Plus Maze (EPM), with High Anxiety-Bred rats spending little time in the open arms of the EPM compared to the less anxious Low Anxiety-Bred rats (Landgraf & Wigger, 2002). Although these lines (as well as our bred HR/LR rats) were developed in different rat strains and selected based on disparate behavioral phenotypes, there is considerable convergence across models such that animals exhibiting relatively higher levels of anxiety-like behavior also show enhanced levels of depression-like behavior in the FST and other tests (Abel, 1991; Abel et al., 1992; Commissaris et al., 1996; Einat et al., 2002; Keck et al., 2003; Liebsch et al., 1998; Muigg et al., 2007; Overstreet, 1986; Overstreet et al., 1992, 1995). Moreover, many of these models report fairly similar neurobiological differences between the "anxious/depressive" and "non-anxious/non-depressive" lines, with several studies pointing to differences in the hippocampus (Corda et al., 1997; Kalisch et al., 2006; Tamborska et al., 1986; Weiss et al., 2008; Whatley et al., 1992), which relates to findings discussed in the next section.

## 4 | NEURAL CIRCUIT DIFFERENCES IN THE HR/LR MODEL: FOCUS ON THE HIPPOCAMPUS AND AMYGDALA

A major goal of creating the bred HR/LR lines was to provide phenotypic predictability in order to study the neurodevelopmental underpinnings of the HR/LR phenotypes. This work began with two transcriptome studies in the developing brains of LR and HR offspring (Clinton, Stead, et al., 2011; McCoy et al., 2016). The experiments focused on four brain regions known to regulate emotional behavior (prefrontal cortex, hippocampus, amygdala, and nucleus accumbens) at three early postnatal time points (P7, P14, and P21). The primary questions addressed in these experiments were as follows: (1) when do HR/LR brains begin to develop differently; and (2) what brain regions vary the most in developing HR/LR rats. Our studies revealed robust HR/LR gene expression differences in the developing hippocampus (Clinton, Stead, et al., 2011) and amygdala (McCoy et al., 2016), particularly during the first two postnatal weeks. Some of the most profound HR/LR differences involved genes critical for synaptogenesis and neuroplasticity, suggesting that innate differences in emotionality may be related to differential formation of hippocampal and amygdalar circuits. The implications of disparate hippocampal and amygdalar development in HR/LR rats will be elaborated on in subsequent sections below.

A surprising result of these transcriptome studies was the lack of HR/LR gene expression differences in the developing nucleus accumbens despite the abundant evidence of differences in psychostimulant reactivity and self-administration in the bred HR/LR animals (Davis et al., 2008; Flagel et al., 2010) and commercially purchased HR/LR rats (Piazza et al., 1989). Several studies point to differences within dopaminergic circuits, particularly accumbal dopamine transmission, that may underlie these effects (Flagel et al., 2011; Hooks et al., 1991, 1994; Hooks & Kalivas, 1995). Notably, all of these studies were conducted in adult animals. Thus, the neurobiological factors contributing to drug-induced behavioral differences may emerge in the accumbens later in life (i.e., post-weaning/puberty), which may explain why there were minimal differential gene expression in this region from P7 to P21. Furthermore, major HR/LR gene expression differences in the nucleus accumbens may become apparent only after exposure to drugs of abuse.

We were similarly surprised to find no HR/LR gene expression differences in the developing prefrontal cortex given that some of their behavioral disparities, such as differences in aggression (Kerman et al., 2011) and impulsivity (Flagel et al., 2009), may stem from divergent prefrontal cortical function. As was the case with the nucleus accumbens, it is possible that HR/LR differences in the prefrontal cortex do not emerge until later developmental periods. Indeed, the timing of our transcriptome study (P7-21) falls during a period when the prefrontal cortex is still developing, as cytoarchitectural organization is ongoing in this region through P18 (van Eden et al., 1990). It is possible that more prominent prefrontal cortical HR/LR differences may become apparent in adolescent and/or adult animals. It is also possible that gene expression differences in HR/LR prefrontal cortex, as well as in other regions, are cell type specific and lost when expression is measured from whole brain tissue.

### 4.1 | Hippocampal differences in the HR/LR model

The hippocampus plays a well-known and long-established role in learning and memory functions (Eichenbaum et al., 1992; Ergorul & Eichenbaum, 2004; Fortin et al., 2002; Shapiro & Eichenbaum, 1999; Squire, 1992), although it is also essential for several functions broadly related to emotional behavior. For instance, the hippocampus plays key roles in detecting novelty, spatial, and contextual information from the environment (Jeewajee et al., 2008; Kumaran & Maguire, 2007; Lever et al., 2006), modulating the HPA stress axis (Jacobson & Sapolsky, 1991; McEwen et al., 1992), and regulating fear and anxiety-like behavior (Bannerman et al., 2004; Bertoglio et al., 2006; Engin & Treit, 2007; Gray & McNaughton, 1996; McNaughton & Gray, 2000; Moser & Moser, 1998). The hippocampus has been suggested to be at the center of a neural "behavioral inhibition system" that is activated during anxiety-provoking or conflict situations to signal whether an organism should approach or avoid a potential threat. Accordingly, it has been postulated that excessive perception of threat and/or exaggerated levels of anxiety-like behavior may derive from aberrant activation of this behavioral inhibition circuit (McNaughton, 1997). Furthermore, data from human neuroimaging studies support the notion that limbic circuitry, including the hippocampus, is dysregulated in patients suffering with anxiety and mood disorders (Brambilla et al., 2002; Bystritsky et al., 2001; Kumari et al., 2007; Mathew & Ho, 2006; Phillips et al., 2003; Rusch et al., 2001; Weniger et al., 2006).

Work in outbred HR/LR rats found altered expression of genes related to dopamine, serotonin, and the HPA axis in the hippocampus (Ballaz et al., 2007b; Kabbaj, 2004; Kabbaj et al., 2000; Rosario & Abercrombie, 1999). Other studies found that LRs had increased cell proliferation and cell number in the dentate gyrus relative to HRs (Lemaire et al., 1999). LRs were also shown to have a larger suprapyramidal mossy fiber terminal field compared to HRs (Isgor et al., 2004), a difference that has been linked to emotionality and anxiety-like behavior (Belzung, 1992; Prior et al., 1997). Our later findings in adult bred HR/LR animals corroborated these findings, showing altered cell proliferation and gene expression (Garcia-Fuster et al., 2010; Perez et al., 2009) in the adult HR/LR hippocampus as well as changes in the early postnatal hippocampus (Clinton, Stead, et al., 2011; Simmons et al., 2012). Moreover, we found that pharmacologically manipulating the hippocampus of "anxiety-prone" bred LR rats during the first week of life (via treatment with the growth factor Fibroblast Growth Factor, FGF) shifted adult behavior, leading bred LRs to display reduced anxiety-like behavior and enhanced behavioral response to novelty (Turner et al., 2011).

Recent hippocampal slice synaptic physiology experiments in adult HR/LR rats found that many aspects of hippocampal function, including long-term depression (LTD), are similar between HR and LR rats. However, in contrast to bred HRs, LR rats show significantly diminished N-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) and decreased spine density in the hippocampus (Widman et al., 2019). Due to known HR/LR differences in stress reactivity (Clinton et al., 2008; Kerman, Clinton, et al., 2012) and the effects of stress on LTP at CA3-CA1 synapses (Miller et al., 2018; Shors & Thompson, 1992), it is possible that HR/LR differences in LTP are related to these factors. Transcriptome profiling the in adult HR/LR hippocampus identified changes in the metabotropic glutamate receptor mGlur5. Other studies have demonstrated an association between mGlur5 expression and positive stress coping and stress resilience (Piers et al., 2012; Sun et al., 2017), so it is conceivable that HR rats' increased mGlur5 levels relative to LRs may be associated with their proactive coping styles (Cohen, Ata, et al., 2017). Additionally, the increased mGlur5 levels in HR hippocampus may contribute to other neurophysiological differences, such as changes in mGluR-dependent LTD. Other studies have demonstrated enhanced mGluR-dependent LTD in young, congenitally learned helpless rats (Pignatelli et al., 2013) and after acute treatment with CORT (Chaouloff et al., 2008), suggesting high mGluR receptor activation in animals with more depressive-relevant behaviors.

Taken together, these data suggest that differences in the formation and/or functioning of the HR versus LR hippocampus contribute to the distinctive ways that LR and HR animals interact with their environment and respond to novelty and exposure to stress. The volume of the CA region of the hippocampus rapidly expands from gestational day 21 through P1 (Bayer, 1980a,b), with its dendritic system rapidly expanding throughout the first 3 postnatal weeks (Pokorny & Trojan, 1986). In the dentate gyrus, a fraction of granule cells are generated around gestational day 16–17, but the majority (85%) of the cells are generated from P0 to P19 and migrate from P10 to P25. Our transcriptome studies in the developing HR/LR hippocampus revealed dramatic HR/LR gene expression differences in the hippocampus at P7 and P14, with minimal changes at P21 (Clinton, Stead, et al., 2011; McCoy et al., 2016). These findings suggest that there is a critical developmental window when hippocampal circuits diverge in HR/LR rats, which may, in turn, contribute to life-long differences in behavior and HPA axis stress reactivity.

### 4.2 | Amygdalar differences in the HR/LR model

Although much of our work in the HR/LR model points to hippocampal differences contributing to the disparate HR/LR phenotypes, recent work from our laboratory highlights

a role for the amygdala as well. The amygdala regulates fear and a number of other emotional behaviors, in part, by processing incoming emotionally salient, sensory information from the environment (LeDoux, 2000; Phelps & Anderson, 1997). The amygdala acts in cooperation with the hippocampus to modulate anxiety-like behavior, and a perturbation of hippocampal–amygdala circuits likely contributes to emotional dysregulation in anxiety and mood disorders (Brambilla et al., 2002; Bystritsky et al., 2001; Kumari et al., 2007; Mathew & Ho, 2006; Phillips et al., 2003; Rusch et al., 2001; Weniger et al., 2006). Dysfunction within either the hippocampus or amygdala, or altered connectivity between these regions, could engender an excessive perception of threat and thus give rise to an overly anxious state (McNaughton, 1997).

Our transcriptome studies in the early postnatal HR/LR brain pointed to marked gene expression differences in the developing amygdala (McCoy et al., 2016), and we later found that myriad HR/LR gene expression changes persisted in the adult amygdala (McCoy et al., 2017). Across these transcriptome studies, we found that cellular metabolism was altered between HR/LR animals. During development, cytochrome c oxidase (COX) activity, which can serve as a measure of ATP production and overall energy consumption, is reduced in LRs relative to HRs in the first 2 weeks of life. Interestingly, this difference disappears by the third week and is not present in adulthood. Although differences in COX were not present in adulthood, we found an increase in the oxygen consumption reserve capacity of mitochondrial isolates from LR amygdala, meaning that LRs have the capacity for a more dynamic working range in energy production to support energetic demands necessary for neurotransmission.

Other work by our group showed that LRs' amygdalar transcriptome was sensitive to the early life manipulation of cross-fostering. Cross-fostering LR pups to HR mothers leads adult offspring to display reduced anxiety-like behavior in the OFT and increased social interaction (Cohen et al., 2015). We conducted a transcriptome study in the amygdala and hippocampus of LRs raised by their biological LR mother, a LR foster mother, or a HR foster mother. We found that cross-fostering LR pups to HR mothers dramatically changed the developing amygdalar transcriptome but did not affect LRs' hippocampal gene expression.

A number of studies by the Sullivan laboratory and others have elegantly described the ontogeny of amygdalar circuits, amygdala-regulated fear, and stress responses, and how perturbations of the developing rodent amygdala elicit lasting changes in emotional behavior (Landers & Sullivan, 2012; Tallot et al., 2016; Thompson et al., 2008). For example, excitotoxic lesions of the P7 rat amygdala impair juvenile play and adult social behavior, increase novelty-induced ambulation (Daenen et al., 2002; Wolterink et al., 2001), and exacerbate amphetamine-induced locomotion (Solis et al., 2009). Lasting neural consequences of P7 amygdala lesions include altered dopamine receptor density in the adult nucleus accumbens, olfactory tubercle, substantia nigra, and central grey (Bouwmeester et al., 2007), as well as decreased cerebral glucose utilization in several limbic brain regions, including the cingulate cortex, lateral septum, anterior hippocampus, and the amygdala itself (Gerrits et al., 2006). It is interesting to consider whether HR/LR differences in amygdala development contribute to their disparate biobehavioral phenotypes. Although our findings in the HR/LR model do not perfectly align with the P7 amygdala lesion literature, HR/LR rats show divergent social behavior as well as novelty- and psychostimulant-induced locomotion (Clinton et al., 2012; Cohen et al., 2015; Stead et al., 2006). They also exhibit a number of dopamine system anomalies, including altered expression of dopamine receptor transcripts in the nucleus accumbens and striatum, and a greater number of spontaneous dopamine release events in the nucleus accumbens (for review, see Flagel et al., (2014) and the section below on Dopamine markers in HR vs. LR brain). New studies should examine anatomical and functional differences in the developing HR/LR amygdala that may contribute to their disparate behavioral phenotypes. Future experiments should also examine the impact of manipulating the amygdala of HR/LR rats during early postnatal development to determine its effects on their adult phenotypes.

## 5 | MOLECULAR ALTERATIONS THAT CONTRIBUTE TO INDIVIDUAL DIFFERENCES IN EMOTIONAL BEHAVIOR

Myriad abnormalities in brain structure, function, epigenetic regulation, and gene expression occur in individuals with psychiatric disorders like depression and anxiety (Bagot et al., 2016; Bernstein et al., 2010; Duman et al., 1997; Nestler, 2015; Nestler et al., 2002; Sabunciyan et al., 2012; Tsankova et al., 2007). Although there is abundant evidence for epigenome and transcriptome alterations in the brains of psychiatric patients, it remains unclear whether such changes represent inborn aberrations that trigger psychopathology, are secondary to factors like stress exposure, or are a combination of both. Model organisms like the selectively bred HR/LR rats offer a tool to dissect these factors and inform work in humans. A range of molecular pathways differ at baseline in the brains of the bred HR/LR rats, ranging from classic neurotransmitter systems like serotonin (Clinton, Kerman, et al., 2011; Kerman et al., 2011) and dopamine (Flagel et al., 2010, 2011), to melanin-concentrating hormone (Garcia-Fuster et al., 2011), the Fibroblast Growth Factor system (Clinton et al., 2012; Perez et al., 2009), and epigenetic processes such as microRNAs and DNA methylation (McCoy

et al., 2017, 2019). Below we discuss a few examples of molecular alterations relevant to depression that may contribute to the emergence of the HR/LR phenotypic differences, first focusing on classical neurotransmitter systems serotonin and dopamine, and then epigenetic processes such as DNA methylation and microRNAs that differ in the LR versus HR brain.

## 5.1 | Overview of serotonin and dopamine system changes in clinical depression

Two major brain areas that synthesize dopamine in the brain are the ventral tegmental area (VTA), which supplies dopamine to the nucleus accumbens and forebrain, and the substantia nigra pars compacta, which supplies dopamine to the basal ganglia. Serotonin in the brain is synthesized by neurons within the raphe nuclei located along the midline throughout much of the rostro-caudal extent of the brainstem (Jacobs & Azmitia, 1992). Extensive evidence points a critical role for monoamine deficiency in the pathophysiology of depression, including abnormalities of the dopamine and serotonin systems (Belujon & Grace, 2017; Dunlop & Nemeroff, 2007). For example, reduced levels of dopamine metabolites have been reported in cerebrospinal fluid of depressed patients (Ogawa et al., 2018), and alterations of tyrosine hydroxylase (key enzyme required for dopamine synthesis), monoamine oxidase (an enzyme involved in dopamine degradation), the dopamine reuptake transporter (DAT), and D2 dopamine receptors have been found in the brains of depressed patients (Dunlop & Nemeroff, 2007; Meyer et al., 2006, 2009; Pizzagalli et al., 2019). Abnormal dopamine function could play a role in several symptoms of depression, including anhedonia, psychomotor retardation, amotivation, and impaired concentration (Dunlop & Nemeroff, 2007; Nestler & Carlezon, 2006). Alterations of the serotonin system include morphological changes within raphe nuclei and altered serotonin-related gene expression, protein levels, and serotonin receptor binding (Bunney & Davis, 1965; Coppen, 1968; Hirschfeld, 2000; Kerman, Bernard, et al., 2012; Lapin & Oxenkrug, 1969; Leonard, 2000; Mann, 1999; Ressler & Nemeroff, 2000; Schildkraut, 1965). The monoamine hypothesis of depression is further supported by the fact that first-line treatments for depression, including selective serotonin reuptake inhibitor (SSRI) antidepressants, increase synaptic serotonin levels (Blier, 2001; Hamon & Blier, 2013; Leonard, 2000; Papakostas et al., 2007), while depletion of serotonin and other monoaminergic transmitters can induce a depressive state (Ruhe et al., 2007).

## 5.2 | Serotonin system alterations in HR/LR rats

Adult male HR/LR rats exhibit a number of serotonin system differences that resemble some of the serotoninergic alterations reported in depressed patients. For example, compared to HRs, LR rats exhibit lower mRNA expression of tryptophan hydroxylase 2 (rate-limiting enzyme for serotonin synthesis) and the serotonin reuptake transporter in the dorsal raphe, median raphe, and B9 cell group (Kerman et al., 2011). LRs also show lower levels of the serotonin metabolite 5-hydroxyindoleacetic acid in the cerebrospinal fluid together with higher *5ht1a* receptor mRNA levels in the cingulate, lateral septum, and CA1 region of the hippocampus compared to HRs (Clinton, Kerman, et al., 2011). Other studies report increased *5ht2a* (Calvo et al., 2011), *5ht6,* and *5ht7* (Ballaz et al., 2007a) receptor mRNA in multiple forebrain regions of LR versus HR rats.

Based on these HR/LR serotonin system differences and work showing that serotonin neurotransmission modulates behaviors such as stress coping style that are known to differ in HR/LR rats (Chung et al., 2000; Gardner et al., 2005), we used c-fos immunocytochemistry to examine neuronal activation within the dorsal raphe of HR/LR rats following the defensive burying test (Cohen, Ata, et al., 2017). As noted earlier, HR rats exhibit high levels of active coping (probe burying) in this task while LRs show a reactive/passive coping response (freezing) (Cohen, Ata, et al., 2017). We hypothesized that these distinct HR versus LR stress coping styles would be accompanied by distinct stress-induced activation of raphe nuclei. Within several sub-regions of the dorsal raphe we found greater overall serotonin cell activation in HR vs. LR rats (both at baseline as well as following shock exposure). These rostral raphe groups project to regions important to dopaminergic signaling, including the substantia nigra, nucleus accumbens, and striatum (Commons, 2015, 2016; van der Kooy & Hattori, 1980; Stratford & Wirtshafter, 1990). Such differences could contribute to HR/LR dopamine system differences and related behaviors (detailed in the next section). Within the caudal aspect of the dorsal raphe nucleus, dorsal part (cDRD), there were no baseline HR/LR differences, but shock exposure in the defensive burying task elicited diminished activation of cDRD cells in LRs compared to HRs. The cDRD targets several structures of the limbic system including the septum, hippocampus, and the central amygdala (Abrams et al., 2005; Commons, 2015, 2016; Commons et al., 2003; Steinbusch et al., 1980; Waterhouse et al., 1986). While the central amygdala has been considered a minor target of the cDRD, it is possible that HR/LR differences in serotoninergic activation in the cDRD following shock exposure contribute to their distinct coping styles through modulation of this region.

These c-fos findings in HR/LR rats following the defensive burying task are generally consistent with work showing that reducing serotoninergic levels promotes a reactive coping response in the defensive burying test (Lopez-Rubalcava et al., 1996, 1999), while increasing serotoninergic signaling triggers proactive coping in the task (Frye & Seliga, 2003).

Likewise, other studies show that increasing serotoninergic signaling triggers proactive coping in the FST, while diminished serotonin levels are associated with passive coping (immobility) in the FST (Abrams et al., 2005; Cryan et al., 2005; Overstreet & Griebel, 2004; Piras et al., 2010, 2013). As noted above, HR/LR rats exhibit distinct coping strategies in the FST, with LRs typically showing high levels of immobility while HRs spend more time swimming and climbing (Clinton et al., 2014; Cohen et al., 2015; Garcia-Fuster et al., 2011; Stedenfeld et al., 2011; Turner et al., 2011). These behavioral differences are likely linked to disparate serotonin levels as 4 weeks of treatment with the SSRI paroxetine (10 mg/kg p.o.) effectively reduced LR rats' high levels of FST immobility (Glover et al., 2015).

### 5.3 | Dopamine system changes in HR/LR rats

Numerous studies in outbred HR/LR rats demonstrate that LRs exhibit a "hypo-dopaminergic state" relative to HRs (Hooks et al., 1992; Hooks & Kalivas, 1994; Marinelli & White, 2000; Piazza et al., 1991), and work in the bred HR/LR rats found similar dopamine system differences. Bred LR rats have lower tyrosine hydroxylase mRNA levels in the VTA compared to bred HRs, but no differences in the substantia nigra (Clinton et al., 2012). Fast-scan cyclic voltammetry experiments revealed fewer spontaneous dopamine release events in the nucleus accumbens core of bred LR versus bred HR rats (Flagel et al., 2010), and in vivo microdialysis showed diminished basal and cocaine-evoked dopamine levels in the nucleus accumbens of bred LR versus HR rats (Mabrouk et al., 2018). The bred HR/LR rats likewise exhibit differences in dopamine receptor expression, with LRs having decreased D1 receptor mRNA expression in the accumbens core and shell relative to HRs, but increased D2 receptor mRNA levels in accumbens and caudate putamen (Clinton et al., 2012).

The observed HR/LR dopamine system differences likely contribute to multiple aspects of their disparate behavioral phenotypes, including differences in behavioral response to novelty (Hooks & Kalivas, 1994; Li, Illenberger, et al., 2019) as well as bred LR rats' diminished acute and sensitized locomotor response to cocaine (Clinton et al., 2012; Cummings et al., 2011; Davis et al., 2008; Flagel et al., 2010; Garcia-Fuster et al., 2009, 2010) and reduced cocaine self-administration relative to bred HRs (Cummings et al., 2011; Davis et al., 2008; Flagel et al., 2016). Bred LR rats also show signs of increased anhedonia relative to bred HRs, including reduced sucrose preference (Stedenfeld et al., 2011) and diminished sexual behavior (Cummings et al., 2013). Altered dopamine function within VTA-accumbal circuitry has been hypothesized to play a role in anhedonic symptoms of depression (Belujon & Grace, 2017; Nestler & Carlezon, 2006), and rodent studies support this notion, showing that chronic stress-induced increases in anhedonia in the sucrose preference test are accompanied by transcriptome changes in the VTA (Warren et al., 2013) as well as diminished firing of VTA neurons (Chaudhury et al., 2013). The disparate HR versus LR stress coping styles may also stem from dopaminergic system differences given that passive coping (such as high immobility in the FST) has been associated with diminished dopaminergic activity (Molendijk & de Kloet, 2019). For example, optogenetic approaches have shown that inhibition of dopamine-producing VTA neurons leads to increased FST immobility while activation of those cells triggers active coping (swimming, climbing, and escape attempts) (Tye et al., 2013). Thus, alterations in dopaminergic system function in HR/LR animals likely contribute not only to their contrasting responses to novelty but also to behavioral and affective domains relevant to clinical depression as well.

### 5.4 | Epigenetic changes in human psychiatric illness—focus on DNA methylation and microRNAs

Epigenetic mechanisms are reversible modifications of DNA that regulate gene expression and/or genomic structure without changing the nucleotide sequence. These processes, which include DNA methylation, histone modifications, and noncoding RNAs like microRNAs, can be stable and heritable, but also can be altered by natural and environmental cues, such as changes during brain development, aging, learning, and stress (Bedrosian et al., 2018; LaSalle, 2011; McGowan et al., 2009; Murgatroyd et al., 2009; Naumova et al., 2012; Roth et al., 2009). There is growing evidence of abnormal epigenetic processes playing a role in the neurobiology of psychiatric disorders, although the precise nature of these anomalies remains largely unknown (Bagot et al., 2014; Lutz et al., 2015; Mill & Petronis, 2007). Because epigenetic mechanisms simultaneously regulate myriad genes, perturbation of a regulatory system such as DNA methylation and microRNAs could induce many gene expression changes and downstream biobehavioral effects that are observed in the brains of individuals suffering psychiatric disorders like depression.

DNA methylation involves the addition of a methyl group to 5' carbon of the cytosine nucleotides by DNA methyltransferases (DNMTs) (Russo et al., 1996). There are three main DNMTs found in the brain: DNMT1, DNMT3a, and DNMT3b. DNMT1 is considered a maintenance enzyme that, during replication, methylates sites on the daughter DNA strand to match the methylated cytosines on the parent DNA strand. DNMT3a and DNMT3b are considered de novo

enzymes due as they are active at unmethylated sites on both the daughter and parent DNA strands and outside of the DNA replication process (Buck-Koehntop & Defossez, 2013). DNA methylation serves a multitude of functions in cells, including controlling gene expression, gene splicing, DNA repair, and chromatin remodeling (Chahrour et al., 2008; Georgel et al., 2003; Horike et al., 2005; Squillaro et al., 2010; Young et al., 2005). Several studies have reported genome-wide and loci-specific DNA methylation changes in depressed patients, including changes within peripheral blood samples (Li, D'Arcy, et al., 2019; Palma-Gudiel et al., 2020) and brain tissue (typically sub-regions of prefrontal cortex) (Aberg et al., 2020; Haghighi et al., 2014; Murphy et al., 2017; Nagy et al., 2015; Oh et al., 2015).

MicroRNAs (miRNAs) represent another major epigenetic mechanism that potentially regulates emotional reactivity and vulnerability to emotional disorders (O'Connor et al., 2013; Rinaldi et al., 2010; Smalheiser et al., 2012). These are short noncoding RNAs that control mRNA target translation and/or stability by binding to a 6 to 8 base pair complimentary "seed region" on the 3' UTR of the mRNA (Bartel, 2004). Because each microRNA potentially targets several genes, co-expression of only a few microRNAs exerts powerful control over large gene networks (Xiong et al., 2011). Acute and chronic stress alter microRNA expression in the brain (Rinaldi et al., 2010), and microRNA differences have been linked to adaptive and maladaptive stress coping as well as susceptibility to depression (Smalheiser et al., 2011, 2012). Likewise, there is growing evidence for altered microRNA expression in brain tissue from subjects that suffered with major depression (Dwivedi, 2011; Serafini et al., 2014).

Studies in humans document DNA methylation, microRNA, and other epigenetic alterations in the brain in patients suffering mood disorders such as depression, although precise mechanisms that link these changes to functional changes in brain and behavior remain elusive. Another key knowledge gap in the field relates to the developmental timeline when such epigenetic changes unfold because most studies in psychiatric patients and model animals relevant to psychopathology focus on changes in the adult brain. Examining epigenetic abnormalities that contribute to the emergence of emotional dysfunction may shed light on the pathogenesis of psychiatric disorders. Moreover, as epigenetic processes are largely modifiable through pharmacological approaches or even dietary factors, epigenetic-targeted therapies offer attractive new treatment opportunities (Szarc vel Szic et al., 2010). Model organisms like the HR/LR rat lines offer a tool to dissect these factors and inform work in humans. The following sections describe our studies to date describing DNA methylation and microRNA differences in the HR/LR brain. This information is also summarized in Table 2.

## 5.5 | DNA methylation alterations in the HR/LR model

Later sections describe our laboratory's transcriptome studies that revealed vast gene expression differences in the developing hippocampus and amygdala of bred HR versus LR rats (Clinton, Stead, et al., 2011; McCoy et al., 2016). Among the many genes that were differentially expressed in the bred HR versus LR limbic amygdala and hippocampus were molecules involved in epigenetic processes, particularly DNA methylation. This finding pointed to a regulatory mechanism that could underlie widespread HR/LR differences in gene expression and ultimately behavior. Based on these findings, we hypothesized that HR/LR differences in neural DNA methylation patterns constitute a key phenotype-driving molecular mechanism and that manipulating methylation in the developing brain (to recapitulate an "HR-like" methylation pattern) can modulate adult emotional behavior. To address these questions, we first examined mRNA and protein levels of DNA methyltransferases DNMT1, DNMT3a, and DNMT3b (enzymes that mediate DNA methylation), as well as global DNA methylation (5-methylcytosine) levels, in the early postnatal HR versus LR brain. We found increased expression of DNMT mRNAs in the P7 and P14 amygdala and hippocampus (Simmons et al., 2012) as well as increased DNMT1 and DNMT3B protein and increased global DNA methylation levels in the P7 and P14 amygdala of LR versus HR pups (McCoy et al., 2019). Using a next-generation sequencing approach, we analyzed gene-specific methylation differences in the P7 HR versus LR amygdala. This assessment revealed a preponderance of genomic sites that were hypermethylated in LR versus HR samples, which was consistent with our finding of increased global methylation in the P7 LR versus HR amygdala. Over half of the differentially methylated sites occurred within 10 kb of genes, suggesting that they could play a role in transcriptional activity. The top molecular pathways that showed evidence of differential DNA methylation involved genes related to glutamate neurotransmission, synaptic plasticity, Rap1 signaling, and PI3K-Akt signaling. These changes coupled with our previous transcriptome findings in the early postnatal HR versus LR amygdala suggest that amygdala circuits may develop differently in HR/LR rats, contributing to their disparate emotional behavior phenotypes (McCoy et al., 2019).

Based on findings of increased DNA methylation levels in the developing amygdala of LR versus HR pups, we next investigated the role of these methylation differences in shaping disparate HR/LR behavioral phenotypes in adulthood. We hypothesized that LRs' elevated DNA methylation levels in the early postnatal amygdala (relative to HRs) contribute to differences in limbic brain development and ultimately to their disparate anxiety-/depression-like behaviors. We found that decreasing DNA methylation in the early postnatal

**TABLE 2** Summary of classic neurotransmitter system (serotonin, dopamine) and epigenetic (DNA methylation, microRNA) changes in the HR versus LR brain. Abbreviations: DNA methyltransferase 1 (Dnmt1); dopamine (DA); Enhancer of zeste homolog 2 (EZH2); histone H3 lysine 27 tri-methylation (H3K27-me3); microRNA-101a (miR-101a); next-generation sequencing (NGS); postnatal day (P); serotonin transporter (Sert); Tryptophan hydroxylase 2 (Tph2); Tyrosine hydroxylase (Th)

| Molecular pathway | Phenotypic difference | Brain region | Reference(s) |
|---|---|---|---|
| Serotonin | LR < HR *Tph2* and *Sert* mRNA | Dorsal raphe, median raphe, B9 | (Kerman et al., 2011) |
| | LR < HR stress-induced *cfos* activation | Caudal dorsal raphe | (Cohen, Ata, et al., 2017) |
| | LR > HR *5ht1a* receptor mRNA | Cingulate, lateral septum, hippocampus | (Clinton, Kerman, et al., 2011) |
| | LR > HR *5ht2a* receptor mRNA | Parietal cortex | (Calvo et al., 2011) |
| | LR > HR *5ht6*, *5ht7* receptor mRNA | Mediodorsal thalamus | (Ballaz et al., 2007a) |
| Dopamine | LR < HR *Th* mRNA | Ventral tegmental area | (Clinton et al., 2012) |
| | LR < HR D1 receptor mRNA | Nucleus accumbens core/shell | (Clinton et al., 2012) |
| | LR > HR D2 receptor mRNA | Nucleus accumbens, caudate putamen | (Clinton et al., 2012) |
| | LR < HR spontaneous DA release events | Nucleus accumbens core | (Flagel et al., 2010) |
| | LR < HR basal and cocaine-evoked DA | Nucleus accumbens | (Mabrouk et al., 2018) |
| DNA methylation | LR > HR *Dnmt1* mRNA and DNMT protein | P7 amygdala<br>P7 amygdala | (McCoy et al., 2019; Simmons et al., 2012) |
| | LR > HR 5-methylcytosine levels | P7 amygdala | (McCoy et al., 2019) |
| | LR > HR number hypermethylated genomic sites detected in NGS sequencing analysis | Adult amygdala | (McCoy et al., 2019) |
| | LR < HR number hypermethylated genomic sites detected in NGS sequencing analysis | | (McCoy et al., 2017) |
| microRNA | NGS sequencing revealed numerous microRNAs differing in LR versus HR | Dorsal raphe and amygdala | (Cohen, Ata, et al., 2017) |
| | LR < HR miR−101a, LR > HR EZH2, and H3K27-me3 | Dorsal raphe | (Cohen, Ata, et al., 2017; Cohen, Jackson, et al., 2017) |
| | LR > HR miR−101a, LR < HR EZH2, and H3K27-me3 | Amygdala | (Cohen, Ata, et al., 2017; Cohen, Jackson, et al., 2017) |

amygdala (via a dietary manipulation or siRNA-mediated approach) led to improved anxiety- and/or depression-like behavior in adulthood (McCoy et al., 2019).

Our findings of altered gene expression and DNA methylation in the early postnatal HR versus LR amygdala led us next to examine whether these changes persist into adulthood (McCoy et al., 2017). To test this, we performed transcriptome profiling and examined several DNA methylation markers in the adult HR versus LR amygdala. Although we found similar levels of DNMT proteins in the adult HR/LR amygdala, next-generation sequencing methylome analysis revealed 793 differentially methylated genomic sites between the groups. Most of the differentially methylated sites were hypermethylated in HR versus LR, so we next tested the hypothesis that enhancing DNA methylation in LRs would improve their anxiety/depression-like phenotype. We found that increasing DNA methylation in LRs (via increased dietary methyl donor content) improved their anxiety-like behavior and decreased their typically high levels of FST immobility; however, dietary methyl donor depletion exacerbated LRs' high FST immobility. These data are generally consistent with findings in depressed patients showing that treatment with DNA methylation-promoting agents improves depressive symptoms (Papakostas et al., 2012; Papakostas, Shelton, et al., 2012), and highlights epigenetic mechanisms that may contribute to individual differences in risk for emotional dysfunction.

## 5.6 | MicroRNA changes in HR/LR brain

Earlier, we described a study that examined expression of the immediate early gene c-fos to reveal distinct stress-induced activation of the dorsal raphe and amygdala of HR/LR rats following the defensive burying test. To interrogate molecular pathways that contribute to HR/LR differences in defensive burying behavior and shock-induced neuronal activation in the dorsal raphe and amygdala, we conducted a next-generation sequencing study to assess mRNA and microRNA expression in these brain regions. Our analysis identified numerous mRNA and microRNA species that were differentially expressed in HR/LR dorsal raphe and amygdala (Cohen, Ata, et al., 2017). As the observed HR/LR differences in gene expression could be mediated by microRNAs, we also evaluated potential microRNA–mRNA networks by correlating and clustering mRNA and microRNA expression

in the HR/LR dorsal raphe and amygdala. Pathway analysis of these dysregulated networks revealed cellular processes that may be involved in the HR/LR phenotype. A network that was enriched for down-regulated microRNA and up-regulated mRNA in the dorsal raphe of LR rats shows gene ontology enrichment for terms related to immune activation and microglial function. The microRNAs miR-378a-3p, miR-206-3p, and miR-3559-5p were negatively correlated with expression of the genes Cd74, Cyth4, Nckap1l, and Rac2, all of which are implicated in immune and microglial function and up-regulated in the dorsal raphe of LR rats. In the amygdala, several of the differentially regulated networks displayed enrichment for terms associated with regulation of gene expression and chromatin function. One microRNA, miR-101a-3p, which was up-regulated in the amygdala of LR rats and is known for its role in regulating the PRC2, was identified for further investigation (Cohen, Jackson, et al., 2017).

Although several microRNAs were altered in the HR versus LR dorsal raphe and amygdala, miR-101a emerged as a microRNA that was differentially expressed between HR/LR rats in both brain regions. Interestingly, there were opposing HR/LR findings in these regions, with LRs having lower miR-101a levels relative to HRs in the raphe but higher levels in amygdala (Cohen, Jackson, et al., 2017). We therefore conducted another series of studies to investigate the role of the miR-101a in driving aspects of HR/LR behavior differences.

One of the major gene targets of miR-101a is the histone methyltransferase EZH2 (Enhancer of zeste homolog 2) (Friedman et al., 2009; Varambally et al., 2008). EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) that silences gene expression via tri-methylation of histone H3 at lysine 27 (H3K27-me3) (Cao et al., 2002; Kuzmichev et al., 2002). Few studies have examined EZH2/PRC2 and H3K27-me3 in the adult brain. PRC2 function is required for a number of developmental processes (Chakrabarty et al., 2007; Marchesi et al., 2014). PRC2 mediates repression of *Bdnf* expression in the VTA (Koo et al., 2015) and hippocampus (Qi et al., 2014), and affects neurogenesis and memory processes in adult hippocampus (Zhang et al., 2014).

Because miR-101a represses EZH2 expression (and downstream Polycomb Repressive Complex 2-mediated H3K27 trimethylation), and because we previously found HR/LR differences in miR-101a in the dorsal raphe and amygdala, we hypothesized and indeed found disparate *Ezh2* mRNA and H3K27-me3 in the HR/LR raphe and amygdala (Cohen, Jackson, et al., 2017). Using a viral vector approach, we then demonstrated that manipulating the expression of miR-101 and its gene target *Ezh2* in the amygdala of HR rats increased their typically low levels of anxiety-like behavior (Cohen, Jackson, et al., 2017).

## 6 | RDOC-GUIDED WORKING MODEL TO IDENTIFY NOVEL CIRCUITS AND MOLECULES RELEVANT TO ACUTE THREAT SUB-DOMAIN OF NEGATIVE VALENCE DOMAIN

The RDoC framework was developed to help identify novel circuits and molecules that mediate transdiagnostic behavioral domains. The HR/LR model is particularly well-suited for this task. As summarized above, the HR/LR model has been used to gain insight into the neurobiological underpinnings of differences in temperament, emotionality, substance abuse, and related behaviors (Flagel et al., 2014). Utilizing this model within the context of RDoC provides a powerful framework to elucidate novel mechanisms that mediate transdiagnostic behavioral domains. One such example is the Acute Threat sub-construct of the Negative Valence domain. We used the defensive buying test to demonstrate increased susceptibility of LR rats to acute threat, demonstrated by their high levels of passive stress coping in response to shock. The shock exposure also lead to distinct activation of serotoninergic cells in the dorsal raphe of LR rats compared to HRs (Cohen, Ata, et al., 2017). The dorsal raphe is comprised several discrete sub-regions that each have unique cytoarchitecture and projections (Lowry et al., 2008). Shock exposure triggered robust activation of the caudal dorsal subdivision of the dorsal raphe (cDRD) of HR rats, with minimal activation in this region of LRs (Cohen, Ata, et al., 2017). The increased serotonin neuron activation in the cDRD of HRs was accompanied by reduced activation in the amygdala, whereas the opposite was found in LRs (Cohen, Ata, et al., 2017). Activation of serotonergic cDRD neurons was negatively correlated with the activation of the central nucleus of amygdala neurons of HR rats, indicating potential communication between these regions.

The pattern of high dorsal raphe activation/low amygdala activation in proactive coping HR animals is congruent with the notion that serotonergic-limbic circuits govern stress coping behaviors (Puglisi-Allegra & Andolina, 2015). For example, optogenetic stimulation of glutamatergic neurons in the basolateral amygdala elicits freezing and anxiety-like behavior (Felix-Ortiz et al., 2013; Yiu et al., 2014), while stimulating GABAergic parvalbumin cells in the basolateral amygdala attenuates conditioned freezing (Wolff et al., 2014). Furthermore, serotonergic afferents inhibit the activity of glutamatergic neurons that project from the basolateral amygdala to the central nucleus of amygdala (LeDoux, 2000; Rainnie, 1999). These effects are due to the serotonergic modulation of the GABAergic basolateral amygdala interneurons, but may also be due to the direct actions of serotonin on the basolateral amygdala projections (Rainnie, 1999). Activation of the central nucleus of amygdala, in turn, results

in disinhibition of the ventrolateral periaqueductal gray, which targets premotor nuclei of the medulla, and causes freezing behavior (Tovote et al., 2016). Consequently, the LR rats' high levels of freezing in response to electric shock may be mediated by reduced serotonergic signaling from cDRD to the basolateral amygdala and increased activation of the central nucleus of amygdala. Figure 3 illustrates our proposed model of the dorsal raphe–amygdalar circuitry that may mediate HR/LR differences in unconditioned fear that is part of the Acute Threat construct of the Negative Valence domain. We propose that LR rats' enhanced freezing following electric shock is mediated by decreased activity of the serotonergic cDRD-basolateral amygdala projection. This could produce enhanced glutamatergic drive from the basolateral amygdala to the central nucleus of amygdala, in turn, driving freezing via projections to hypothalamus and brainstem projections.

Using this working model, we focused on identifying molecules and genes that mediate behavioral responses to Acute or Potential Threat. We performed next-generation sequencing to measure genome-wide mRNA and miRNA expression in the dorsal raphe and amygdala of HR/LR rats (Cohen, Ata, et al., 2017). These analyses identified 21 miRNA–mRNA networks in the dorsal raphe and 19 miRNA–mRNA networks in the amygdala that differed in HR/LR rats. Networks that were enriched for miRNA species up- or down-regulated in the LR brain were also enriched for down- or up- regulated mRNA species, respectively (which was expected as miRNAs typically act to suppress expression of mRNA targets). We found miR-101a-3p to be one of the most abundant miRNAs expressed in amygdala, and found it to be expressed at lower levels in HR versus LR amygdala (Cohen, Jackson, et al., 2017). As noted above, a major target miR-101a-3p is the histone methyltransferase EZH2 that silences gene expression via H3K27-me3 (Cao et al., 2002).

Our follow-up studies confirmed decreased miR-101a-3p levels in the HR (vs. LR) amygdala as well as increased levels of EZH2 and H3K27me3 (Cohen, Jackson, et al., 2017). We also showed that viral-mediated

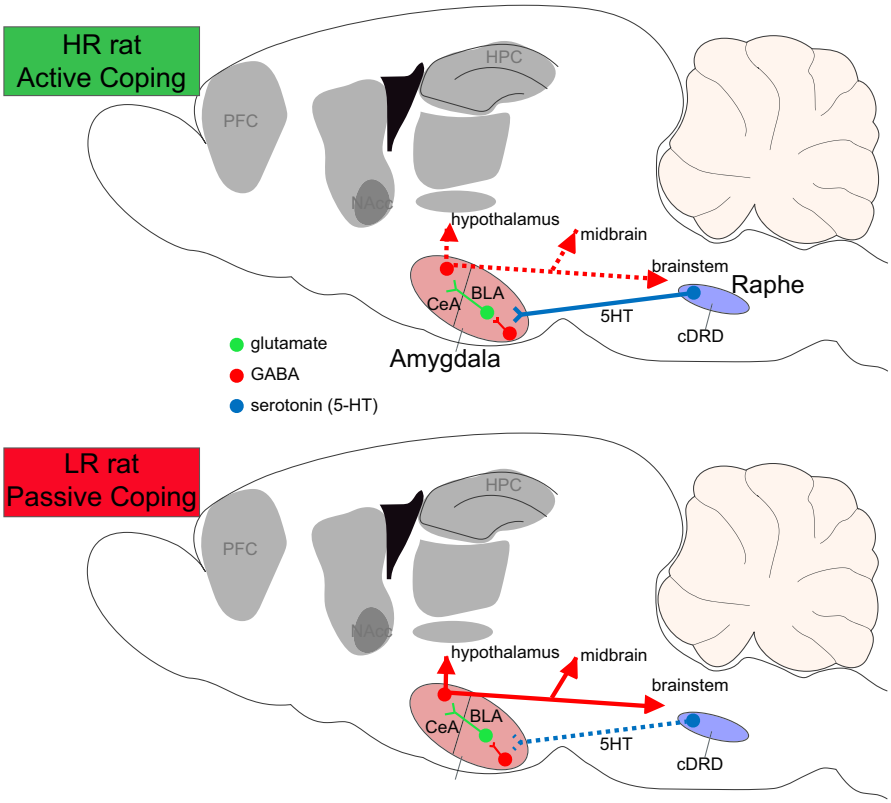


**FIGURE 3** Proposed working model of dorsal raphe–amygdala circuitry that may mediate individual differences in fear behavior in High Responder (HR)/Low Responder (LR) rats. Diagrams depict simplified circuitry of the caudal dorsal subdivision of the dorsal raphe (cDRD) sending serotonin (5-hydroxytryptamine, 5HT) projections to the basolateral amygdala (BLA). 5HT has been shown to modulate activity of both GABAergic neurons and glutamatergic neurons in the BLA that influence neurons in the central nucleus of the amygdala (CeA). Efferents from the CeA target regions of the hypothalamus, midbrain, and brainstem to mediate behaviors related to Acute Threat, such as freezing. During the defensive burying task, the stress of shock exposure elicited robust activation of serotonergic cDRD neurons in HR rats (indicated by solid blue line), diminished activation in the CeA (indicated by dashed red line), and little to no freezing behavior. In LR rats, on the other hand, shock exposure leads to minimal activation of cDRD (indicated by dashed blue line), strong activation in CeA (indicated by solid red line), and high levels of freezing. The strong activation of CeA within LR rats likely contributes to their high levels of freezing behavior in response to shock

miR-101a overexpression in the amygdala leads to predictable decreases in EZH2 and H3K27me3 together with enhanced anxiety-like behavior and threat assessment (Cohen, Jackson, et al., 2017). Similar effects were observed with siRNA-mediated knockdown of Ezh2 in HR amygdala, including increased anxiety-like behavior on the elevated plus maze and decreased H3K27me3 (Cohen, Jackson, et al., 2017). Taken together, these studies suggest that a novel serotonergic projection from the dorsal raphe to the amygdala may regulate behavioral responses to Acute Threat. The molecular machinery that mediates responses to Potential Threat, include the miRNA miR-101a-3p, PRC2, Ezh2, and H3K27me3. Future studies will be required to determine the overlap in circuitry among the Acute Threat and Potential Threat behaviors. Future efforts will also focus on other miRNA candidates identified by our sequencing studies as potential mediators of Acute Threat behaviors within this circuitry.

## 7 | CONCLUSIONS AND FUTURE DIRECTIONS

Animal models are an essential tool in biomedical research that allow us to replicate behavioral phenomena observed in clinical psychiatric states and investigate the neural and molecular underpinnings potentially at work in psychopathological conditions. The selectively bred HR/LR rat lines, bred for their behavioral responses to novelty, display distinct emotional behavior phenotypes relevant to clinical depression and anxiety disorders. These phenotypes are heritable and emerge in the second week of life, and include high levels of avoidance, conditioned fear, passive stress coping, and vulnerability to a range of stressors. These phenotypes are broadly applicable to several behavioral features of mood disorders that are described in sub-constructs of the RDoC Negative Valence Domain (and others). We took advantage of the heritable nature of HR/LR behavioral phenotypes to fulfill a key objectives of the RDoC framework: examining molecular and brain circuit anomalies that drive disparate patterns of emotional behavior and how these neurobiological changes unfold across the developmental trajectory. An important new direction for this work is expand our neurobiological studies in the bred HR/LR rats to females. Most of our genome-wide methylation, miRNA, and transcriptome studies to date have focused solely on male HR/LR bred rats. In our basic behavioral characterization of the bred HR/LR lines, we report similar phenotypic differences with bred HR females showing high novelty-induced activity, high behavioral response to psychostimulants, low levels of anxiety-related behavior, and active stress coping compared to bred LR females (Cummings et al., 2011; Davis et al., 2008). We have limited information, though, on neurobiological differences in the bred HR/LR females and would be particularly interested to examine brain development in the HR/LR females to determine whether they exhibit similar differences that appeared in the studies on male offspring. With regard to our DNA methylation studies, existent data show sex differences in epigenetic markers in the brain (McCarthy & Nugent, 2015; Menger et al., 2010), so it would be interested to interrogate epigenetic differences in HR/LR bred females and test whether such differences impact both their baseline behavioral phenotypes as well as susceptibility (or resilience) to experiences such as chronic stress. Studies of this nature in the bred HR/LR model can potentially shed light on neurobiological mechanisms that increase risk for high versus low propensity to an anxiety/depression-like phenotype.

Our molecular studies reveal HR/LR differences in dopamine and serotonin transmission that are consistent with known neurochemical abnormalities that occur in human emotional disorders. One of the more innovative aspects of our molecular work has been to identify epigenetic changes in the developing and adult brain of HR versus LR rats, including altered DNA methylation levels in the early postnatal amygdala and microRNA expression in in the adult amygdala. Using this model, we identified a novel serotonergic dorsal raphe–amygdala circuit that likely mediates aspects of behavioral response to Acute Threat. We were also successful in identifying novel molecular components within this circuitry that contribute to behavioral response to Acute and Potential Threat sub-domains of the Negative Valence domain. Future studies will continue to leverage the HR/LR model to determine unique and shared circuits and molecules that drive distinct components of the RDoC Negative Valence domain. Understanding these neurobiological mechanisms, both at the levels of circuits and molecules, may open up avenues for the development of more efficacious therapeutics. Such novel treatments would target anxiety and trauma-related symptomatology that cut across multiple neuropsychiatric disorders.

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### CONFLICT OF INTEREST

All of the authors (SMC, EAS, MEG, KAU, CRM, JLC, and IAK) have contributed to original HRLR studies included in the review. SMC wrote the initial draft of the article and each additional author participated in revision. All authors approved the final draft.

## AUTHOR CONTRIBUTIONS

All of the authors (SMC, EAS, MEG, KAU, CRM, JLC, and IAK) have contributed to original HRLR studies included in the review. SMC wrote the initial draft of the article and each additional author participated in revision. All authors approved the final draft.

## PEER REVIEW

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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