# Investigation of somatomotor-sympathetic brain circuit abnormalities in two rat models featuring inborn differences in emotional behavior

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Neuroscience

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June 21, 2023
Blacksburg, Virginia

Keywords: somatomotor, autonomic, brain circuits, depression, rat behavior

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## ABSTRACT

Major depressive disorder (MDD) features symptoms spanning cognitive, affective, behavioral, and physiological domains. While many of the neural circuit disruptions mediating emotional and cognitive disturbances in depression have been described, far fewer studies have explored neurobiological mechanisms underlying its associated motor or physiological impairments. Emotionally motivated behaviors, including responses to stress, are characterized by concomitant somatomotor actions and autonomic changes that require intricate coordination of the motor and autonomic systems. Prior investigations by our group used a pseudorabies virus (PRV)-mediated retrograde tract-tracing approach to identify brain regions with parallel descending premotor and presympathetic efferents that play a role in integrating somatomotor and sympathetic functions. Several nodes of this circuitry, including the hypothalamic paraventricular nucleus (PVN), locus coeruleus (LC), and periaqueductal gray (PAG), are implicated in responses to stressful and emotionally salient stimuli. Based on this observation, it was hypothesized that these parallel descending circuits shape responses to diverse stressors and are altered in clinical depression and comorbid anxiety disorders. To explore this possibility, the experiments in this dissertation used two recombinant PRV strains to trace polysynaptic premotor and presympathetic pathways innervating sympathectomized skeletal muscle and adrenal gland, respectively, in two rat models with heritable differences in emotionality and stress reactivity: the Wistar-Kyoto (WKY) rat and the selectively bred Low Novelty Responder (bLR) rat. During our initial neuroanatomical investigations in the PVN, we observed that both WKY and bLR rats displayed significant decreases in the quantity of PVN neurons with premotor projections to skeletal muscle compared to their respective control strains. Labeling of neurons with presympathetic projections to adrenal gland or dual-labeled polysynaptic projections to both motor and sympathetic targets was not altered in either model. Our subsequent neuroanatomical studies focused on comparing premotor efferent projections from LC and PAG. In LC, fewer premotor efferent projections to skeletal muscle were observed in both models. There were also reductions in the number of premotor efferents in the four subdivisions of the PAG. WKY rats had significantly fewer premotor projections in the dorsomedial (DMPAG), lateral (LPAG), and ventrolateral (VLPAG) subdivisions, while bLR rats had significantly fewer premotor efferents in dorsolateral (DL)PAG. The final experiments in this dissertation sought to determine whether one potential therapeutic intervention, environmental enrichment during late childhood and adolescence, can improve emotional behavior disturbances and reverse premotor circuit alterations in bLR rats. Rearing young bLR rats in conditions with increased environmental complexity partially but incompletely improved aspects of depression- and anxiety-relevant behaviors and their corresponding PVN premotor circuit abnormalities. Cumulatively, these findings highlight somatomotor circuits in several brain structures involved in responses to stress and emotional stimuli that could be implicated in mediating motor-related impairments in clinical depression.

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## GENERAL AUDIENCE ABSTRACT

Depression is a common and complex illness that features many types of impairing symptoms. Some of these symptoms involve functions regulated by the somatic motor system, which controls movement, and the autonomic nervous system, which regulates many basic bodily functions (for example, heart rate and blood pressure) that occur outside of our conscious control. The ability to coordinate the actions of these two systems is important for many behaviors, including how we respond to emotional or stressful situations. Past experiments in our laboratory used a type of virus (pseudorabies virus, PRV) that travels backwards through neural circuits containing multiple neurons and allows us to label parts of the brain that project to peripheral areas regulated by the somatic motor system (i.e., hindlimb skeletal muscle), the autonomic nervous system (i.e., adrenal gland), or both. These labeling experiments identified neurons in these motor and autonomic circuits in several parts of the brain, including the paraventricular nucleus of the hypothalamus (PVN), locus coeruleus (LC), and periaqueductal gray (PAG). Of note, all of these structures are involved in regulating responses to stressful or emotional situations. This observation led us to hypothesize that motor and autonomic projections from these areas of the brain are important for regulating how we respond to stress and might be altered in individuals suffering from depression. To test this idea, we labeled motor- and autonomic-projections with PRV in two separate rat models with a genetic disposition for emotional behaviors that resemble symptoms of clinical depression or anxiety. When we analyzed the PVN, LC, and PAG of rats with depression-relevant behaviors, we discovered that each of these brain areas contained fewer labeled neurons with motor projections to skeletal muscle. Based on these findings, we were interested in exploring whether enriching or stimulating experiences during early life had the potential to reverse deficits in the PVN motor projections and improve emotional behavior in one of our rat models for depression. Although enrichment partially improved behavioral and circuit-level outcomes, it was not fully effective. Taken together, our experimental findings highlight disruptions of motor projecting circuits in several brain structures implicated in mediating responses to stressful or emotional stimuli in two rat models relevant to depression and anxiety disorders. These motor circuit disruptions could be implicated in mediating motor-related symptoms observed in clinically depressed patients.

## ACKNOWLEDGEMENTS

To my advisor, Sarah – I am so grateful for the mentorship, patience, generosity, and kindness you have extended to me throughout my graduate school years. Thank you for allowing me to explore new scientific avenues as a trainee and for always supporting my personal and professional goals. I could not have asked for a better advisor to navigate me through these years.

To my committee members – Thank you for your wisdom and insightfulness throughout my time as a graduate student. Your intellect and scientific expertise have been invaluable.

To Ilan – Thank you for serving as an unofficial co-advisor to me in the lab. I have learned so much from you throughout these years. This dissertation would not have been possible without your pioneering intellectual and experimental contributions.

To my former labmates, Keaton, Matt, and Chelsea – Thank you for being amazing colleagues, mentors, and friends during your years at Virginia Tech. I will always cherish the time we spent together in the lab and beyond the bench.

To my husband, Jay – Words cannot express how grateful I am to have you as my partner on this journey. Your love and support mean the world to me, and I could not have done this without you.

To my family and friends – Thank you for your unwavering faith in and support of me throughout the years. Your encouragement has kept me going in difficult times.

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# CHAPTER 1: INTRODUCTION

## 1.1. Epidemiology and clinical presentation of major depressive disorder

Major depressive disorder (MDD; depression) is among the most prevalent and debilitating psychiatric illnesses, affecting one in five adults U.S. adults at some point during their lifetime[1]. It is almost twice as likely to occur in women than in men[2,3]. Depression is associated with greater risk of functional impairment, disability, and all-cause mortality[4,5]. Depressed patients often experience health comorbidities with other psychiatric conditions (e.g., anxiety disorders, post-traumatic stress disorder (PTSD), and substance use disorders) as well as chronic physical diseases (e.g., cardiovascular disease, cancer, and diabetes)[1,6,7]. Altogether, impairments associated with depressive disorders contribute to significant personal and societal costs[1,8].

As a complex and heterogeneous syndrome, MDD is characterized by disturbances in various psychological and physiological domains. Cognitive and affective disturbances documented in depressed patients are numerous and include excessive rumination, concentration difficulties, indecisiveness, anhedonia, feelings of worthlessness, and depressed mood. Depressed mood and anhedonia are core emotional features of depression that must be present to receive a clinical diagnosis[9]. In addition, depression is characterized by behavioral and somatic impairments, including fatigue, lack of energy, sleep-related issues, psychomotor retardation, and altered sympathetic tone and stress reactivity[3,9,10].

Symptoms of depression and other emotional disorders begin to emerge around the onset of puberty and present during adolescence and early adulthood. The etiology of these disorders is complex and stems from a combination of environmental and genetic factors[11]. Depressive

episodes are often triggered by stressful events, and early childhood adversity can amplify individual risk of developing depression in response to proximal stressors[3,12]. Certain personality traits or temperaments that predispose individual vulnerability to stress may also contribute to augmented risk for developing psychiatric disorders like depression or anxiety[13–15]. Animal models have been used extensively to recapitulate the biological and environmental elements that contribute to disordered psychiatric states. These models have proven critical for understanding neurobiological mechanisms underlying emotional behaviors in humans.

### 1.2 Rat models with genetic disposition for depression- and anxiety-relevant behavioral phenotypes and biological abnormalities

Major depression has a strong genetic component. Early epidemiological studies estimate that additive genetic effects contribute to 31-42% of liability for heritability of MDD[16]. Paternal and maternal depression are both significant predictors of offspring psychopathology, influencing susceptibility to certain temperaments, internalizing and externalizing symptoms, and dysregulation of stress-induced cortisol reactivity[17–19]. Given the heterogeneity of clinical manifestations of depression, studying heritability of depression is exceedingly complex. Meta-analyses of genome-wide association studies (GWAS) support the notion that MDD is a polygenic disorder influenced by numerous genetic variants, including genes and gene pathways involved in synaptic structure and neurotransmission[20,21]. No single gene mutation in isolation can recapitulate the genetic causation of MDD.

Available genetic models for depression and anxiety-relevant behaviors in rodents vary widely[22]. Certain transgenic lines have been created that target genes implicated in regulation of serotonergic, noradrenergic, or other neurotransmitter systems. Gene mutations for

hypothalamic-pituitary-adrenocortical (HPA) axis regulatory genes have also been used in this context. Still other lines are selected based on their sensitivity to stress (e.g., Wistar-Kyoto (WKY) rats and high-reactive mice[23,24]) or on behavioral endpoints that are relevant to clinical pathologies (e.g., locomotor response to novelty, as with the Selectively Bred Low Novelty Responder (bLR) rat[25]). It is important to acknowledge that while these models are immensely useful for studying and modeling the underlying neurobiology of these psychopathologies, they cannot completely recapitulate the vast complexities inherent to such heterogeneous disorders.

In the following subsections, this dissertation will expand on phenotypic characterizations of two rat models with genetic predisposition for depression- and anxiety-relevant behavioral phenotypes. The first section (**1.2.1**) will summarize observations from the bLR rat line, while the second (**1.2.2**) will describe findings in the WKY rat.

### *1.2.1 Selectively Bred Low Novelty Responder rats*

Inborn differences in personality and emotional reactivity influence individual responses to stress and susceptibility for depression and anxiety-related disorders[26]. Numerous studies support the idea that inheritance of specific traits from one's parents contributes to heightened risk of psychopathology in vulnerable children[27–30]. Behavioral responses to novelty, for instance, correlate with numerous other measures of emotionality and substance use[31–37]. In toddlers, higher levels of behavioral inhibition (i.e., showing restraint, wariness, or fearfulness in novel situations) are associated with greater risk for developing anxiety-related disorders and depression later in childhood, adolescence, and/or adulthood[38–46]. During early childhood, this trait is also associated with greater cortisol secretion and stress reactivity[42,43,46]. By contrast, impulsivity and decreased behavioral inhibition are linked to greater risk of antisocial behavior

and substance use[47]. Thus, behavioral response to novelty may represent a unique way to explore how inborn differences in temperament and emotional reactivity influence individual susceptibility for stress-related mood disorders.

To emulate and study these dynamics within an animal model, the laboratory of Huda Akil at the University of Michigan developed a model using Sprague Dawley rats selectively bred for divergent responses to novelty[36]. Like humans, some rats show extensive exploratory behavior in a novel environment ("behavioral disinhibition"), while others display considerably lower levels of exploration ("behavioral overinhibition"). By selecting for rats reflecting extremes (top and bottom 20%) of novelty-induced locomotor activity across several generations of breeding, it is possible to generate lines of rats that interface with their environment in fundamentally different ways[15,36]. Earlier work in this model demonstrated that greater locomotor responses to novelty predicted propensity for psychostimulant self-administration, making bHRs an attractive model for studying addiction[35]. Follow-up experiments in the bHR line demonstrated that these rats also display greater novelty-seeking[36,48–55], impulsivity toward reward-associated cues[56], aggression[57,58], and proclivity for drug-seeking behavior and drug addiction[52,59–63]. These behaviors are inversely correlated with spontaneous anxiety and depression-related behaviors[64].

Subsequent studies to those in the bHR line showed that decreased novelty-induced locomotion, as observed in bLR rats, predicted several other behaviors resembling aspects of clinical anxiety and depression. The bLR rat line display greater behavioral inhibition and anxiety-relevant behaviors in several assessments used to probe this phenotype in animal models (i.e., elevated plus maze (EPM), open field test (OFT), light-dark box test, and novelty-suppressed feeding test)[25,33,36,49,51,52,65]. The bLR rat also shows passive stress-coping behaviors

in the forced swim test (FST) and defensive burying task[25,49,51,53,55,66]. In addition, these rats display diminished social behavior[51], reduced sexual motivation and performance[67], and stress-induced anhedonia[25]. Furthermore, bLR rats show decreased basal locomotor activity during the dark (active) phase of the circadian cycle, reflective of a possible psychomotor disturbance in these animals[68]. Although most of these behaviors were primarily documented in males, many of the same behavioral profiles are also observed in females[15].

Differences in the HPA axis have also been described in the bHR/bLR model. HPA axis dysregulation is likewise noted in depressed patients, although the directionality of this effect differs among clinical depression subtypes[22]. While bHR and bLR rats appear to display overall similar patterns of basal corticosterone (CORT) secretion over the diurnal period, bLRs appear to have a shift in peak CORT secretion, with bLRs showing a two-hour delay in peak CORT levels during the active period[68]. In response to novelty stress, bLR rats display blunted CORT responses[33,69] and distinct patterns of neuronal activation[70] relative to bHR rats. However, bHR and bLR rats show similar CORT levels following restraint stress[70]. Such disparities may be attributable to factors such as the overall magnitude of stress elicited by the stressor and the controllability of the stressor[70]. Individual responses to stress in the bHR/bLR model at various developmental stages are sensitive to environmental manipulations, including prenatal stress, maternal separation, cross-fostering, and chronic mild variable stressors[15,25,48,49,51,71].

### *1.2.2 Wistar-Kyoto rats*

The WKY line was initially derived from the Wistar rat strain as a normotensive control for the spontaneously hypertensive (SHR) rat[72]. Over the course of experiments characterizing behavioral features of the SHR model, it was observed that WKY rats, relative to SHR, Fisher-

344, and Wistar rats, showed behavioral abnormalities with similarity to symptoms of clinical depression[73–76]. In the FST and shuttle box escape test, WKY rats displayed more passive stress-coping or avoidance responses. Additionally, WKY rats displayed exaggerated responses to stress and greater susceptibility for developing stress-induced stomach ulcers[74–79]. Collectively, these observations led researchers to consider WKY rats an attractive model for studying endogenous depression.

Subsequent investigations in this strain have revealed additional behavioral phenotypes relevant to clinical depression and anxiety disorders. Male WKY rats display reduced locomotor activity, a feature similar to clinical psychomotor retardation, as well as reduced sociability[80,81]. Male WKY rats also display behavioral inhibition, hyponeophagia, neophobia, and anxiety-relevant behaviors[73,80,81]. Behavioral phenotypes relevant to anxiety- and depression in the OFT, EPM, and FST emerge as early as adolescence (postnatal days (P)35-39)[82].

Notably, many of the more recent behavioral characterizations in the WKY model have yielded mixed findings. For example, anhedonia, a core feature of clinical depression, has been reported in male WKY rats in some studies[81,83,84], but not others[80,85]. Further, recent experiments show that females of this strain do not show anhedonia, suggesting a possible sex difference in the model. Inconsistent findings on this measure in male WKY rats may reflect variations in experimental protocols among researchers[81]. In addition, many studies have found that adult male WKY rats were resistant to treatment with the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and escitalopram[86–88], which are among the first line antidepressant interventions used today[89]. However, treatment responses to another pharmacological intervention, tricyclic antidepressants (TCAs), are mixed in their efficacy of alleviating depressive-like symptoms in male WKY rats[86,90–92]. Perhaps reassuringly, two treatments that are

effective for alleviating treatment-resistant depression in clinical populations – deep brain stimulation (DBS) and ketamine – are also effective in mitigating emotional behavior deficits in the WKY model[93–96].

Although comparatively few studies have characterized correlates of emotional behavior dysregulation in female WKY rats, the literature in this area is growing. Reports in adolescent and adult animals show that female WKY rats, like males, display elevated anxiety- and depression-relevant behaviors in the OFT, EPM, and FST[81,82,97,98]. However, many studies have also reported disparities in the behavioral profile of female WKY rats. In addition to the absence of anhedonia, manifestations of anxiety-relevant behavior differ in female relative to male WKY rats[73,81]. In particular, female WKY rats display increased locomotor activity in the home cage, a tendency toward increased burying in the marble burying test, and a lower propensity for novelty-induced hyponeophagia[81].

Neurobiological assessments in the WKY model have also outlined features observed in clinical neuropsychiatric syndromes. Hypercortisolemia is reported a subset of depressed patients, notably in those with melancholic and psychotic depression[99]. Exaggerated neuroendocrine responses to stress are likewise noted in the WKY model[78,79,100], contrasting with the attenuated stress reactivity of the neuroendocrine axis in bLR rats. Elevations in basal adrenocorticotropin-releasing hormone (ACTH) and CORT levels are also observed in male WKY rats at the peak of the diurnal CORT cycle, mirroring increases in basal ACTH and cortisol observed in clinically depressed patients[73,101,102]. (Differences in basal ACTH and CORT are not reported at the trough of the diurnal cycle[79,80,100].) In addition to neuroendocrine axis changes, perturbations of the locus coeruleus (LC)–norepinephrine (NE) system, which mediates arousal as well as cognitive and behavioral aspects of the stress response[103], are also described in

male WKY rats. Basal and burst (phasic) firing activity of LC noradrenergic neurons are higher in WKY rats compared to control strains (Wistar and Sprague Dawley), and inhibitory control of inputs onto LC neurons is dysregulated in this strain[104,105]. Microarray analysis also reveals that genes involved in NE synthesis and metabolism (NE turnover) are elevated in the LC of WKY rats relative to Sprague Dawley rats[106]. Considered with the observation that WKY rats display attenuated NE release and tyrosine hydroxylase (TH) mRNA in response to acute immobilization stress[102], it has been proposed that increased NE turnover in this strain could explain its passive phenotype[106].

### 1.3 The importance of motor and autonomic circuitry in MDD symptomatology and responses to emotionally salient stimuli

Extensive literature has chronicled dysfunction of both motor and autonomic functions in depressed patients. Psychomotor retardation is one of the core symptoms of depression[9] and is characterized by a wide array of deficits in gross locomotor activity, motor aspects of speech production, execution of simple motor tasks with minimal cognitive demand, and execution of willful movements[107–113]. Psychomotor disturbances may distinguish the melancholic depression subtype from others[111,114,115]. Motor disturbances in depressed patients have predictive value for determining severity and treatment outcomes[113]. In addition to motor-related disturbances, sympathetic arousal – a symptom that is not included in the DSM-5 – is among the most central symptoms in MDD[116]. Several observations consistent with increased sympathetic tone are evident in MDD patients, including elevated baseline NE levels, greater NE spillover in response to a mild psychological stressor, and higher reactivity of the sympathetic nervous system during stress[10,117,118]. It is notable that motor and autonomic impairments can persist after antidepressant

treatment even when affective and cognitive symptoms are alleviated[119–122]. This observation suggests that neural circuits underlying these deficits in depressed patients are likely distinct from those underlying cognitive and emotional perturbations.

While many of the neural circuit disruptions governing discrete affective and cognitive disturbances relevant to MDD and other psychopathologies have been described[123,124], far fewer studies have explored or identified neurobiological mechanisms governing physiological or motor impairments in MDD. Emotionally motivated behaviors, including responses to stress, require intricate coordination of motor and autonomic systems. A prominent example of this is the classic fight-or-flight response, an active stress coping strategy that engages both somatomotor actions (e.g., sudden attack behavior) and autonomic changes (e.g., elevated blood pressure)[112,125–127]. Movements elicited during emotionally motivated behaviors are mediated by the emotional motor system, which is organized similarly to the somatic motor system that innervates skeletal muscles[128–132]. Emotional-somatomotor circuits in the emotional motor system receive strong afferents from integrative limbic structures such as the hypothalamus and bed nucleus of the stria terminalis (BNST)[112,129]. Importantly, structures in the emotional motor system also contain neuronal populations capable of eliciting autonomic responses during stressful situations[133–136]. Collectively, this work suggests that several key brain regions involved in regulating stress responses may be able to coordinate both somatomotor and autonomic activation during stress. It remains unclear, however, whether somatomotor-sympathetic integration is achieved by discrete neuronal populations with separate motor or autonomic functions or by a single population of neurons with dual functions.

### 1.4 Circuits and systems involved in stress regulation

The ability of an organism to respond to changes in its environment is central to survival. Hans Selye, an early pioneer in the field, defined stress as "the non-specific response of the body to any demand"[137]. Although much of the ongoing work in the stress field focuses on the impacts of negative stressors (i.e., stimuli that are aversive, adverse, or noxious), many of the same physiological responses are also observed following pleasurable or appetitive events[138,139]. The HPA axis and the sympathoadrenomedullary (SAM) axis are two major systems recruited by stressors. Both axes mobilize energy reserves in the liver to allow the organism to adequately respond to challenges[139].

As a key integrator of endocrine, autonomic, and behavioral responses to stress, the hypothalamic paraventricular nucleus (PVN) contains and receives input from numerous neuroanatomical sites with direct or indirect involvement in these roles[139–141]. Information about psychogenic and systemic stressors is relayed from several limbic areas, including the prefrontal cortex (PFC), amygdala, and hippocampus[142]. Corticotropin-releasing hormone (CRH)-expressing neurons in the PVN play a central role in stimulating glucocorticoid release from the adrenal cortex as part of the neuroendocrine stress response[140]. PVN CRH neurons are capable of encoding the positive and negative valence of stimuli and can modulate the selection of active or passive defensive strategies[143,144]. A subset of PVN CRH neurons send projections to the spinal cord[145], and CRH has been shown to elicit sympathetically-mediated tachycardia and increased locomotor activity in rats[146].

CRH neurons of the PVN share bidirectional communication with noradrenergic neurons of the LC-NE system[147–149]. NE can excite PVN CRH cells to activate the HPA axis. Conversely, stress-induced CRH activates noradrenergic neurons in the LC, shifting their physiological

properties to bias towards increased tonic firing (and decreased phasic firing) to alter sympathetic arousal and attention[148,150]. The LC-NE system is thought to mediate hyperarousal in patients with depression[150,151].

### 1.5 Transsynaptic tract-tracing of somatomotor and sympathetic efferents involved in stress regulation

The synchronized activity of motor and autonomic efferents is critical for mediating responses to stress. To delineate the organization of descending premotor and presympathetic circuits recruited during stress, prior work by our laboratory optimized a virally mediated transsynaptic tract-tracing approach using genetically modified strains of pseudorabies virus (PRV). PRVs are neurotropic viruses that infect neuronal circuits via transneuronal spread across synapses[152,153]. These viruses have been commonly implemented in studies characterizing central nervous system connectivity to peripheral organs[154,155]. Our previous investigations employed the viral recombinants PRV-152 and PRV-BaBlu[156–158] (**Figure 1**). PRV-152 contains the gene coding for enhanced green fluorescent protein (eGFP) at the gG locus[159], while PRV-BaBlu contains the *lacZ* gene at the gG locus and produces β-galactosidase (β-gal)[160]. These recombinants are derived from PRV-Bartha, an attenuated form of the parental strain PRV-Becker[153]. PRV-152 and PRV-BaBlu are selectively transported in a retrograde fashion and capable of simultaneously co-infecting the same neuronal population[153,156,157,159,161–165] (**Figure 2**).

Premotor and presympathetic circuits were identified by injecting PRV-152 into sympathectomized gastrocnemius muscle and PRV-BaBlu into adrenal gland[112,156,157] (**Figure 1**). Premotor and presympathetic circuits have the potential to independently regulate activity of

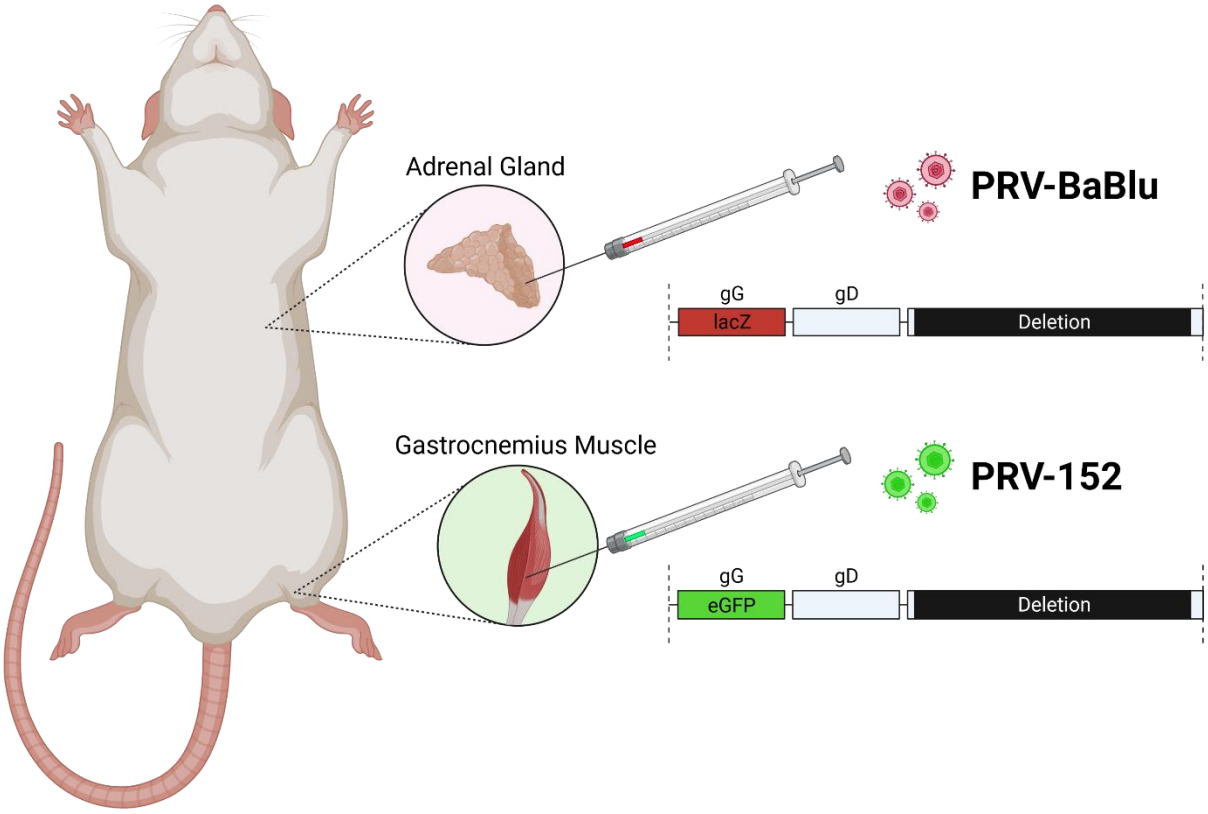


**Figure 1**. Pseudorabies virus (PRV) approach for retrograde tract-tracing of premotor and presympathetic circuits in the rat. Prior studies in our laboratory have used two PRV recombinants derived from PRV-Bartha: PRV-152, which is injected into sympathectomized gastrocnemius muscle and expresses enhanced green fluorescent protein (eGFP), and PRV-BaBlu, which is injected into the adrenal gland and produces β-galactosidase. Created with BioRender.

skeletal muscle or adrenal gland, respectively, while premotor-presympathetic projections have the potential to simultaneously engage both targets. In typically behaving animals, our group observed that key nodes in these integrated circuits included areas known for mediating stress-related responses, including the PVN and LC[112,156,157]. Furthermore, a considerable portion of these neurons overlapped with neurotransmitter and peptide populations that regulate affective responses in rodents and humans (e.g., NE, serotonin, vasopressin, oxytocin)[112,156–158,166,167]. However, a critical knowledge gap remained in our understanding of how the organization of these circuits is altered in stress-related emotional disorders.

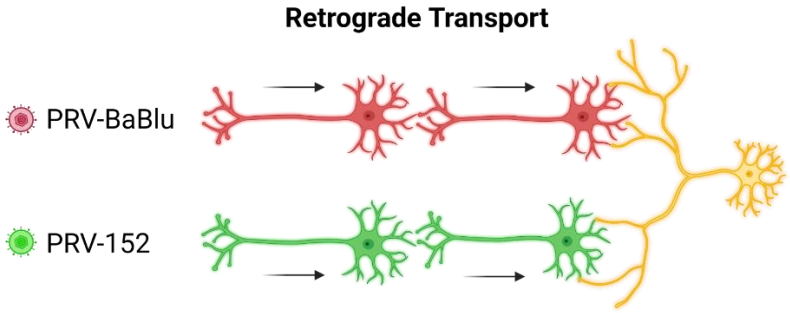


**Figure 2**. Dynamics of pseudorabies virus (PRV)-152 and PRV-BaBlu infection in the peripheral and central nervous systems. PRVs are capable of infecting neurons within a circuit through transneuronal spread across synapses. PRV-152 and PRV-BaBlu, which are both derived from PRV-Bartha, are selectively transported in a retrograde fashion and capable of simultaneously co-infecting the same neuronal population. Created with BioRender.

### 1.6 Goals for dissertation research

Emotionally motivated behaviors, including responses to stress, require the parallel coordination of systems involved in regulating motor and autonomic functions. Dysregulation of motor and autonomic functions, in addition to affective and cognitive impairments, is well documented in MDD and contributes to the significant burden of disease experienced by depressed patients. Despite this, far less is understood about the underlying neural mechanisms governing motor and autonomic impairments in depression. Antidepressant treatments such as SSRIs, when successful, improve affective and cognitive symptoms associated with MDD but are less effective at ameliorating autonomic and motor disturbances. Therefore, a major goal of this dissertation was to identify central neural circuits that may be involved in mediating these impairments in depression. This work may provide invaluable insights into the potential links between these circuits and certain clinical features of MDD.

The subsequent chapters of this dissertation will outline studies in two rat models with inborn emotional behavior characteristics relevant to clinical depression and co-morbid anxiety. These experiments employed retrograde tract-tracing with PRV-152 and PRV-BaBlu to discern

alterations in somatomotor (gastrocnemius-projecting) and sympathetic (adrenal-projecting) circuits that may underlie the distinct behavioral phenotypes in these models. Chapter 2 describes assessments in the WKY and bLR rat lines to validate prior emotional behavioral observations as well as neuroanatomical investigations of somatomotor, sympathetic, and somatomotor-sympathetic projections in the PVN. Chapter 3 extends these neuroanatomical investigations to include the LC and periaqueductal gray (PAG). These structures are pivotal in mediating behavioral and physiological responses to stress and other emotional stimuli and, like the PVN, were previously observed to contain populations of somatomotor-sympathetic neurons. Finally, Chapter 4 describes experiments to interrogate whether behavioral and selected neuroanatomical alterations in the bLR model are modifiable by enriching environmental stimuli during early life. Altogether, these findings will (1) further our understanding of the neural circuits involved in mediating motor and autonomic dysfunction in rat models with depression-relevant phenotypes, and (2) examine the efficacy of non-pharmacological interventions on emotional behavior abnormalities observed in these models.

# CHAPTER 2: INBORN DIFFERENCES IN EMOTIONAL BEHAVIOR COINCIDE WITH ALTERATIONS IN HYPOTHALAMIC PARAVENTRICULAR MOTOR PROJECTIONS

## 2.1 Introduction

Millions of individuals in the United States suffer from affective disorders such as depression and anxiety, with estimated lifetime prevalence of these illnesses as high as 20% and 33%, respectively[1,168]. Mood disorder symptoms span multiple domains, involving disturbances of cognition (e.g., rumination, inability to concentrate), emotion (e.g., apathy, anhedonia), behavior (e.g., restlessness, social isolation), and physiology (e.g., altered stress responsivity, cardiovascular anomalies). Improved understanding of neural circuits that mediate these diverse symptoms can inform the pathophysiology of stress-related mood disorders and point to novel therapeutic interventions.

The paraventricular nucleus of the hypothalamus (PVN) plays an essential role in governing behavioral and physiological responses to stress. Organisms react to stress in a variety of ways, with individuals exhibiting distinct coping styles (i.e., active versus passive coping) and showing differential vulnerability to the negative effects of stress. Individual differences in stress reactivity are shaped by early life environment and genetics[169–173] and can, in turn, influence risk for emotional disorders[170,174–176].

Emotionally motivated behaviors require an intricate coordination of motor and autonomic systems[112,125–127,157]. For example, active stress coping behaviors such as the defensive-aggressive fight-or-flight response involve a combination of somatomotor actions (e.g., hissing, sudden attack behavior) and autonomic changes (e.g., elevated blood pressure, increased cardiac output)[125–127,157]. By contrast, passive stress coping mechanisms feature their

own distinct behavioral and physiological elements, including immobilization, low maternal and sex drives, and increased corticosteroid secretion[125,157]. Holstege and colleagues were the first to describe an emotional motor system that coordinates somatic motor neuron activity relevant to emotionally motivated behaviors, with fundamental organization similar to the somatic motor system[112,128,129,131]. Brain structures in the emotional motor system receive strong afferents from integrative limbic structures including the hypothalamus, amygdala, and bed nucleus of the stria terminalis[112,129], all of which are regions known to be altered in mood disorders[177–180].

Previous work by our group used a pseudorabies virus (PRV) transsynaptic tract-tracing approach to delineate the organization of premotor and presympathetic circuits, which send parallel descending polysynaptic projections to skeletal muscle and adrenal gland. Premotor and presympathetic circuits have the potential to independently regulate activity of either skeletal muscle or the adrenal gland, respectively, while the premotor-presympathetic projection has the potential to simultaneously engage both targets. Our previous studies noted that key nodes in these circuits included hypothalamic nuclei well-known to mediate stress-related responses, including the PVN[112,157]. Other important nodes in the circuitry, including dorsomedial hypothalamus, periaqueductal gray, and rostral ventromedial medulla, also mediate a host of emotional behaviors such as panic, anxiety, active coping, passive coping, and pain[112]. Additionally, we observed that a considerable portion of such neurons overlapped with neurotransmitter and peptide populations that regulate affective responses in rodents and humans, including serotonin, norepinephrine, melanin-concentrating hormone, orexin, urocortin-1, vasopressin, and oxytocin[112,156–158,166,181].

Based on these earlier observations, we proposed that these parallel descending neural circuits participate in shaping physiological responses to diverse stressors and facilitate stress

coping. The goal of the present study was to test the hypothesis that organization of the PVN descending projections to skeletal muscle and/or the adrenal gland differ in organisms that exhibit individual differences in emotionality and stress reactivity. To address this question, we used two rat models featuring heritable differences in emotional reactivity: (1) the Wistar Kyoto (WKY) rat, a line known to display altered function within domains relevant to depression (i.e., behavioral inhibition, passive stress coping, and physiological dysfunction)[23,80,81], and (2) the selectively-bred Low Novelty Responder (bLR) rat, a model that exhibits a highly inhibited, anxious phenotype and passive stress coping[25,36,50,182,183]. Our first experiment assessed emotional behavior of WKY rats versus outbred Sprague Dawley rats, and then used PRV transsynaptic tract-tracing to compare descending PVN premotor, presympathetic, and premotor-presympathetic circuits between the two strains. A parallel experiment contrasted behavior and descending PVN neural circuits in bLR rats versus selectively bred High Novelty Responder (bHR) rats. We hypothesized that WKY rats and bLR rats, due to their relatively similar phenotypes, would likewise exhibit comparable alterations in the descending PVN projections mediating emotional behavior.

## 2.2 Methods

All experiments were approved by the local Institutional Committee on the Use and Care of Animals. This work was performed in accordance with the National Institutes of Health (USA, 2011) guidelines on animal research.

*Animals*

For Experiment 1 studies, adult male WKY and Sprague Dawley rats were purchased from Charles River Laboratories (Kingston, NY, USA). Experiment 2 studies were conducted in bLR and bHR male rats obtained from the 17th, 18th, 20th, or 21st generations of the in-house colony. A detailed description of the creation and characterization of the bred bHR/bLR lines can be found in prior publications[36,61,184]. Housing was maintained at 21-23°C and humidity at 50-55% on a 12:12 light-dark cycle (lights on/off at 6:00 a.m./6:00 p.m.). Rats were housed in groups of 2-3 per cage with food and water *ad libitum* for all studies. The number of animals used in each experiment are noted within each subsection below.

***Experiment 1: Emotional behavior assessment and viral tract-tracing studies in WKY rats versus outbred Sprague Dawley rats***

*Behavioral testing*

We compared behavior of adult male WKY and Sprague Dawley rats ($n$ = 9-12 per strain) in a test of novelty-induced locomotor activity, the Light-Dark Box test, and Porsolt's Forced Swim Test (FST). These tests were chosen because they powerfully and reliably engage motor and autonomic systems to shape distinct stress coping strategies[185–189]. All testing was performed between 8:00 a.m. – 11:30 a.m. and conducted under dim lighting (30 lux) except where otherwise noted. The same animals were assessed across the three tests with at least two days of rest between tests.

*Locomotor Response to Novelty.* Animals were screened for locomotor response to a novel environment as previously described[36]. Briefly, each rat was placed into a standard-sized (43 x 21.5 x 24.5 cm) clear acrylic cage with a mesh floor in a different room from where the animals had been housed. Locomotor activity was monitored in 5-min increments over a 1-hr

period by two panels of photocells to record horizontal locomotion and rearing behavior. Final locomotion scores were determined by summing horizontal and rearing activities.

*Light-Dark Box Test.* Rats were next subjected to the Light-Dark Box as previously described[36]. The apparatus was a 30 × 60 × 30 cm Plexiglas shuttle-box divided into two equal-sized compartments by a wall with a 12-cm-wide open door. One compartment was painted white and brightly illuminated (70 lux), and the other compartment was painted black with dim light (5 lux). Rows of five photocells located 2.5 cm above the stainless steel grid floor monitored locomotor activity and time spent in each compartment. A microprocessor recorded the latency to first exit the dark compartment where each rat was initially placed, the number of photocell beams interrupted, and the time spent in each compartment during the 5-minute test.

*Forced Swim Test.* Porsolt's FST was performed as previously described with 30-cm deep 25°C water in Plexiglas containers (45 cm high x 20 cm diameter)[80,190]. On FST day 1, rats were placed (1/cylinder) into the water for 15 mins. After 24 hr, the rats were returned to the water-filled cylinder and tested for another 5 mins. Water was changed after every swim session. Rats were videotaped during both test days and immobility was scored using the Ethovision ® XT 8.0 software (Noldus, Wageningen, The Netherlands).

*Viral tract-tracing*

A separate cohort of WKY and Sprague Dawley rats ($n$ = 3-13 per group) was used in a viral tract-tracing study to examine potential differences within neural circuits that govern sympathetic and/or motor output. To this end, rats received injections of transgenic recombinants of an attenuated PRV strain, PRV-Bartha, for transneuronal tracing of multisynaptic pathways innervating skeletal muscle [gastrocnemius muscle, our selected motor target] and the adrenal

gland [our selected sympathetically-innervated target]. The viral recombinants used were PRV-152, which carries the gene coding for enhanced green fluorescent protein (eGFP), and PRV-BaBlu, which contains the lac Z gene at the gG locus and produces β-galactosidase (β-gal)[159,160]. Previous studies by our group and others demonstrated that PRV-152 and PRV-BaBlu are transported transsynaptically in a retrograde manner, and that the two recombinants are capable of simultaneously co-infecting the same neuronal population[156,161,163,164,166,181].

*Sympathectomy and viral injections:* Animals were anesthetized with 5% isoflurane vaporized in 1.0-1.5 L/min of O2 and maintained at 1.5-2.5%. Surgical plane of anesthesia was achieved such that there was no spontaneous movement and no withdrawal responses to tail and/or foot pinch. Prior to PRV injections, surgical sympathectomy was performed as previously described[156,157,191] to remove sympathetic innervation of the hindlimb musculature. Briefly, a ventral laparotomy was performed and a segment of the lumbar sympathetic nerve from the level of the renal artery to the aortic bifurcation was extirpated. Neural plexuses along the abdominal aorta were stripped off under microscopic observation using fine forceps, and the aorta was swabbed with a 10% phenol solution. Our group has performed this procedure successfully in numerous prior studies[156–158,166,181].

Viral stocks were harvested from pig kidney cell cultures at a titer of 108-109 pfu/mL, aliquoted into 50 μL volumes, and stored at −80°C until the time of inoculations when they were rapidly thawed in a 37°C water bath. Following a 2-10 day recovery period after the surgical sympathectomy, animals were injected with PRV-152 and PRV-BaBlu as previously described[112,156–158,166,181,191]. Briefly, PRV-152 was injected throughout the lateral head of the gastrocnemius muscle in 1-μl volumes (totaling 30 μl) using a 10-μl glass syringe (Hamilton Company, Reno, NV). The surgical incision was then closed and the animal returned to its home

cage. After 24 hours, PRV-BaBlu was similarly injected using a Hamilton syringe with a glass pipette attached to the tip with wax. A total of 2-4 μl of PRV-BaBlu was injected into the ipsilateral adrenal gland. We previously determined that a separation of 12-24 hours between the gastrocnemius and adrenal injections is required to match the temporal transport of the two viruses to the PVN[156,157]. Analgesic buprenorphine (0.05-0.1 mg/kg, s.c.) was administered during all surgical procedures, and carpofen (5 mg/kg, s.c.) was administered once daily for post-operative pain relief.

Rats were allowed to survive either 120 hr ($n$ = 3 per group) or 132 hr ($n$ = 9 per group) after initial PRV injections. At the end of the designated survival period, animals were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with 100-150 mL of physiological saline (0.9% NaCl) followed by 400-500 mL of paraformaldehyde L-lysine sodium metaperiodate (PLP) fixative[192].

*Tissue collection and processing:* After transcardial perfusion, brains and spinal cords were extracted, post-fixed overnight, and immersed in 30% sucrose overnight on the following day. Brains were sectioned coronally on a freezing microtome at a thickness of 40 μm and then stored at −20°C in cryoprotectant (30% ethylene glycol, 1% polyvinyl-pyrrolidone, 30% sucrose in 0.1M sodium phosphate buffer[156]) until immunohistochemical processing. Spinal cords were extracted in three segments: T1-T7, T8-T13, and L1-L6. They were postfixed and cyroprotected as described above, and then sectioned horizontally at a thickness of 40 μm and stored at -20°C in cryoprotectant.

Brains from PRV-injected animals were processed for double immunofluorescent detection of eGFP and β-gal. Free-floating brain sections were rinsed with 0.1M phosphate buffer (PB; pH 7.4) several times at room temperature and then incubated for 1 hr in blocking

buffer containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton X-100 (TX-100) in 0.1M PB. Sections were then reacted with a cocktail of primary antibodies: chicken anti-GFP IgY (Cat. No. 13970, Abcam, Cambridge, MA, USA) at 1:2,000; and mouse anti-β-galactosidase IgG (Cat. No. G4644, Sigma-Aldrich, St. Louis, MO, USA) at 1:1,000 in a solution containing 1% NGS, 1% BSA, and 0.3% TX-100. Following overnight incubation at 4°C, the tissue was rinsed with 0.1M PB and reacted with a secondary antibody cocktail of Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and AlexaFluor 488-conjugated goat anti-chicken IgG (1:200; Molecular Probes, Eugene, OR, USA), dissolved in 1% NGS, 1% BSA, and 0.3% TX-100 in 0.1M PB. Thus, PRV-152-infected cells appeared green whereas those infected with PRV-BaBlu were red, and double-infected cells appeared yellow or orange depending on the balance of colors. Spinal cord sections were processed for GFP single-label immunofluorescence using the same protocol as above, but excluding antibodies for the detection of β-gal. Following processing, tissue sections were mounted on glass slides (SuperFrost slides, Fisher Scientific, Waltham, MA, USA) then coverslipped with Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA, USA).

*Antibody characterization:* The chicken anti-GFP antibody (Cat. No. 13970, Abcam, Cambridge, MA, USA) was raised against recombinant full-length protein. This antibody yields a single band on Western blot and detects GFP in transgenic mice expressing GFP in lamina II of the spinal cord (manufacturer's technical information). The mouse anti-β-galactosidase antibody (Cat. No. G4644, Sigma-Aldrich, St. Louis, MO, USA) was developed in mouse peritoneal cavities using β-galactosidase purified from E. coli as the immunogen. Using Western blot, this antibody was shown to be specific for β-gal in its native form (116 kD), and it reacts only with β-

galactosidase from E. coli (manufacturer's technical information). We previously validated specificity of these primary antibodies in immunofluorescent staining[156,157].

*Tissue analysis*: Immunofluorescently labeled tissue was examined by using a Leica DMR photomicroscope (Wetzlar, Germany). The presence of each fluorophore was detected by using specific filter sets (Chroma Technology, Brattleboro, VT, USA) with the following respective excitation and emission ranges: Alexa Fluor 488, 440-520 nm and 500-555 nm (green fluorescence) and CY3, 535-560 nm and 545-625 nm (red fluorescence). Swanson's (2004) rat atlas was used as a reference for anatomical classification[193]. Adjacent black-and-white images of the PVN were digitized under a 20× objective; images were then stitched using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA). Each of these large stitched images corresponded to a different fluorescent filter set, with GFP-positive neurons pseudocolored as green and β-gal-containing cells pseudocolored as red. These pseudocolored images were then overlaid, and co-localization of fluorophores was determined by turning each layer on and off to determine location and color of each cell. Double-labeled neurons appeared yellow or orange, depending on color balance. Individual PRV-positive PVN neurons were distinguishable by eye and manually counted from stitched micrographs digitized under 20× objective. Each field of view was digitized using specific filter sets for the green and red fluorescence. These signals were digitally overlaid, and the presence of either or both of the fluorophores was verified by manually turning on and off each virtual layer. Bilateral quantification was performed in each section, sampled at 240-μm intervals, through the PVN. Each labeled neuron was included in the counts. Adobe Photoshop CS5.1 (Adobe Systems, San Jose, CA, USA) was used to optimize brightness and contrast of the exported images. Figures were prepared using Adobe Photoshop and Illustrator CS5.1 (Adobe Systems).

***Experiment 2: Emotional behavior assessment and viral tract-tracing studies in selectively-bred HR versus LR rats***

Similar to the study in WKY and outbred Sprague Dawley rats, we evaluated behavior in adult male bHR and bLR rats ($n$ = 9-12 per group) using the same test battery: locomotor response to novelty, light-dark box test, and the FST. We then used PRV-152 and PRV-BaBlu for a tract-tracing study in a second cohort of bHR and bLR rats ($n$ = 5-9 per group) to examine possible differences in neural circuits that govern motor and autonomic output. PRV injections were made in an identical manner as in Experiment 1. Rats survived 120 hrs post-injection, and were euthanized as described above. Tissue processing was and analyses were conducted as in Experiment 1.

*Statistical analysis*

Data from behavioral and anatomical studies were analyzed using GraphPad Prism Software (Version 8.3.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Data sets were first verified to be normally distributed using the D'Agostino & Pearson omnibus normality test. Data were then analyzed with two-tailed Student's t-tests (behavior) or two-way ANOVA (PRV immunolabeling). Significance was set at $p < 0.05$, and results are presented as mean ± SEM. If significant treatment effects were observed by ANOVA, differences between groups were discerned by post hoc testing (Fisher's Least Significant Difference (LSD)).

## 2.3 Results

***Experiment 1***

*WKY rats display high levels of behavioral inhibition and passive stress coping compared to outbred Sprague Dawley rats*

Numerous previous studies have reported the distinct behavioral phenotype of WKY rats relative to other rat strains, including high levels of behavioral inhibition, anxiety-like behavior, and passive stress coping[23,80,81]. Our present behavioral findings were consistent with past work, with male WKY rats displaying a distinct behavioral phenotype relative to outbred male Sprague Dawley rats. WKY rats showed diminished locomotor activity (i.e., horizontal movement and rearing) in a novel cage compared to Sprague Dawley rats ($t(16) = 6.752$, $p < 0.0001$; **Figure 3A**) and increased immobility in the FST ($t(20) = 3.785$, $p = 0.0012$; **Figure 3B**). In the Light-Dark box text, WKY rats showed a trend for a higher latency to initially explore the lighted compartment than Sprague Dawley rats ($t(20) = 1.946$, $p = 0.0658$; **Figure 3C**) and spent overall less time in the lighted compartment ($t(20) = 2.153$, $p = 0.0437$; **Figure 3D**).

*Quantitative analyses of PRV immunolabeling in WKY versus outbred Sprague Dawley rats*

Next, we examined potential differences within neural circuits governing motor and sympathetic functions by quantifying immunolabeling of muscle-connected (PRV-152), adrenal-connected (PRV-BaBlu), and dual projection (presympathetic-premotor neurons, PSPMNs) in the PVN of WKY and outbred Sprague Dawley rats (**Figure 4**). At 120 hrs post-infection, we observed significant main effects of rat strain ($F(1,15) = 15.74$, $p = 0.0012$) and PRV strain ($F(2,15) = 20.83$, $p < 0.0001$), as well as an interaction of these factors ($F(2,15) = 8.256$, $p = 0.0038$), on the number of PRV-labeled cells (**Figure 4B**). Post hoc comparisons between the two rat strains revealed a significant decrease in the number of PRV-152-labeled cells in WKY rats compared

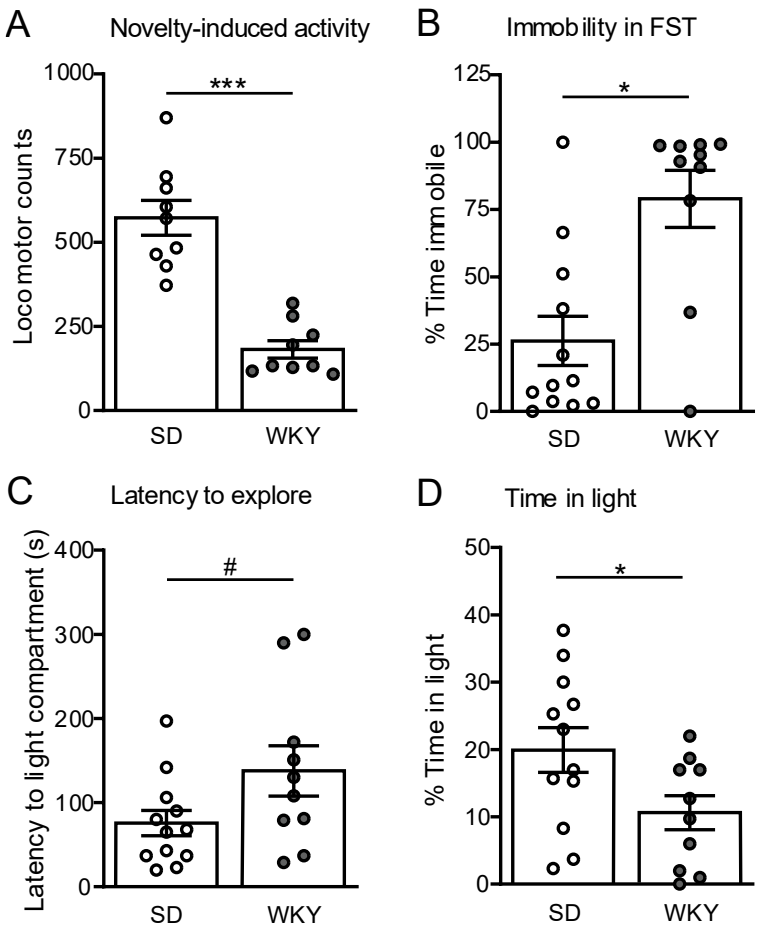


**Figure 3**. Emotional behavioral comparison between adult male Wistar Kyoto (WKY) rats and outbred Sprague Dawley (SD) rats. (**A**) WKY rats ($n = 9$) displayed markedly reduced locomotor responses compared to SD rats ($n = 9$) when placed in a novel test cage for 1 hr. (**B**) Relative to SD rats ($n = 12$), WKY rats ($n = 10$) show high levels of passive coping (immobility) in the Forced Swim Test. (**C–D**) WKY rats ($n = 10$) also display high levels of anxiety-like behavior in the Light-Dark Box Test, showing a trend for higher latency to initially enter the light compartment and spending significantly less time in the lighted compartment compared to SD rats ($n = 12$). Bars represent mean ± SEM. Statistically significant differences at ***$p < 0.0001$; *$p < 0.05$. Trend for statistical significance at #$p < 0.10$.

to Sprague Dawley rats ($p < 0.0001$). There were no differences in the numbers of neurons infected with PRV-BaBlu ($p = 0.5857$) or dually-infected with both viral strains ($p = 0.4901$). At 132 hrs post-infection, we also observed significant main effects of rat and PRV strains and a significant interaction between these two factors (rat strain: $F(1,48) = 11.92$, $p = 0.0012$; PRV strain: $F(2,48) = 23.08$, $p < 0.0001$; Interaction: $F(2,48) = 5.960$, $p = 0.0049$; **Figure 4C**). Post

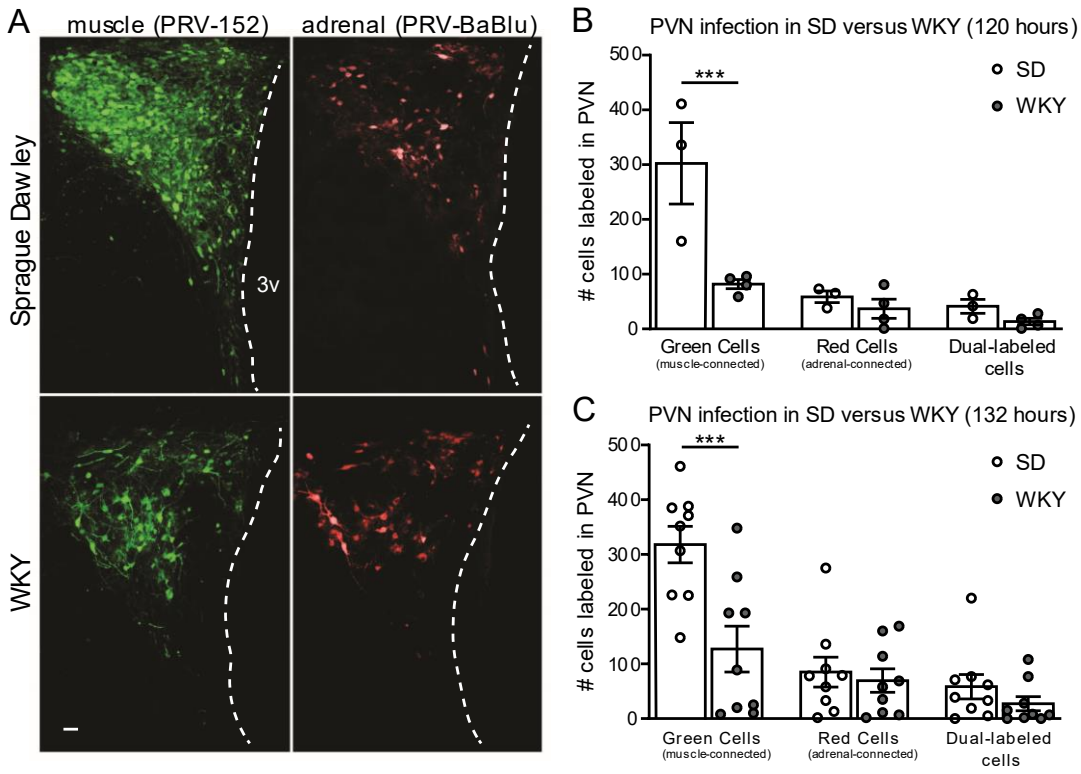


**Figure 4**. Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) and presympathetic (PRV-BaBlu) cells in the paraventricular nucleus of the hypothalamus (PVN) of adult male Wistar Kyoto (WKY) rats and outbred Sprague Dawley (SD) rats. (**A**) Transsynaptically labeled cells infected with PRV-152, which was injected in the gastrocnemius muscle, appear green, while cells labeled with PRV-BaBlu, which was injected into the adrenal gland, appear red. Images are from representative SD and WKY rats that survived 120 hr after injections with PRV-152 and PRV-BaBlu. Dashed lines show the border of the third ventricle. Scale bars, 50 µm. (**B**) At 120 hr post-infection, a substantially higher quantity of PRV-152-infected, muscle-connected cells in the PVN were observed in SD ($n$ = 3) vs. WKY ($n$ = 4) rats, with no significant differences in the quantity of PRV-BaBlu-infected, adrenal-connected cells or dual-labeled cells. (**C**) At 132 hr post-infection, a similar pattern persisted wherein SD rats ($n$ = 9) continued to show markedly higher numbers of PRV-152-infected cells in the PVN relative to WKY rats ($n$ = 9), with no differences in the number of PRV-BaBlu-infected or dual-labeled cells. Bars represent mean ± SEM. Statistically significant differences at ***$p < 0.0001$.

hoc comparisons between rat strains again revealed a significant reduction in the quantity of PRV-152-labeled cells in WKY rats relative to Sprague Dawley rats ($p < 0.0001$). As before, there were no differences in the numbers of neurons infected with PRV-BaBlu ($p = 0.6972$) or

dually-infected with both viral strains ($p = 0.4360$). Because the extent of PRV immunolabeling did not differ between post-infection time points for any rat or viral strain (120 hr versus 132 hr; Student's t-tests; $p > 0.05$), we will report immunolabeling data obtained at 120 hr post-infection for subsequent experiments.

Differences in the numbers of PRV-152-infected PVN neurons between Sprague Dawley and WKY rats were not explained by the greater efficiency of virus uptake by and entry into the first-order neurons (i.e., gastrocnemius motoneurons). We did not observe differences in the numbers of gastrocnemius motoneurons in spinal cord infected with PRV-152 between Sprague Dawley and WKY rats at 120 hr (Sprague Dawley: 18.5 ± 4.9; WKY: 15.6 ± 4.0; $t(21) = 0.2393$, $p = 0.8132$). The increased number of muscle-connected PVN neurons in Sprague Dawley rats correlates with their propensity for active coping rather than potential differences in virus uptake.

***Experiment 2***

*Behavioral similarities of selectively bred LR and WKY rats: High levels of behavioral inhibition and passive stress coping*

Previous studies have delineated the diverse behavioral phenotypes of bHR and bLR rats, two selectively-bred lines of Sprague Dawley rats bred based on extremes in novelty-induced exploratory behavior[36,50,184]. Our present findings are consistent with the earlier reports demonstrating that bLR rats show behavioral responses comparable to WKY rats, while bHR rats behave more similarly to outbred Sprague Dawley rats (**Figure 5**). In comparison to bHR rats, bLR rats showed significantly reduced locomotor activity in a novel cage ($t(16) = 15.25$, $p < 0.0001$; **Figure 5A**) and increased immobility in the FST ($t(20) = 4.469$, $p = 0.0002$; **Figure 5B**). In the Light-Dark Box Test, bLR rats showed a higher latency to initially explore the lighted

compartment ($t(20) = 5.027$, $p < 0.0001$; **Figure 5C**) and spent significantly less time overall in the lighted compartment relative to bHR rats ($t(20) = 5.357$, $p < 0.0001$; **Figure 5D**).

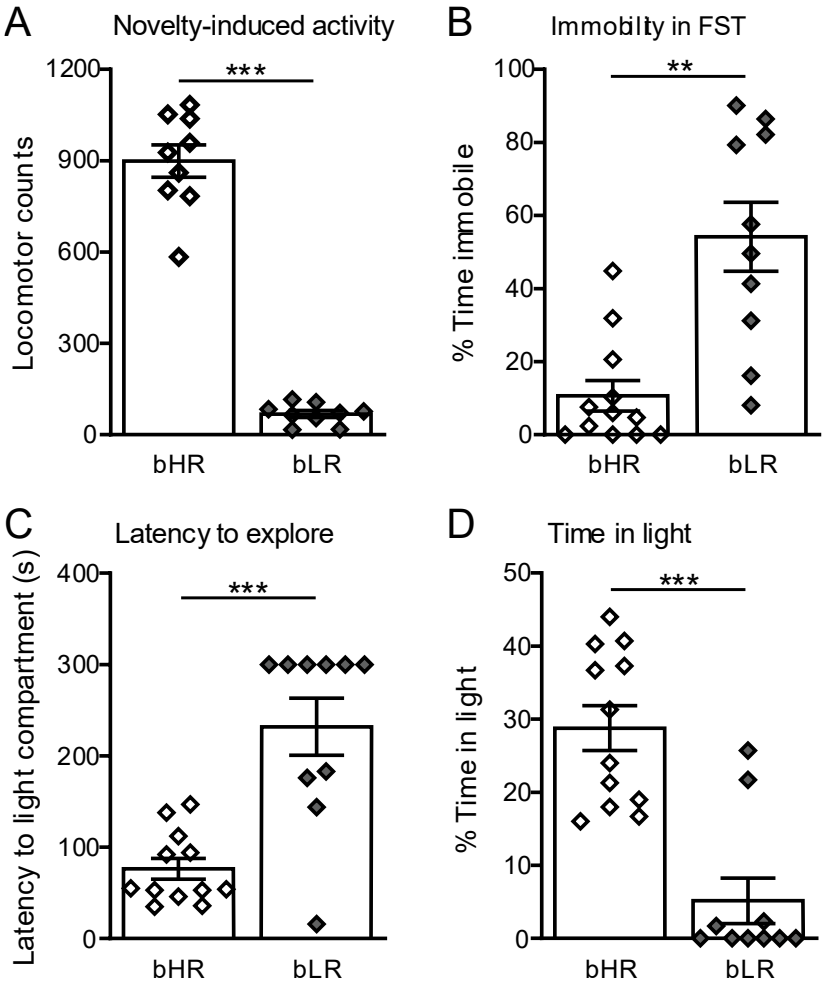


**Figure 5**. Comparison of emotional behavior between adult male rats selectively bred for high (bHR) or low (bLR) behavioral responses to novelty. (**A**) bLR rats ($n = 9$) showed significantly attenuated locomotor activity compared to bHR rats ($n = 9$) when placed in a novel test cage for 1 hr. (**B**) In the FST, bLR rats ($n = 10$) showed markedly higher levels of passive coping (immobility) relative to bHR rats ($n = 12$). (**C–D**) In the Light-Dark Box Test, bLR rats ($n = 10$) had a higher latency to begin exploring the lighted compartment and spent less time overall in the lighted area than bHR rats ($n = 12$). Bars represent mean ± SEM. Statistically significant differences at ***$p < 0.0001$; **$p < 0.01$.

*Quantitative analyses of PRV immunolabeling in bLR versus bHR rats*

As a follow-up to our previous experiment in WKY and Sprague Dawley rats, we next quantified PRV-labeled cells in the PVN of bHR and bLR rats to discern whether variations between

emotional motor circuits in these lines are similar to those observed in outbred Sprague Dawley and WKY rats (**Figure 6**). Indeed, our findings in the selectively bred bHR and bLR rat lines were similar to those described above. At 120 hrs post-infection, there were main effects of rat strain ($F(1,27) = 4.954$, $p = 0.0346$) and PRV strain ($F(2,27) = 11.66$, $p = 0.0002$), as well as an interaction of these factors ($F(2,27) = 4.458$, $p = 0.0212$; **Figure 6B**). Post hoc comparisons between bHR and bLR rats identified that differences in PRV immunolabeling occurred specifically in PRV-152-infected cells, with bLR rats showing a dramatic reduction in labeled premotor cells relative to bHR rats ($p = 0.0009$). There were no differences in the numbers of neurons infected with PRV-BaBlu ($p = 0.9430$) or dually-infected with both viral strains ($p = 0.9527$). Selectively bred bHR and bLR rats showed similar levels of PRV infection in labeled motor neurons of the spinal cord (bLR: 45.7 ± 2.9; bHR: 43.1 ± 1.9; $t(16) = 0.7314$, $p = 0.4751$).

### 2.4 Discussion

The present study examined differences in polysynaptic PVN projections to skeletal muscle and the adrenal gland in rats that exhibit innate differences in emotionality and stress reactivity. We first demonstrated altered emotional behaviors in male selectively bred LR (versus bHR) and WKY (versus outbred Sprague Dawley) rats, including substantially reduced locomotor activity in a novel environment, elevated anxiety-like behavior, and increased passive stress coping. These findings are consistent with prior studies in the bLR and WKY models documenting behavioral anomalies within domains relevant to depression[23,25,36,80,183]. Subsequent PRV tract-tracing revealed a decrease specifically in muscle-projecting PVN neurons within bLR and WKY animals relative to control strains. We did not detect differences in the quantities of adrenal-connected neurons or neurons with polysynaptic collateral projections to both targets. This

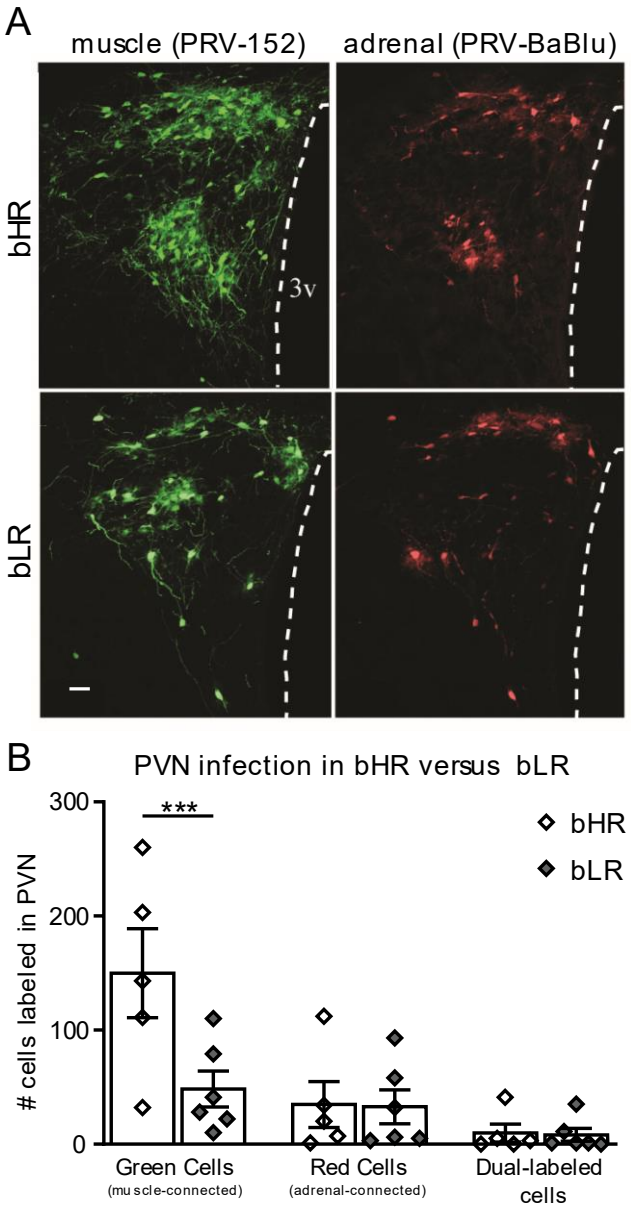


**Figure 6**. Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) and presympathetic (PRV-BaBlu) cells in the paraventricular nucleus of the hypothalamus (PVN) of adult male rats selectively bred for high (bHR) or low (bLR) behavioral responses to novelty. (**A**) Transsynaptically labeled cells infected with PRV-152, which was injected in the gastrocnemius muscle, appear green, while cells labeled with PRV-BaBlu, which was injected into the adrenal, appear red. Images are from representative bHR and bLR rats that survived 120 hr after injections with PRV-152 and PRV-BaBlu. Dashed lines show the border of the third ventricle. Scale bars, 50 µm. (**B**) At 120 hr post-infection, a substantially higher quantity of PRV-152-infected, muscle-connected cells in the PVN were observed in bHR ($n$ = 5) vs. bLR ($n$ = 6) rats, with no significant differences in the quantity of PRV-BaBlu-infected, adrenal-connected cells or dual-labeled cells. Bars represent mean ± SEM. Statistically significant differences at ***$p < 0.0001$.

neurobiological difference may underpin their distinct motoric responses under stressful conditions.

The bLR and WKY rat lines are extensively used animal models that replicate key physiological and behavioral responses described in clinically depressed patient populations[50,71,80,182,184,194]. Major depressive disorder, in addition to its cognitive and mood-related symptoms, is also characterized by debilitating physical symptoms and motor deficits. The latter entails a wide array of impairments, not limited to execution of simple motor tasks with minimal cognitive demand, motor aspects of speech production, and execution of willful movements[107,108,111,112,195]. We have previously postulated that premotor and presympathetic manifestations of depression are likely related to one another, with clinical data raising the possibility that premotor-presympathetic circuits (also termed somatomotor-sympathetic circuits) may be dysregulated in depressed patients and contribute to the presentation of physical symptoms[112].

Our group has identified that the PVN likely plays a major role in somatomotor-sympathetic integration[112,156,157] in addition to its well-defined role in integrating endocrine, autonomic, and behavioral responses to stress[112,196,197]. The present study expands upon our previous work delineating central premotor-presympathetic neural circuits in male Sprague Dawley rats by examining this circuitry in two animal models with heritable differences in stress-induced emotionally motivated behaviors. Intriguingly, in male bLR and WKY rats, differences in emotional motor circuitry appear to be specific to muscle-connected premotor PVN cells, as there were no relative differences in the labeling of adrenal-connected presympathetic cells or dual-function presympathetic-premotor neurons (PSPMNs) between WKY or bLR rats and their respective controls. These findings are generally in agreement with

our prior work showing that that a higher proportion of PRV-labeled cells consist of premotor- rather than presympathetic-projecting cells[157]. Under non-stressful conditions, male WKY and bLR rats, relative to Sprague Dawley and bHR rats, show no differences in basal locomotor activity during the light period, coinciding with the timing of behavioral testing in this study [68,81]. Our findings suggest that deficits in the PVN premotor node of the emotional motor circuit may drive inherited differences in emotionally motivated locomotor behavior.

The absence of a difference in labeled presympathetic PVN projections or dual-function PSPMNs between rats with inborn emotional behavior differences and their respective controls may be surprising given that each of these models have demonstrated perturbations in behavioral and hormonal responses to stress. While these strains feature similar behavioral profiles, as demonstrated by our present work as well as that of others[25,36,50,80,81,182,183], WKY and bLR rats do show some differences in hypothalamic-pituitary-adrenal (HPA) axis activity and reactivity. Whereas WKY rats show exaggerated adrenocorticotropic hormone (ACTH) and corticosterone (CORT) responses to acute and chronic stress and elevated basal CORT at the peak of the diurnal cycle[23,80,101], bLR rats show reduced activity and reactivity of the HPA axis[48,68]. In both of these cases, however, stress-induced CORT responses are primarily modulated by neuroendocrine mechanisms. Upon stress exposure, release of corticotropin-releasing hormone (CRH) from the PVN eventually results in glucocorticoid release from the adrenal cortex[198]. However, through sympathetic mechanisms, stressors also induce sympathetic PVN projections to drive norepinephrine and epinephrine release from the adrenal medulla[198–200]. In this manner, both hormonal and sympathetic-mediated mechanisms contribute to stress responses in rats and other organisms. The present findings suggest that sympathetic regulation of the stress response may not be altered in male WKY or bLR rats relative to their respective controls. However, the

neurobiological bases underlying differential neuroendocrine responses to stress in these models was not tested in and is outside the scope of the present study.

Despite the lack of observed differences in adrenal-connected neurons or dual-projecting PSPMNs in the PVN of WKY and bLR rats, it is possible that such neural circuit perturbations exist in other regions involved in emotional behavior regulation. Two possible candidates for future investigations into this area are the dorsomedial hypothalamus and ventral periaqueductal gray, two prominent nodes in the premotor-presympathetic circuitry[112,156,157,191] that are both implicated in shaping behavioral and autonomic responses to panic[201–203]. Pre-autonomic emotional motor circuits in rats have previously been demonstrated to be altered by early environmental perturbations – namely, alterations in maternal care during early postnatal development[169,171]. Selectively-bred HR and LR rat dams show distinct differences in maternal style, and indeed, patterns of maternal care impact offspring neurodevelopment and emotional behavior outcomes[204–206]. Interestingly, the act of cross-fostering bLR rat offspring to bHR mothers improves adult anxiety-like behavior and enhances social interaction[51], suggesting that parental behavior itself may have the ability to shape not only emotional behavior but also the neural circuits contributing to the expression of depressive-like behaviors in adult rodents.

As in prior work, the experiments outlined above used PRV-152 and PRV-BaBlu, which are both derived from PRV-Bartha. Each strain is capable of effectively co-infecting the same neuronal population simultaneously[156,157,159,161–165,191]. It should be acknowledged, however, that infection with one virus can make it less likely that a neuron will be infected with a different PRV strain[207], and that PRV-152 is more invasive and virulent than PRV-BaBlu[154]. Although it is unlikely that these limitations would account for relative differences in immunolabeling of adrenal-connected presympathetic neurons between WKY versus Sprague Dawley rats and bLR

versus bHR rats, they may have led us to underestimate the total quantity of adrenal-connected neurons in the PVN of each rat strain studied here. Constraints with respect to properly matching the temporal transport of the two PRV strains to the PVN[156,157] necessitated the methodological decision to inject PRV-152 in gastrocnemius prior to PRV-BaBlu in adrenal gland in these experiments. Our observation that PRV-152 infection of gastrocnemius motoneurons in spinal cord is comparable between WKY versus Sprague Dawley rats and bLR versus bHR rats suggests equivalent uptake of PRV by first-order neurons. Thus, differences between rat strains in PRV-152 immunolabeling are likely not explained by differences in motoneurons between rat strains. We cannot exclude the possibility, however, that there may be differences in the timing of viral spread such that PRV is transported more quickly to afferent neurons in Sprague Dawley or bHR rats versus WKY and bLR rats, respectively, which may result in differences in PVN immunolabeling. This possibility would need to be addressed using alternative polysynaptic viruses or classic monosynaptic tracers.

### 2.5 Conclusions

In conclusion, the present studies provide evidence to suggest that, in male subjects in two rodent models, heritable differences in emotional temperament and stress reactivity are accompanied by drastic reductions in polysynaptic projections to skeletal muscle from the PVN. These emotional motor circuit differences may underlie locomotor disturbances associated with altered emotional states. Such findings have implications for individuals experiencing depressive or anxiety-related disorders, which feature symptoms such as psychomotor retardation and behavioral inhibition in novel contexts. Future studies should aim to further characterize the nature of the descending PVN neurons in these animal models. Pertinent investigations might include elucidating sex

differences in emotional motor circuitry and determining the expression profiles of relevant neuromodulators and neuroactive peptides (e.g., vasopressin, oxytocin) within these cell populations.

**Note**: The contents of Chapter 2 were published previously in *European Journal of Neuroscience*. The respective contributions of Ilan Kerman, M.D., Ph.D., Sarah Clinton, Ph.D., Matthew Glover, Ph.D., and Keaton Unroe, Ph.D., to this work are gratefully acknowledged. The study authors also thank Elizabeth Bauer, Ph.D., and David Krolewski, Ph.D., for their thorough and thoughtful peer review of the manuscript prior to publication. Figure numbers have been adapted from the original publication to reflect their order of appearance in this dissertation.

# CHAPTER 3: MOTOR PROJECTIONS FROM THE PERIAQUEDUCTAL GREY AND LOCUS COERULEUS ARE ALTERED IN TWO RAT MODELS WITH INBORN EMOTIONAL BEHAVIOR DIFFERENCES

## 3.1 Introduction

Numerous behaviors in humans and animals depend upon intricate coordination of the somatic motor and autonomic nervous systems. While plenty of these behaviors occur in the absence of stress, this synchronized outflow of motor and autonomic efferents is often engaged as part of a broader response to changes in an organism's environment. A cornerstone example of this phenomenon is the classic fight-or-flight response, an emotionally motivated stress response in which an organism encounters a predator or rival and must prepare to fight or flee to ensure survival. The defensive-aggressive fight-or-flight response is distinguished by active stress coping strategies involving a combination of somatomotor actions (e.g., alerting, hissing, and sudden attack behavior) and concomitant autonomic changes (e.g., elevated blood pressure and cardiac output)[125–127,157]. The contrasting actions involved in a passive stress coping approach are characterized by their own distinct behavioral and physiological features, including immobilization, enhanced corticosteroid secretion, and changes in blood pressure[125,157]. Together, these examples and others underscore the necessity for organisms to have a means of integrating the parallel activities of the somatic motor and autonomic nervous systems[112].

Mechanisms governing central control of motor and autonomic outflows were traditionally considered to be quite distinct from one another[112]. Actions of the somatic motor system, which innervates and commands skeletal muscle fibers, were thought to be largely voluntary[208]. By contrast, autonomic functions are thus named because they occur automatically and without conscious control[209,210]. These assumptions have since been challenged in

anatomical studies by Holstege and colleagues, who were the first to describe an emotional motor system responsible for coordinating somatomotor activity during emotionally motivated behaviors[128,129,131]. Although this system is organized similarly to the somatic motor system, structures comprising the emotional motor system also receive afferents from several integrative limbic structures and contain neurons that regulate autonomic functions[112,129,211,212].

Our laboratory group sought to follow up on these observations by determining whether integration of somatomotor-sympathetic efferents was achieved by populations of neurons with singular functional roles in common neuroanatomical nodes of this circuit or by populations of neurons with dual functions. Using typically behaving Sprague Dawley rats, we adopted a retrograde viral tract-tracing strategy to separately label transsynaptic connections from peripheral motor and sympathetic targets[112,156,157]. Two genetically modified strains of pseudorabies virus (PRV), PRV-152 and PRV-BaBlu, were injected into our selected motor target (sympathectomized hindlimb gastrocnemius muscle) and our sympathetically innervated target (adrenal gland), respectively. These experiments revealed the presence of discrete somatomotor and sympathetic neurons, as well as putative dual-function somatomotor-sympathetic neurons (SMSNs) infected by both recombinant PRV strains, throughout the central rostral-caudal axis[112]. The majority of caudal SMSNs were concentrated in subareas of the ventral medulla, although a small number of caudal SMSNs were also located in locus coeruleus (LC) and the A5 and A7 noradrenergic cell groups[156]. Rostrally located SMSNs were observed in several regions, with a majority concentrated in subnuclei of the periaqueductal gray (PAG; caudal and intermediate ventrolateral portions) and hypothalamus (dorsomedial (DMH), dorsolateral lateral (dlLH), and medial parvocellular ventral subdivision of the paraventricular nucleus (PVNmpv))[157]. In agreement with prior work using classical tract-tracing methods, these

studies found that ventromedial medulla, ventrolateral PAG (VLPAG), PVN, and LH contained direct projections to spinal cord. Higher-order SMSNs were distributed in several additional areas, including DMH (which has projections to PVN, VLPAG, and ventromedial medulla) and other subdivisions of the PAG[112,156,157].

A notable finding that emerged from these experiments was that several areas involved in somatomotor-sympathetic integration were key structures involved in the regulation of organismal responses to stress. Perturbations of the stress response are commonly observed in patients with mood and anxiety disorders[3,213]. Recent work by our laboratory investigated whether somatomotor-sympathetic circuitry of the PVN, an key integrator of behavioral, endocrine, and autonomic responses to stress[196], is altered in rodent models for emotional behavior dysregulation[214]. Our PRV-mediated neuroanatomical investigations in male Wistar-Kyoto (WKY) and selectively bred Low Novelty Responder (bLR) rats, two models featuring inborn differences in emotionality and stress reactivity[25,36,80,81,183], revealed that both strains display fewer descending somatomotor projections from the PVN, potentially contributing to the locomotor disturbances associated with their altered emotional states[214]. In the present study, we continued our investigations in the WKY and bLR models to explore the extent to which premotor midbrain and brainstem circuitry differ in these models relative to their respective controls. Using PRV transsynaptic tract-tracing, the following experiments evaluated descending somatomotor circuits in two additional structures relevant to stress and emotional regulation: the PAG and LC. Based on our previous observations, we hypothesized that fewer premotor projections to hindlimb skeletal muscle would be observed in subdivisions of the PAG and LC of WKY and bLR rats.

## 3.2 Methods

Experiments described in this report were approved by the Institutional Committee on the Use and Care of Animals (IACUC) and performed in accordance with the National Institutes of Health guidelines on research in animals (USA, 2011).

*Animals*

Adult male WKY and Sprague Dawley rats were purchased from Charles River Laboratories (Kingston, NY, USA). Adult male selectively bred Low and High Responder rats were obtained from the fifteenth generation of the in-house breeding colony. The process of generating and characterizing the selectively bred HR and LR lines has been described elsewhere[36,61]. Validation of the emotional behavior phenotypes in these strains was performed previously[214]. Housing was maintained under conditions of standard temperature (21-23ºC) and humidity (50-55%) in a 12:12 hr light-dark cycle (lights on at 6:00 a.m.). Rats were housed in groups of 2-3 animals per cage and provided *ad libitum* chow and water.

***Experiment 1: Viral tract-tracing of PAG and LC premotor efferents in Wistar-Kyoto rats versus outbred Sprague Dawley rats***

*Viral tract-tracing overview*

The first experiment employed retrograde viral tract-tracing to label premotor and presympathetic efferents in a cohort of WKY and Sprague Dawley rats ($n$ = 9 per strain). To this end, two transgenic recombinants of an attenuated PRV strain, PRV-Bartha, were used: (1) PRV-152, which contains the gene encoding enhanced green fluorescent protein (eGFP), and (2) PRV-BaBlu, which carries the lac Z gene at the gG locus and codes for β-galactosidase (β-gal)[159,160].

In the present study, PRV-152 was injected into sympathectomized gastrocnemius muscle (i.e., skeletal muscle; selected motor target) while PRV-BaBlu was injected into the adrenal gland (i.e., selected sympathetic target) as described in earlier work by our laboratory[112,157,158,214]. Although both PRV strains were used to keep in line with prior methodological choices, the analyses in this study centered on premotor circuitry based on our recent observation that PVN somatomotor projections, but not sympathetic or somatomotor-sympathetic projections, differ among two distinct rat strains with endogenous emotional behavior differences[214].

*Sympathectomy and viral injections*

The procedures performed during surgical sympathectomy of the hindlimb gastrocnemius muscle and subsequent injections with PRV-152 and PRV-BaBlu in WKY and Sprague Dawley rats were performed as described in Chapter 2 of this dissertation, in line with prior work by our laboratory[156,157,215]. Rats were allowed to survive 132 hrs ($n$ = 9 per group) after the initial injections with PRV-152 and sacrificed in the manner described before[214].

*Tissue processing*

Following the designated survival period, collection and processing of brain and spinal cord tissue were carried out as outlined in Chapter 2 of this dissertation. In the present study, brains of PRV-injected rats were processed for immunofluorescent detection of eGFP ($n$ = 5 per strain). Free-floating coronal brain sections spanning Bregma levels -6.72 mm to -14.76 mm were washed several times at room temperature with 1X phosphate buffered saline (PBS; pH 7.4) followed by incubation for one hour in blocking solution (0.2% TX-100 and 2% gelatin from cold water fish skin in 1X PBS). Sections were then incubated in primary antibody solution

(chicken anti-GFP (Cat. No. ab13970, Abcam, Cambridge, MA, USA), 1:2,000 in blocking solution, for one hour at room temperature before incubating overnight at 4°C. The next day, tissue was rinsed several times in 1X PBS at room temperature before incubating in secondary antibody solution for two hours (AlexaFluor 488 goat anti-chicken IgY H+L (Cat. No. A11039, Invitrogen/Life Technologies Corporation, Eugene, OR, USA), 1:200, in blocking solution). Following additional washes in 1X PBS, tissue was mounted to glass slides and coverslipped with VectaShield Antifade Mounting Medium with DAPI (Cat. No. H-1200, Vector Laboratories, Newark, CA, USA) and stored at 4°C.

*Tissue analysis*

Whole brain sections were imaged using a Keyence BZ-X810 All-in-One Fluorescence Microscope equipped with a computer-controlled motorized stage (Keyence Corporation of America, Itasca, IL, USA). Images were digitized under a 20× objective with specific filter sets corresponding to DAPI (excitation and emission ranges 325-375 nm and 435-485 nm, respectively; Cat. No. 49000-UF1, Chroma Technology Corporation, Bellows Falls, VT, USA) and eGFP (excitation and emission ranges 450-490 nm and 500-550 nm, respectively; Cat. No. 49002-UF1, Chroma Technology Corporation). Images were acquired in multiple z-stacks and stitched together under Full Focus in BZ-X Analyzer software (Keyence).

The Rat Brain in Stereotaxic Coordinates was used as a reference for anatomical classification of individual regions of interest[216]. Bilateral quantification of cells expressing eGFP and measurements of area ($mm^2$) was performed manually in FIJI software[217] for consecutive sections sampled at 240-µm intervals. Where necessary, co-expression of DAPI was used to confirm the location of discrete cell somas. GFP-expressing cells were quantified in the

LC (Bregma levels -9.48 mm to -10.32 mm) and PAG (Bregma levels -6.72 to -8.64). Discrete quantifications of GFP-expressing cells were performed for four subdivisions of the PAG: (1) dorsomedial (DMPAG; Bregma levels -6.72 mm to -8.76 mm); (2) dorsolateral (DLPAG; Bregma levels -6.72 mm to -8.16 mm); (3) lateral (LPAG; Bregma levels -6.72 mm to -8.64 mm); and (4) ventrolateral (VLPAG; Bregma levels -6.72 mm to -8.76 mm). One WKY sample was excluded from analysis due to poor tissue quality after processing.

Figures were prepared using BZ-X Analyzer software and Adobe Illustrator 2023 (Adobe Inc., San Jose, CA).

### ***Experiment 2: Viral tract-tracing of PAG and LC premotor efferents in selectively bred Low Novelty Responder versus High Responder rats***

In complement to Experiment 1, a separate cohort of bHR and bLR rats ($n$ = 7 per group) were used in a parallel PRV tract-tracing study. PRV injections were performed as previously described for Experiment 1. Rats were allowed to survive 120 hrs post-injection. Subsequent procedures for euthanasia, tissue collection, and tissue processing were performed in a manner identical to those described for Experiment 1. GFP immunofluorescence was carried out in a subset of tissue samples ($n$ = 5 per group). Two samples ($n$ = 1 per group) were excluded from final analysis due to insufficient PRV immunolabeling.

#### *Statistical analysis*

GraphPad Prism Version 9.5.1 (GraphPad Software) was used for statistical analysis. Cell counts were calculated as the sum of total bilateral counts from all sections divided by the total area ($mm^2$) over which counts were performed to account for disparities in the number of tissue

sections surveyed between samples. Data were assessed for normality prior to analysis using the Shapiro-Wilk test. Data were excluded only if determined to be an outlier using both the Grubbs test and the ROUT method. When data were normally distributed, differences between WKY versus SD rats and bHR versus bLR rats were analyzed using two-tailed Welch's t-tests. The Mann-Whitney U test (two-tailed) was used when data were not normally distributed. All results are expressed as mean ± SEM. Significance was set at $p < 0.05$.

### 3.3 Results

#### *Experiment 1*

*Quantitative analyses of PRV-152 immunolabeling in WKY versus Sprague Dawley rats*

We first examined eGFP immunolabeling of cells with premotor projections to gastrocnemius muscle in the dorsomedial (DMPAG), dorsolateral (DLPAG), lateral (LPAG), and ventrolateral (VLPAG) subdivisions of the PAG in WKY and Sprague Dawley rats (**Figure 7**). Relative to Sprague Dawley rats, WKY rats showed significant reductions in the quantity of premotor projecting cells in the DMPAG (Mann-Whitney test, $p = 0.0159$; **Figure 7B**), LPAG ($t(6.954) = 3.194$, $p = 0.0153$; **Figure 7D**), and VLPAG ($t(6.496) = 3.028$, $p = 0.0210$; **Figure 7E**). Although a smaller quantity of premotor efferent cells were also observed in the DLPAG of WKY rats relative to Sprague Dawley rats, the data did not meet the threshold for significance ($t(4.249) = 2.463$, $p = 0.0658$; **Figure 7C**). In both strains, immunolabeling of somatomotor projections in the DLPAG was sparse and appeared to be limited to the more caudal extent of the PAG.

Next, we quantified immunolabeling of muscle-connected cells in the LC (**Figure 8**).

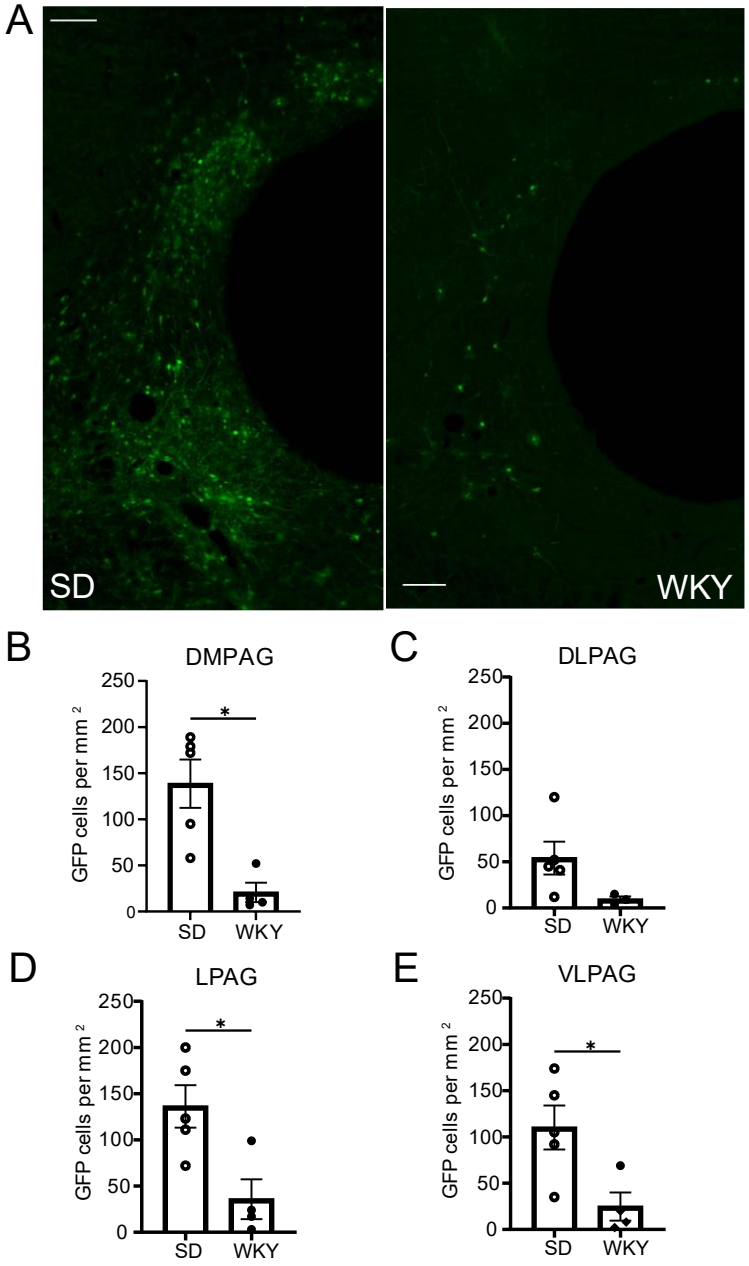


**Figure 7.** Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) cells in the periaqueductal gray (PAG) of adult male Sprague Dawley (SD) and Wistar-Kyoto (WKY) rats. (**A**) Representative images of transsynaptically labeled cells infected with PRV-152 in subdivisions of the PAG in SD and WKY rats that survived 132 hrs after injections with PRV-152 and PRV-BaBlu. The aqueduct (Aq) is oriented to the right side of each image. Scale bar, 200 µm. (**B**) Quantification of somatomotor projecting cells in dorsomedial (DM)PAG. (**C**) Quantification of somatomotor projecting cells in dorsolateral (DL)PAG. (**D**) Quantification of somatomotor projecting cells in lateral (L)PAG. (**E**) Quantification of somatomotor projecting cells in ventrolateral (VL)PAG. Bars represent mean ± SEM. Statistically significant differences at $*p < 0.05$.

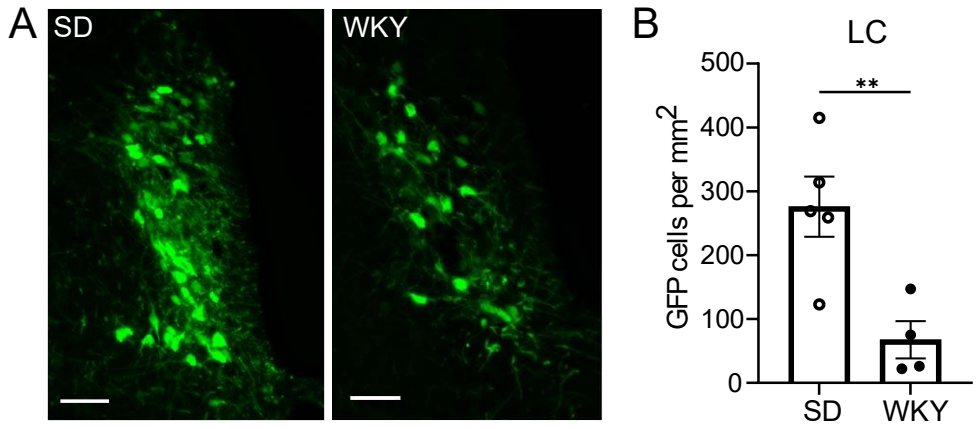


**Figure 8.** Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) cells in the locus coeruleus (LC) of adult male Sprague Dawley (SD) and Wistar-Kyoto (WKY) rats. (**A**) Representative images of transsynaptically labeled cells infected with PRV-152 in the LC of SD and WKY rats that survived 132 hrs after injections with PRV-152 and PRV-BaBlu. The fourth ventricle (4V) is oriented to the right side of each image. Scale bar, 100 µm. (**B**) Quantification of somatomotor projecting cells in LC. Bars represent mean ± SEM. Statistically significant differences at $*p < 0.05$.

Mirroring the findings in PAG, the LC contained fewer premotor efferent cells in WKY rats compared to Sprague Dawley rats ($t(6.391) = 3.761$, $p = 0.0084$; **Figure 8B**). Immunolabeling of cells infected with PRV-152 in both strains was predominantly observed in the rostral and intermediate levels of LC.

***Experiment 2:***

*Quantitative analyses of PRV-152 immunolabeling in bLR versus bHR rats*

In complement to the assessments performed in Experiment 1, immunolabeling of premotor efferents was first quantified in subdivisions of the PAG in bLR rats relative to bHR rats (**Figure 9**). Significantly fewer premotor projections to gastrocnemius muscle were observed in the DLPAG of bLR rats compared to bHR rats (Mann-Whitney test, $p = 0.0286$; **Figure 9C**). Trends for a significant decrease in immunolabeling of premotor efferents were observed in LPAG (Mann-Whitney test, $p = 0.0571$; **Figure 9D**) and VLPAG (Mann-Whitney test, $p = 0.0571$;

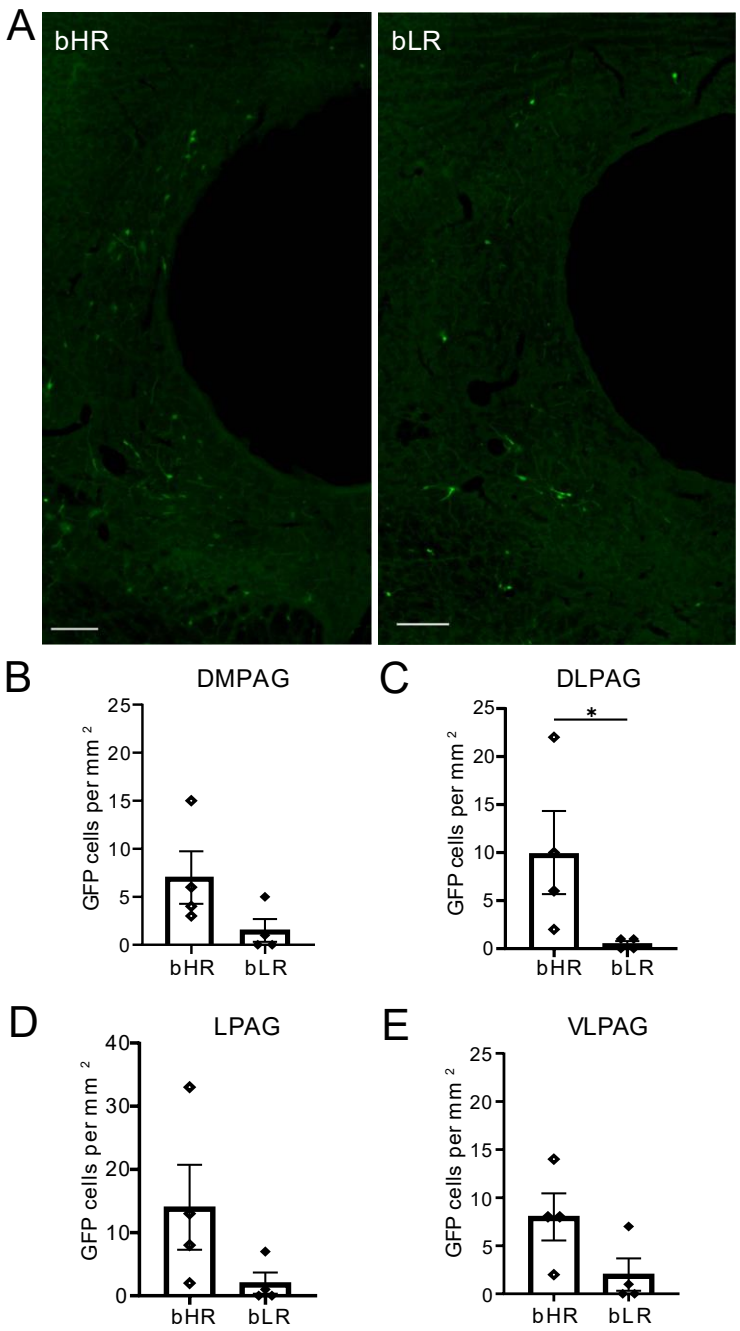


**Figure 9.** Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) cells in the periaqueductal gray (PAG) of adult male selectively bred High Novelty Responder (bHR) and Low Novelty Responder (bLR) rats. (**A**) Representative images of transsynaptically labeled cells infected with PRV-152 in subregions of the PAG in bHR and bLR rats that survived 120 hrs after injections with PRV-152 and PRV-BaBlu. The aqueduct (Aq) is oriented to the right side of each image. Scale bar, 200 µm. (**B**) Quantification of somatomotor projecting cells in dorsomedial (DM)PAG. (**C**) Quantification of somatomotor projecting cells in dorsolateral (DL)PAG. (**D**) Quantification of somatomotor projecting cells in lateral (L)PAG. (**E**) Quantification of somatomotor projecting cells in ventrolateral (VL)PAG. Bars represent mean ± SEM. Statistically significant differences at $*p < 0.05$.

**Figure 9E**). Significant differences in immunolabeling of premotor efferents were not observed in the DMPAG ($t(4.094) = 1.842$, $p = 0.1376$; **Figure 9B**).

Finally, immunolabeling of premotor projections in LC was examined for bHR and bLR rats (**Figure 10**). Relative to bHR rats, bLR rats had significantly fewer PRV-152-infected cells in the LC ($t(3.954) = 3.612$, $p = 0.0230$; **Figure 10B**). As in WKY and Sprague Dawley rats, eGFP-expressing cells in the LC of bHR and bLR rats were primarily observed at the rostral and intermediate levels of this structure.

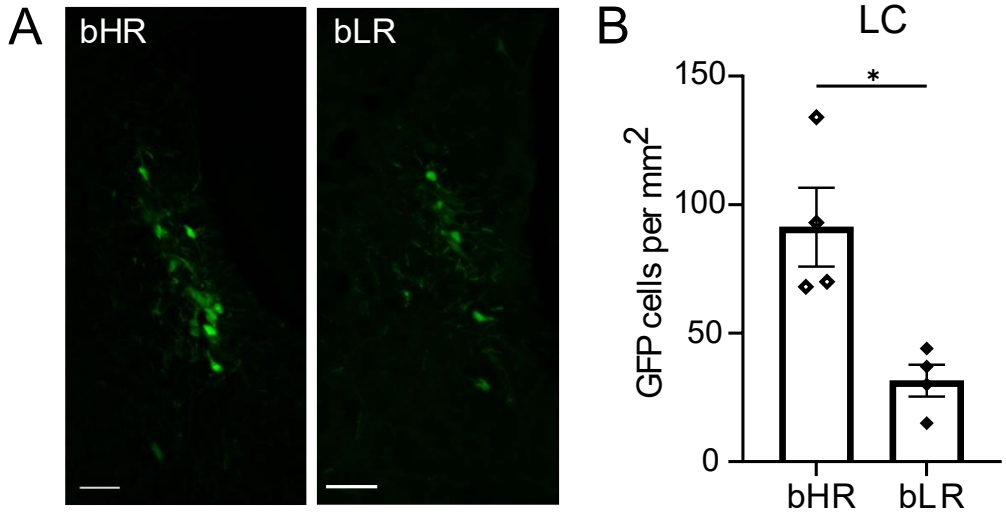


**Figure 10.** Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) cells in the locus coeruleus (LC) of adult male adult male selectively bred High Novelty Responder (bHR) and Low Novelty Responder (bLR) rats. (**A**) Representative images of transsynaptically labeled cells infected with PRV-152 in the LC of bHR and bLR rats that survived 120 hrs after injections with PRV-152 and PRV-BaBlu. The fourth ventricle (4V) is oriented to the right side of each image. Scale bar, 100 µm. (**B**) Quantification of somatomotor projecting cells in LC. Bars represent mean ± SEM. Statistically significant differences at $*p < 0.05$.

### 3.4 Discussion

The experiments in this chapter extended our neuroanatomical investigations of polysynaptic somatomotor projections from central circuits to skeletal muscle in two rat models that innately display behavioral and physiological traits relevant to emotional disorders. Using a PRV tract-

tracing approach, we demonstrated that male WKY and bLR rats both have fewer descending somatomotor projections from LC and various subdivisions of the PAG. Specifically, male WKY rats have fewer muscle-projecting neurons from the LC, DMPAG, LPAG, and VLPAG (relative to outbred Sprague Dawley rats), and male bLR rats have fewer muscle-projecting neurons from the LC and DLPAG (compared to bHR rats). Despite these models being derived from separate genetic backgrounds, these findings indicate that both strains share an additional premotor circuit abnormality beyond that documented previously by our group[214]. The viral tract-tracing data in PAG also suggest the possibility that some nodes of the somatomotor-sympathetic circuitry could be differentially regulated between these two model systems, which might contribute to observed variations between these strains.

Prior retrograde tract-tracing experiments by our laboratory demonstrated that the LC contains a small but notable quantity of neurons with dual premotor and presympathetic outflows (in addition to single-function neurons with these roles)[156]. The LC modulates sympathetic arousal, cognitive and behavioral responses to stress, and other functions via noradrenergic signaling throughout the forebrain, brainstem, and spinal cord[150,218]. LC neurons can shift their mode of activity between tonic and phasic firing, allowing an organism to shift attentional strategies (i.e., survey its surroundings or sustain focused attention) to adapt to changes in its environment[219,220]. When the hypothalamic-pituitary-adrenocortical (HPA) axis is recruited during stress, corticotropin-releasing hormone (CRH) release by the PVN engages LC neurons to favor increased tonic firing (and decreased phasic firing) to promote greater sympathetic arousal and cognitive flexibility[218]. A recent study shows that chemogenetic activation of the LC in mice promotes anxiety-relevant behavior in the open field, reduces exploratory locomotor behavior, and enhances brain-wide functional connectivity[221].

Although investigations of the LC-norephinephine (NE) system in the bLR model are not well-documented, some data are available in the WKY model. Electrophysiological investigations in WKY rats show that basal and phasic firing activity are elevated in these animals[105]. Additionally, WKY rats show attenuated NE release during acute stress as well as molecular signatures consistent with greater NE turnover[102,106]. Considered alongside our laboratory's recent findings that fewer premotor connections exist in the LC and PVN of male WKY (and bLR) rats, it may be possible to speculate explanations for their propensities for passive stress coping and attenuated locomotor response to novelty. While our neuroanatomical work thus far is unable to conjecture the existence of indirect premotor projections from PVN to skeletal muscle via the LC, other neuroanatomical studies in the Sprague Dawley rat brain have shown that a subset of CRH-immunoreactive neurons from PVN send monosynaptic projections to LC[222]. If this population of LC-projecting PVN neurons contains indirect premotor projections to skeletal muscle, the observation that WKY and bLR rats have fewer premotor projections in both PVN and LC could potentially contribute to a more passive behavioral approach (i.e., reduced locomotor activity) in response to stressful or novel situations by way of decreased anatomical resources to engage an adequate locomotor response. Perhaps more likely, however, is that the role of the LC in promoting a passive stress coping strategy in these animals is more related to observed deficits in stress-induced noradrenergic reactivity[105,106], independent of premotor efferent projections.

In comparison to the LC, the PAG appears to be a more prominent node of the premotor-presympathetic circuitry[112,156]. The PAG is implicated in autonomic and behavioral responses to panic and threat as well as modulation and perception of pain[201]. This structure consists of dorsal (DLPAG, LPAG) and ventrolateral (VLPAG) columns that span the rostral-caudal extent and

mediate distinct stress coping strategies[223]. Early functional studies in the PAG demonstrated that the caudal two-thirds of the dorsal PAG are involved in active defensive reactions, which are characterized by escape behaviors and elevated blood pressure, among other features. Whereas active coping strategies mediated by the DLPAG appear to be engaged by escapable psychological stressors, the activities of the LPAG are likely engaged by escapable physical stressors[223]. By contrast, the ventrolateral portion of the caudal PAG promotes manifestations of passive stress coping (i.e., immobility, hypotension, and hypovigilance) in response to both physical and psychological stressors[223]. Our prior work has described VLPAG as a node of the somatomotor-sympathetic circuitry that likely contains direct projections to spinal cord[112,157]. Although small numbers of somatomotor-sympathetic projections are also observed in DLPAG and DMPAG, immunolabeling of these efferents is not observed until later survival times when viral infection reveals more upstream components of this circuitry[112].

In the present study, our PRV tract-tracing investigation revealed that WKY rats (compared to Sprague Dawley rats) display significantly reduced numbers of somatomotor efferents in several subdivisions of the PAG (dorsomedial, lateral, and ventrolateral), as well as a trend for a decrease in these efferents in DLPAG. In bLR rats (compared to bHR rats), significant reductions in immunolabeling of premotor efferents were limited to the DLPAG (although trending decreases were also observed in LPAG and VLPAG). It is pertinent to note, however, that interpretation of the findings in bLR rats are complicated by the overall modest levels of viral labeling in these animals. Still, it remains tempting to speculate how broader attenuation of premotor projections throughout the PAG may impact behavior in these models. One possibility is that the tendency of these strains to display passive stress coping behaviors reflects impairments in the ability to mount an active coping strategy (as opposed to favoring a

passive strategy) due to a reduction in functional connections that can sufficiently engage peripheral motor targets following psychogenic or physical stressors. In the context of existing behavioral assessments in rodent models, active stress coping strategies (i.e., increased swimming or climbing) in the forced swim test (FST) by typically behaving rodents may be influenced by psychological (via the DLPAG) or physical/homeostatic (via the LPAG) challenges associated with the testing experience, or potentially a combination of these factors.

The relevance of reduced DMPAG or VLPAG premotor efferents in male WKY rats, as described in the present study, is less clear. The DMPAG is involved in mediating defensive responses to social threats[224,225], which do not appear to be studied in the WKY rat strain. (Decreased sociability under non-stressful conditions is already a reported feature in WKY rats[80].) The VLPAG, in addition to its role in mediating passive coping to inescapable stressors[223], is also implicated in rapid eye movement sleep regulation[226], food intake[227], and pain modulation[228]. While disturbances relevant to all of these functional domains are reported in depressed patients, it is not possible to identify how altered premotor circuits from DMPAG or VLPAG may contribute to these specific perturbations in the WKY model based on the scope of our current work.

The findings of our PRV tract-tracing investigations in LC and PAG should be interpreted cautiously with consideration of several methodological limitations. Firstly, survival times following initial injections of PRV-152 varied between experiments, with bHR/bLR rats surviving 120 hours post-injection and WKY/SD rats surviving 132 hours post-injection. These survival times correspond to short and intermediate survival times in our group's prior work[157]. Survival times of 120 hours are sufficient to identify somatomotor-sympathetic efferents in PVN, LH, ventrolateral PAG, and ventromedial medulla. These efferents are likely to project directly

to the spinal cord and collateralize to innervate somatic motoneurons and sympathetic preganglionic neurons simultaneously. At intermediate and later survival times, dual-labeled neurons appear in additional brain structures such as the DMH and different subdivisions of the PAG[112]. While the neuroanatomical investigations described here do not intend to draw direct comparisons between the WKY and bLR strains, it should be noted that without examining neuroanatomical characteristics at both time points for each strain, it is not possible here to draw conclusions about whether a particular neuroanatomical area has direct or indirect projections to spinal cord (or to other nodes of the somatomotor-sympathetic circuitry) in each respective strain. Secondly, in contrast to our published data in PVN, cell count data in the present study was reported in relationship to the total area ($mm^2$) over which cell counts were measured. This modification was necessitated by irregularities in the quantity and quality of available brain tissue among samples. The intent was to identify true differences between strains that are attributable to their distinct genetic backgrounds (and behavioral features) as opposed to creating a bias in cell counts based on greater availability of tissue in random samples. Finally, overall immunolabeling of somatomotor circuits in our experiments with bHR and bLR rats was considerably diminished relative to that of WKY and Sprague Dawley rats. Experiments in the bHR/bLR model were carried out later than those in the WKY/SD strains, and disparities in viral labeling are likely due to inevitable decreases in viral titer that naturally occur over extended periods in storage.

### 3.5 Conclusions

In summary, the neuroanatomical data presented in this chapter extend prior observations that premotor efferents in central nervous system structures implicated in somatomotor-sympathetic

integration are altered in two distinct rodent models for heritable differences in stress reactivity and emotional behavior. Specifically, male subjects from the WKY and bLR rat lines display fewer polysynaptic projections to skeletal muscle from the LC and various subregions of the PAG. Each of these areas are involved in coordinating behavioral and autonomic responses to stress. Deficits in premotor projections from these structures, particularly the dorsolateral and lateral PAG, have the potential to contribute to the passive coping strategies adopted by these animals in response to stressful stimuli. More broadly, these results may deepen our understanding of how emotional motor circuits are dysregulated in individuals suffering from clinical depression or anxiety-related disorders.

# CHAPTER 4: IMPACTS OF INCREASED ENVIRONMENTAL COMPLEXITY DURING EARLY LIFE ON EMOTIONAL BEHAVIOR AND HYPOTHALAMIC PARAVENTRICULAR MOTOR PROJECTIONS IN THE SELECTIVELY BRED LOW NOVELTY RESPONDER RAT

## 4.1 Introduction

Fundamentally, both positive and negative features of an organism's external environment have the propensity to shape physical, mental, and emotional well-being. Stressful experiences, including social isolation, low socioeconomic status (SES), and adverse childhood events, are linked with greater risk for and prevalence of mood-related psychiatric disorders (e.g., depression and anxiety disorders)[3,174,175]. Conversely, enriching stimuli and experiences exert numerous cognitive and behavioral benefits[229,230]. Several lines of evidence identify positive impacts of social support and physical exercise, for example, on outcomes in depressed patients[231–236]. In animal models relevant to clinical depression and anxiety disorders, environmental enrichment incorporating elements of both physical and social stimulation have demonstrated anti-depressant, anxiolytic, and neuroprotective effects[229,237]. Given that many traditional pharmacological interventions for depression show limited effectiveness in symptom remission and yield undesirable side effects[89,119–121], the need to identify alternative therapeutic approaches may be critical for improving quality of life in these individuals.

Although early life experiences are pivotal in influencing neural development and individual resilience or vulnerability to stress and stress-related emotional disorders[238], genetic factors also contribute to psychiatric pathologies[15,239]. Extensive prior work by our laboratory and others has used Sprague Dawley rats selectively bred for divergent behavioral responses to novelty to interrogate neural and molecular mechanisms by which genetic and environmental

factors drive the pathogenesis of emotional disorders[15,26]. Bred High Novelty Responder (bHR) rats are defined by their extensive exploration of novel environments, and this phenotype is associated with greater novelty-seeking, impulsivity, and proclivity for drug-seeking behavior. Bred Low Novelty Responders (bLRs), on the other hand, display marked reductions in novelty-induced locomotion, and this trait is linked with additional behavioral perturbations relevant to clinical anxiety and depression (e.g., psychomotor retardation, anhedonia, and passive stress coping)[15,36,61]. Earlier studies in this model indicate that prolonged or repeated exposure to increased environmental complexity (EC) during adulthood leads to attenuated anxiety-relevant behavior and locomotor activity as well as greater positive affect[54,240]. Thus, enriching social and sensory experiences associated with greater EC can improve emotional behavior outcomes in this model. However, further study in bLR rats is needed to determine if emotional behavior perturbations relevant to depression are also impacted by EC and whether the timing of EC has an impact on behavioral and neurobiological outcomes.

Additional lines of investigation outlined in Chapters 2 and 3 of this dissertation suggest that psychomotor deficits in the bLR model may be influenced by alterations of polysynaptic premotor efferents in brain structures implicated in both somatomotor-sympathetic integration and regulation of emotionally motivated behaviors. Given the sensitivity of emotional brain circuits to early experience[15], we were interested in whether EC-mediated improvements in emotional behavior in the bLR model also occur during earlier developmental stages. In the present study, we also sought to expand on our earlier neuroanatomical work by testing the hypothesis that increased EC during early life improves emotional behavior outcomes in the bLR rats by “re-wiring” the organization of descending premotor PVN projections to skeletal muscle, modifying the circuitry in these animals to more closely resemble that of bHR rats. To this end,

we implemented pseudorabies virus (PRV)-mediated transsynaptic tract-tracing strategies employed in our prior work[112,156,157] to identify descending PVN premotor efferent circuitry in bHR and bLR animals reared in standard housing conditions as well as in bLR rats reared in an environment with increased EC.

## 4.2 Methods

The Institutional Animal Care and Use Committee approved all experimental procedures described in this study. Experiments were performed in accordance with National Institutes of Health guidelines for animal research (USA, 2011).

*Animals*

Male bHR and bLR rats were acquired from the thirteenth and fourteenth generations of the in-house breeding colony. The process of generating and characterizing the bred High and Low Novelty Responder rat lines is described elsewhere[36,61]. Housing was maintained at controlled temperature (21-23ºC) and humidity (50-55%) conditions on a 12:12-hour light-dark cycle (lights on at 6:00 a.m.). *Ad libitum* food and water were provided throughout the study. Following weaning at postnatal day (P)21, male bHR and bLR rats were housed by strain in groups of three in standard cages. Experiments were conducted using two cohorts containing 12 bHR and 24 bLR rats each. Half of the bLR rats in each cohort were selected to be housed in conditions of progressively increasing environmental complexity (bLR+EC) during the period of late childhood and adolescence (P25-P50). Group assignments (bHR, bLR, bLR+EC) were equally balanced between cohorts. Animals in both cohorts were subjected to either control or environmentally complex housing conditions and subsequent emotional behavior testing.

Animals from both cohorts were also used for viral tract-tracing experiments to examine whether greater environmental complexity during early life modifies neural circuits governing motor output during stress. **Figure 11** shows the full timeline of experimental procedures.

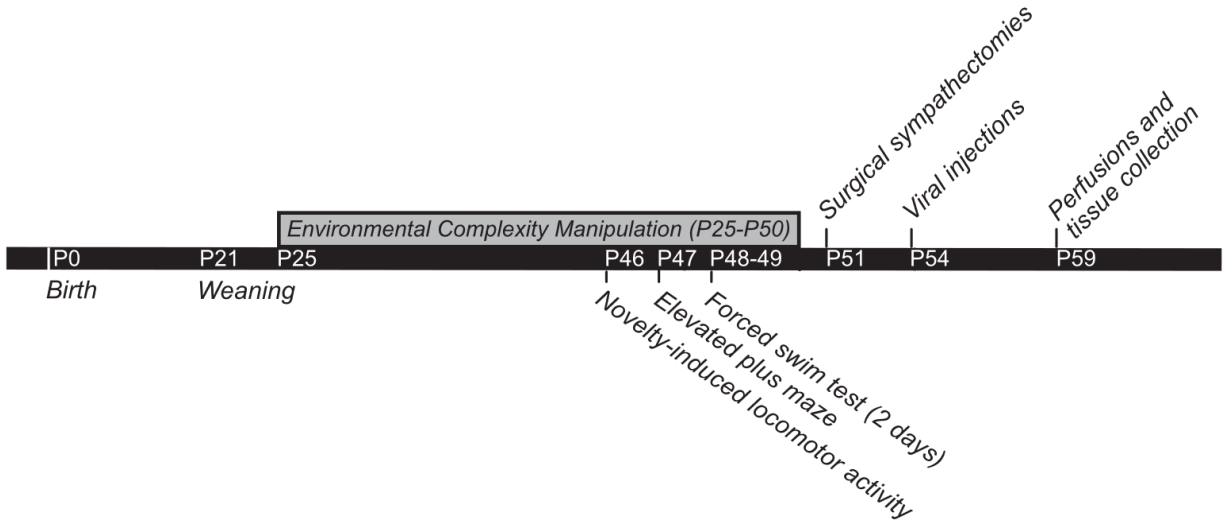


**Figure 11**. Timeline of experimental procedures for studying environmental complexity in the selectively bred High and Low Novelty Responder (bHR/bLR) rat lines. Male bHR and bLR rats were weaned on postnatal day (P)21. For 25 days starting on P25, a subset of bLR rats ($n$ = 24 across two cohorts) were placed in alternative stainless steel home cages outfitted with various enriching environmental stimuli. The quantity of toys and novel objects increased weekly. Control bHR and bLR rats ($n$ = 24 across two cohorts) were handled every other day. Assessments of emotional behavior were performed from P46-P49. Following behavior testing, animals were subjected to our previously published pseudorabies virus (PRV) tract-tracing protocol using PRV-152 and PRV-BaBlu. Brain and spinal cord tissue were collected for subsequent analysis.

*Environmental complexity*

Procedures for increasing complexity of the homecage environment in the bHR/bLR model have been described previously[54]. In the present study, a subset of bLR rats aged P25 ($n$ = 12 per cohort; $N$ = 24) were transferred to separate cages outfitted to provide richer environmental complexity. EC-treated bLR rats were housed in 3 × 3 × 3 ft stainless steel cages ($n$ = 3 per cage) containing various toys, obstacle courses, and other enriching stimuli that were replaced daily to

maintain novelty. Over the course of the 25-day enrichment period, the quantity of new toys and objects in the homecage was increased on a weekly basis. Control bHR and bLR rats ($n$ = 12 per group per cohort; $N$ = 24 per group) were handled once every other day for an equivalent duration of time as the bLR+EC rats.

*Emotional behavior testing*

Novelty-induced locomotion and emotional behavior perturbations were examined in male bHR, bLR, and bLR+EC rats ($n$ = 24 per group across 2 cohorts) at ages P46-P49. Behavioral testing was performed between 8:00 a.m. to 1:30 p.m. under dim lighting (30 lux). Each animal was evaluated in all three tests. Although animals typically receive one day of rest between emotional behavioral assessments in our prior work[214], logistical considerations with regard to timing of subsequent PRV tract-tracing experiments necessitated that this timeline be expedited to ensure adequate immunolabeling. Transneuronal infection of brainstem and lumbar spinal cord motoneurons is strongly negatively correlated with body weight, such that rats weighing less than 300 g display the most robust level of infection following PRV-152 injection into gastrocnemius muscle[156].

*Locomotor response to novelty*. Rats were screened for locomotor response to a novel homecage environment on P46 as previously described[36,214]. Individual rats were placed into a standard-sized clear acrylic homecage (43 × 21.5 × 24.5 cm) outfitted with a mesh floor located in a room separate from where the animals were typically housed. Two panels of photocells recording horizontal locomotion and rearing behavior monitored locomotor activity in 5-min increments over a 1-hr period. Horizontal and rearing activity were combined to determine final locomotion scores.

*Elevated plus maze*. Animals were assessed for anxiety-relevant phenotypes in the EPM on P47 using procedures described elsewhere[54]. The EPM consists of a black Plexiglas apparatus with four arms (45 cm length × 12 cm width) arranged in a cross and elevated 70 cm from the floor. Two opposite arms are enclosed by 45-cm high walls, while the two remaining arms are open to the surroundings. A central 12 × 12 cm square platform at the intersection of the apparatus allows the animal access to all four arms. At the start of the test, the rat is placed at the center of the maze facing a closed arm and allowed to explore the maze freely for 5 min. EthoVision XT 8.0 software (Noldus) was used to record the animal's movements during the trial. Latency to enter the open arms and duration of time spent in the open and closed arms of the apparatus were measured for each animal.

*Forced swim test*. Procedures for the two-day Porsolt's FST were conducted as before[214] on P48-P49. On Day 1 (pretest phase; 15 min), animals were placed individually into 30-cm deep 25$^{o}$C water in Plexiglas containers (45 cm height × 20 cm diameter). After 24 hr, rats were returned to the water-filled cylinders for an additional 5 min (test phase). Water was changed after each session so that each rat was swimming in clean water. Rats were videotaped during both sessions and duration of time spent immobile during the test phase was scored using EthoVision XT 8.0 software (Noldus).

*Viral tract-tracing overview*

Beginning on P51, rats in each cohort underwent our previously published protocol for retrograde viral tract-tracing (**Figure 11**), performed as described in Chapter 2. Rats were injected with PRV-152 into sympathectomized gastrocnemius muscle and PRV-BaBlu into adrenal gland. As also described for the experiments in Chapter 3, neuroanatomical analysis in

this experiment focused on premotor circuitry even though injections with both recombinant strains were performed.

*Sympathectomy and viral injections*

Surgical sympathectomy of the hindlimb gastrocnemius muscle was performed beginning on P51. Injections of PRV-152 and PRV-BaBlu in bHR, bLR, and bLR+EC rats began on P54. These procedures were carried out as described in Chapter 2 of this dissertation in line with prior studies by our group[156,157,214]. Rats were sacrificed 120 hr following the initial injections with PRV-152 using previously described methods[214].

*Tissue collection and processing*

After animals were sacrificed on P59, tissue collection was performed as described in Chapters 2 and 3. Processing of free-floating coronal sections containing PVN for immunofluorescent detection of eGFP and β-galactosidase was carried out in a subset of tissue samples from Cohort 1 animals ($n$ = 5-6 per group) using methods described in Chapter 2.

*Tissue analysis*

Analysis of immunofluorescently-labeled coronal sections containing PVN was performed as described in Chapter 2. Although bilateral quantification of PRV-infected cells was performed for both recombinant strains, only eGFP (PRV-152) immunolabeling data in the PVN are presented in this chapter.

*Statistical analysis*

Behavioral and neuroanatomical data were analyzed using GraphPad Prism Version 9.5.1 (GraphPad Software). All results are expressed as mean ± SEM. Significance was set at $p < 0.05$ for all tests. The Shapiro-Wilk test was used to determine whether data were normally distributed, and the Brown-Forsythe test for homogeneity of variance was used to determine whether the variances of the sample populations were equal. Data were excluded only if determined to be an outlier using both the Grubbs test and the ROUT method. Two-way ANOVA was used to verify that there were no statistically significant differences in behavioral outcomes across the two cohorts. Behavioral data from animals in both cohorts were subsequently combined for final analysis. Data meeting the assumptions of normality and equality of variance were analyzed using one-way ANOVA. Group differences by one-way ANOVA were discerned using Tukey's *post hoc* analysis. When the assumption of equality of variances was not met, data were analyzed using Welch's ANOVA (normally distributed) or Brown-Forsythe ANOVA (non-normally distributed) followed by Dunnett's T3 multiple comparisons test.

### 4.3 Results

*Increased environmental complexity during early life partially rescues emotional behavior deficits in bLR rats*

Prior studies have illustrated the diversity of emotional behavior phenotypes in the bHR and bLR rat lines[26,61] and demonstrate that these phenotypes are improved by exposure to increased environmental complexity during adulthood[54,240]. The present study extends these observations by determining whether exposure to greater environmental complexity during an earlier developmental period can likewise ameliorate emotional behavioral deficits in bLR rats.

Following exposure to greater environmental complexity from P25-P50, significant differences between bHR, bLR, and bLR+EC rats were observed with respect to locomotor activity in a novel environment (Welch's ANOVA, $W(2, 34.37) = 232.5$, $p < 0.0001$; **Figure 12A**). As predicted based on their genetic background, bLR rats displayed significantly less novelty-induced locomotor activity relative to bHR rats ($p < 0.0001$). Bred LR rats exposed to increased environmental complexity from P25-P50 showed an even further reduction in locomotor activity in this assessment relative to both control bLR rats ($p < 0.0001$) and bHR rats ($p < 0.0001$).

Passive coping behavior was also evaluated in the FST (**Figure 12B**). In this test, the percentage of time spent immobile differed significantly among groups (Brown-Forsythe ANOVA, $F^*(2,51.40) = 22.89$, $p < 0.0001$; **Figure 12B**). Compared to bHRs, control bLR rats spent significantly greater time immobile in the FST ($p < 0.0001$), an observation classically interpreted as indicative of increased depression-relevant behavior. Although bLR+EC rats spent significantly less time immobile compared to control bLR rats ($p = 0.0242$), their levels of immobility remained significantly higher than those of bHR controls ($p = 0.0005$).
Finally, indices of anxiety-relevant behavior were assessed in the EPM (**Figure 12C-12D**). Significant differences were observed among groups in latency to enter the open arm of the maze (Brown-Forsythe ANOVA, $F^*(2,27.78) = 7.948$, $p = 0.0019$; **Figure 12C**) as well as the overall percentage of time spent in the open arms (Brown-Forsythe ANOVA, $F^*(2,49.01) = 9.862$, $p = 0.0003$; **Figure 12D**). Relative to bHR rats, bLR rats displayed a significantly higher latency to first approach the open arms of the maze ($p = 0.0339$). The latency of bLR+EC rats to explore the open arms was significantly decreased compared to control bLR rats ($p = 0.0165$) and comparable to that of bHRs ($p = 0.7465$). Control bLR rats also spent a significantly lower

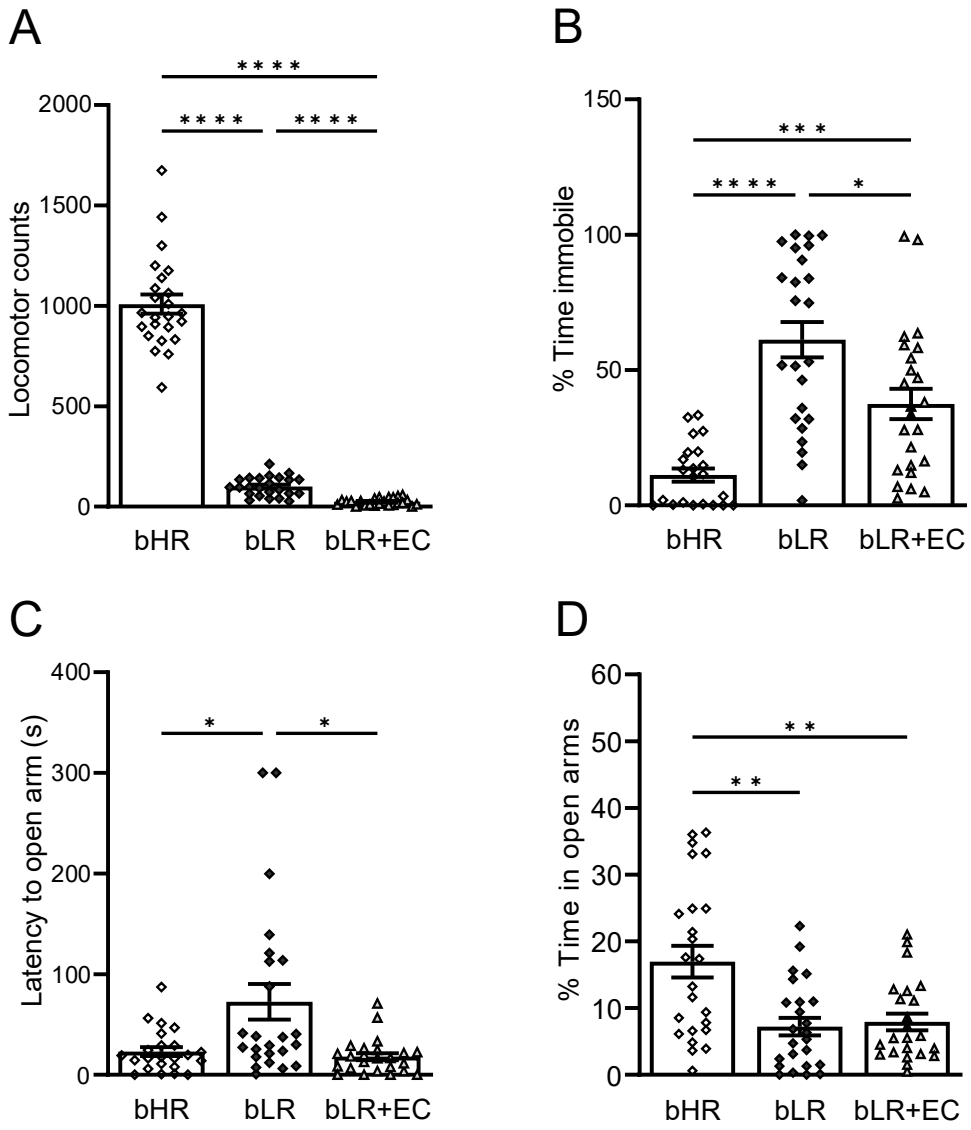


**Figure 12**. Emotional behavior comparison among young adult selectively bred High Novelty Responder (bHR) rats, Low Novelty Responder (bLR) rats, and bLR rats reared in conditions of increased environmental complexity (bLR+EC) from postnatal days (P)25-P50. (**A**) Compared to bHR rats ($n$ = 24), bLR rats ($n$ = 24) exhibit significantly attenuated locomotor activity in a novel homecage environment. Even further reductions in novelty-induced locomotor responses are observed in bLR+EC rats ($n$ = 24) relative to bHR and bLR rats. (**B**) In the forced swim test (FST), bLR rats ($n$ = 24) display significantly greater passive coping (immobile) behavior compared to bHR ($n$ = 22) and bLR+EC ($n$ = 24) rats. The bLR+EC rats display intermediate levels of immobility compared to control bHR and bLR rats. (**C**) In the elevated plus maze (EPM), bLR rats ($n$ = 24) show a higher latency to enter the open arm of the maze compared to bHR ($n$ = 23) and bLR+EC ($n$ = 24) rats. (**D**) Compared to bHR rats ($n$ = 24), bLR ($n$ = 24) and bLR+EC rats ($n$ = 23) both spend significantly less time in the open arms of the EPM, reflecting increased anxiety-like behavior. Bars represent mean ± SEM. Statistically significant differences at **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

duration of time in the open arms of the maze compared to bHR rats ($p$ = 0.0031). Exposure to an enriched homecage environment during early life did not improve deficits in the time spent by bLR rats in the open arms of the EPM (bLR vs. bLR+EC, $p$ = 0.9724; bHR vs. bLR+EC, $p$ = 0.0058).

*Motor projections from PVN are incompletely restored by the experience of increased environmental complexity during early life*

To determine whether positive environmental stimuli in the weeks post-weaning has the capacity to modify neural circuits involved in emotionally mediated behavior, PRV tract-tracing was used to compare premotor projections from the PVN. Our previous work identified that bLR rats have a dramatic reduction in immunolabeling of premotor projecting cells in the PVN relative to bHRs[214]. Here, we hypothesized that increased EC during early life would "re-wire" premotor circuits in the bLR PVN to more closely resemble those of bHR rats. However, quantification of immunolabeled premotor projections in the PVN of bHR, bLR, and bLR+EC rats did not reveal statistically significant differences among these groups ($F(2,13) = 3.124$, $p$ = 0.0780; **Figure 13**).

## 4.4 Discussion

The experiments described in this chapter examined whether the experience of increased environmental complexity during early life improves emotional behavior and modifies neural circuit abnormalities in a rat model selectively bred for distinct locomotor responses to novelty. The findings of our initial behavioral assessments were consistent with prior studies showing that typically reared bLR rats display dramatic reductions in novelty-induced locomotor activity and increases in anxiety- and depression-relevant behaviors[15,26]. Bred LR rats reared in an enriched

environment from P25-P50 displayed further decreases in novelty-induced locomotion, reductions in latency to explore the anxiogenic portion of the EPM, and partial improvements in depression-relevant behavior in the FST. The subsequent neuroanatomical comparisons of muscle-projecting PVN neurons in bLR+EC rats relative to typically reared bHRs and bLRs revealed a trend for changes to premotor circuitry by experience, though ultimately no

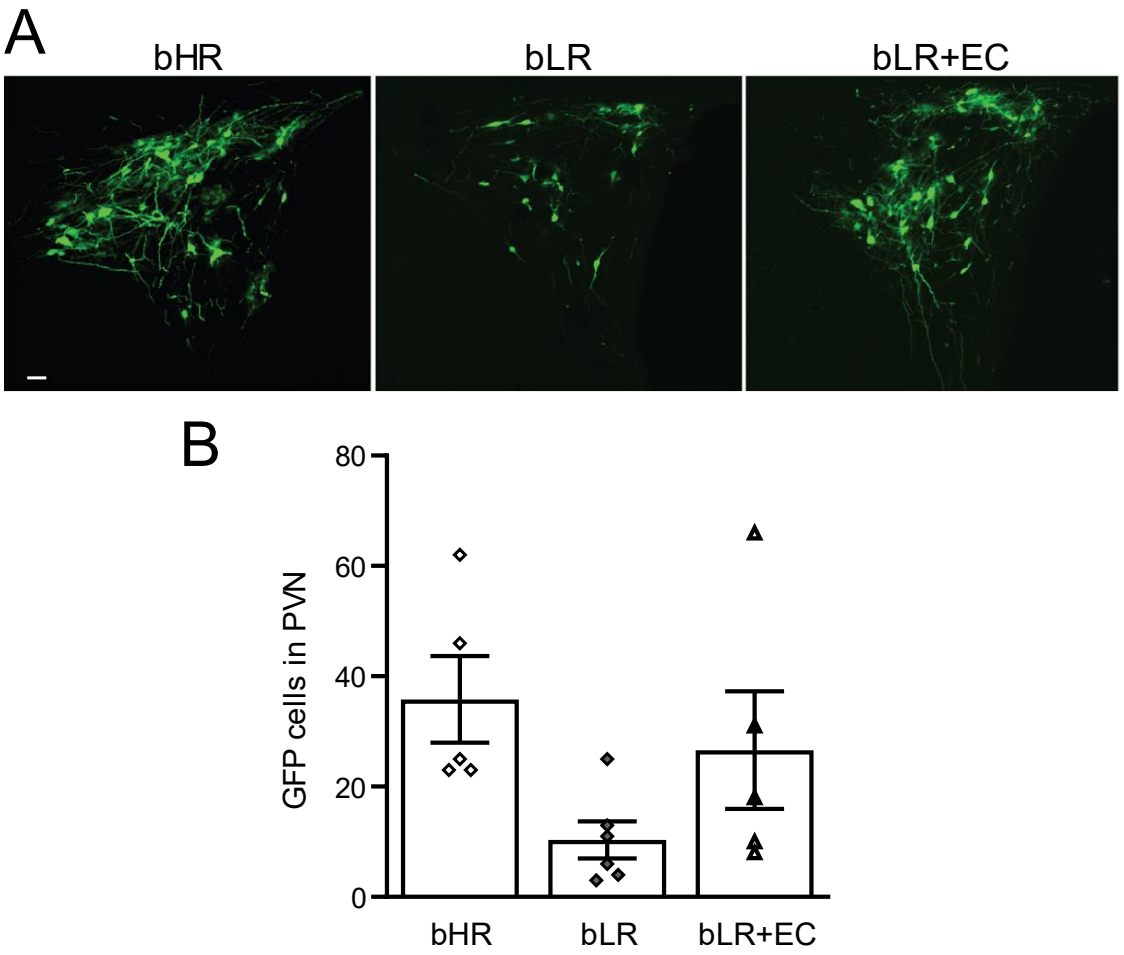


**Figure 13**. Pseudorabies virus (PRV) immunolabeling of premotor (PRV-152) cells in the paraventricular nucleus of the hypothalamus (PVN) of young adult male selectively bred High Novelty Responder (bHR) rats, Low Novelty Responder (bLR) rats, and bLR rats reared in conditions of increased environmental complexity (bLR+EC) from postnatal days (P)25-P50. (**A**) Representative images of transsynaptically labeled cells infected with PRV-152 in PVN of bHR, bLR, and bLR+EC rats that survived 120 hrs after injections with PRV-152 and PRV-BaBlu. Scale bar, 50 µm. (**B**) At 120 hrs following infection with PRV-152, a trend for significant differences ($p = 0.0780$) in the quantity of GFP-positive premotor cells in the PVN was observed among bHR ($n = 5$), bLR ($n = 6$), and bLR+EC rats ($n = 5$).

statistically significant differences in wiring of these circuits were observed among the groups. Collectively, this body of work demonstrates increased environmental complexity from P25-P50 was not sufficient on its own to rescue behavioral or neuroanatomical abnormalities inherent to this strain.

Studies of environmental enrichment in animal models provide housing or exploratory chambers that facilitate enhanced cognitive, motor, sensory, and sometimes social stimulation. Key elements of these paradigms include provisions for increased complexity and novelty. Procedures among laboratories vary with respect to the age of the animal and duration of exposure to enriching conditions[241]. Employing an environmental manipulation for which the experience of novelty is a central component is interesting when considering our chosen animal model: rats selectively bred for distinct locomotor responses to a novel environment. Early EC studies in bHR/bLR rats showed that bLR rats were more behaviorally sensitive to EC exposure during adulthood. While bLR and bHR rats both displayed increased open arm entries in the EPM, improvements in anxiety-relevant behavior in the light-dark box test (i.e., percent time in the lighted compartment) were selectively observed in EC-exposed bLR rats[54]. In follow-up experiments, adulthood EC exposure decreased locomotor activity in bHR, but not bLR, rats[240]. Although our study design did not allow us to examine the effects of early life EC in bHR rats, our findings in bLR rats suggest that the developmental timing of EC exposure influences its impact on novelty-induced locomotor activity in this stress-sensitive strain. Similar observations of novelty-induced hypoactivity after EC have been documented by others and likely reflect more rapid behavioral habituation of enrichment-exposed animals to a novel environment[242–244]. Our findings with respect to anxiety- and depression-relevant behaviors in the EPM and FST indicate that some but not most aspects of emotional behavior abnormalities in the bLR model

are improved by EC during the developmental stages studied in the present experiments. The only parameter that was restored to levels comparable to bHR animals was latency to enter the open arm of the EPM. A possible interpretation of this finding is that following EC exposure, bLR rats may be willing to initially explore their novel surroundings. However, these animals may still retain their sensitivity to the anxiogenic effects of being on the open arm and revert to their ingrained behavioral strategies (i.e., spending less time in the open arms).

Increased environmental complexity drives various alterations to brain structure and function. Cellular and molecular studies have documented experience-dependent increases in neurogenesis and synaptogenesis, neuronal signaling, and synaptic plasticity due to environmental enrichment. These changes are capable of strengthening existing connections within a neural network and promoting the development of new circuits[241]. In the present work, we were interested in understanding whether environmental enrichment during early life can improve emotional behavioral outcomes by driving plastic changes to premotor circuitry involved in mediating responses to stressful stimuli. Our earlier work in bLR rats has documented drastic reductions in premotor efferents from the PVN to skeletal muscle[214], which could be implicated in the documented psychomotor deficits observed in this strain. While rearing in a more complex environment appeared to promote some increase in the volume of somatomotor projections from PVN to gastrocnemius muscle in bLR rats, this effect did not reach the threshold for statistical significance. Given that greater improvements to anxiety-relevant behavior are observed following adulthood EC exposure, it is possible that improvements in PVN premotor circuits would be observed if the timing of the manipulation were later. Testing this possibility, however, would be more technically challenging using the current PRV viral tract-tracing strategy discussed here and may need to be evaluated using

alternative approaches. Rats of a more advanced age at the start and conclusion of rearing in a more complex enrichment would weigh significantly more by the time they underwent subsequent viral infection relative to those tested in the present study. Because motoneuron infection by PRV-152 declines with increased body weight[156], the current PRV approach would be less effective in robustly labeling premotor efferents in older adult animals.

Despite the lack of significant circuit changes reported here, however, it is tempting to speculate that the apparent trend for experience-dependent strengthening of PVN premotor circuits in this model raises the possibility that plasticity-mediated mechanisms can still partially influence "wiring" of emotional motor circuits. Either positive or negative experiences could thus shape these circuits in beneficial or detrimental ways to drive distinct behavioral coping strategies in response to subsequent challenges. Future studies should explore whether other nodes of the emotional motor circuitry that are disrupted in the bLR model, including subdivisions of the periaqueductal grey and locus coeruleus, are modified in response to rearing in more complex housing conditions.

### 4.5 Conclusions

In summary, the behavioral and neuroanatomical findings discussed in this chapter provide evidence to suggest that, in a rat model with inborn differences in temperament and stress reactivity, rearing in a more complex housing environment during early life (P25-P50) only partially improves emotional behavior outcomes in the bLR rat relative to adulthood exposure to this manipulation. These modest behavioral changes are accompanied by a trend for increases in the quantity of polysynaptic projections from PVN to skeletal muscle, which could be functionally involved in mediating locomotor disturbances associated with altered emotional

states. In the context of treating emotional behavior disturbances that manifest prior to young adulthood, therapeutic approaches that incorporate enriching features may need to additionally incorporate increased social stimulation, or be paired with other treatment modalities, to elicit more successful treatment outcomes in this strain.

# CHAPTER 5: GENERAL DISCUSSION

## 5.1 Summary of findings

Emotionally motivated behaviors such as responses to stress rely on the coordinated activity of descending neural circuits involved in motor and autonomic functions. Several neuroanatomical investigations by our laboratory group employed a pseudorabies virus (PRV) tract-tracing approach to delineate the organization of descending premotor and presympathetic efferents from mesencephalic and telencephalic areas as well as midbrain and brainstem. Experiments in typically behaving male Sprague Dawley rats revealed putative "dual-function" populations of neurons in several nodes of the central premotor and presympathetic circuitry that appear to participate in somatomotor-sympathetic integration[112,156,157]. This dissertation sought to add to this body of work by discerning how central nodes involved in somatomotor-sympathetic integration may be implicated in psychomotor disturbances and/or autonomic impairments observed in clinical depression (i.e., major depressive disorder, MDD) and co-morbid anxiety disorders. To address this, experiments outlined in Chapters 2 and 3 of this dissertation examined neuroanatomical differences in male subjects in two distinct rat models with innate behavioral and physiological perturbations resembling aspects of comorbid depression and anxiety: (1) the Wistar-Kyoto (WKY) rat and (2) the selectively bred Low Novelty Responder (bLR) rat. Using retrograde tract-tracing with PRV recombinants injected into sympathectomized gastrocnemius muscle (i.e., skeletal muscle; somatomotor target) and adrenal gland (sympathetic target), we identified that the hypothalamic paraventricular nucleus (PVN) – a key integrator of behavioral, endocrine, and autonomic responses to stress[196] – contains fewer neurons with polysynaptic projections to skeletal muscle in both WKY (compared to Sprague Dawley) and bLR (compared to selectively bred High Novelty Responder) rats. No differences in the quantity of

presympathetic or integrative somatomotor-sympathetic projections were observed in these models relative to their respective control strains. Cumulatively, these observations suggested that perturbations in PVN premotor circuitry could be implicated in mediating psychomotor disturbances observed in emotional disorders.

In Chapter 3, we extended our neuroanatomical investigation of premotor circuit abnormalities in the WKY and bLR rat models to include the locus coeruleus (LC) and periaqueductal gray (PAG). Like the PVN, these structures also contain populations of neurons involved in somatomotor-sympathetic integration[112,156]. Furthermore, the LC and PAG play critical roles in modulating behavioral and physiological responses to stress and other emotionally relevant stimuli. In both WKY and bLR rats (relative to controls), the LC, like the PVN, contains significantly fewer neurons with polysynaptic premotor efferents to skeletal muscle. By contrast, our findings in subregions of the PAG appeared to reveal strain-specific immunolabeling of premotor efferent cells in this area. In WKY rats, significant reductions in premotor efferents to skeletal muscle were observed in the dorsomedial (DMPAG), lateral (LPAG), and ventrolateral (VLPAG) subdivisions, as well as a trend for a significant reduction in premotor efferents from dorsolateral (DLPAG) column. In bLR rats, significant decreases in somatomotor projections to skeletal muscle were only observed in the DLPAG, although trends for a significant decrease in these projections were noted in LPAG and VLPAG. These differential patterns of immunolabeling between the WKY and bLR strains could be a result of their disparate genetic backgrounds. However, interpretation of our neuroanatomical findings in the bLR rat PAG may require more caution in their interpretation for two reasons: (1) bLR/bHR rats were sacrificed after a shorter survival time (120 hrs following initial PRV-152 infection) compared to WKY/SD rats (132 hrs), and (2) decreased viral titers between studies may have

contributed to overall attenuations of PRV-152 immunolabeling in bHR/bLR rats. Overall, the neuroanatomical work described in Chapter 3 appears to support the notion that at least nodes involved in somatomotor-sympathetic integration (i.e., the PVN and LC) are similarly dysregulated across two distinct genetic rat models for depression- and anxiety-relevant phenotypes. These results also suggest that at least one node of this circuitry, the PAG, is differentially regulated between the two models, which could contribute to observed phenotypic or biological variations between these strains.

Finally, the experiments described in Chapter 4 examined whether wiring of skeletal muscle-connected circuits of the PVN are modifiable by enriching experience. To this end, a subset of bLR rats were reared in housing conditions with increased environmental complexity (i.e., increased sensory, motor, and cognitive stimulation) from postnatal days (P)25-P50, corresponding approximately to late childhood and adolescence. Exposure to enriching experiences and stimuli may represent a viable therapeutic alternative for stress-related emotional disorders based on its anxiolytic, antidepressant, and neuroprotective impacts[237]. Our behavioral assessments found that rearing of young bLR rats in a highly complex environment resulted in further attenuation of novelty-induced locomotor activity as well as modest but incomplete improvements of emotional behavior abnormalities in the elevated plus maze (anxiety-like behavior) and forced swim test (depression-like behavior). Parallel neuroanatomical comparisons of bLR rats reared in a more complex environment, relative to bHR and bLR rats reared in standard housing, identified a trend for significant differences in the quantity of premotor efferents from PVN to skeletal muscle among these groups. Similarly to the patterns reflected in our behavioral assessments, greater EC appeared to incompletely reverse the

reductions in PVN premotor efferents to skeletal muscle that we have documented previously in this model[214].

Taken together, the data presented in this dissertation highlight several premotor circuit-level perturbations in central nervous system structures involved in mediating responses to stressful and emotional stimuli. Similar perturbations in two areas, the PVN and LC, are observed in two rat models with innate emotional behavior abnormalities and derived from distinct genetic backgrounds. Patterns of premotor circuit disturbances in a third area, the PAG, appear to differ between bLR and WKY strains. These observations could potentially indicate that certain emotional motor circuit abnormalities are conserved across animal models with depression-relevant traits or clinical depression subtypes, while others may vary depending on an individual's innate genetic predisposition or experience.

### 5.2 How are emotional motor circuits altered in rat models with genetic predisposition for depression- and anxiety-relevant behaviors?

Holstege and colleagues were the first to describe an emotional motor system, which features a similar organization to the somatic motor system[112,245]. This system was thought to contain a distinct set of motor pathways that command somatic, autonomic, and endocrine motor responses and operate in parallel to motor pathways involved in voluntary motor control[130]. Unlike the somatic motor system, components of the emotional motor system are innervated by several integrative limbic structures (i.e., amygdala, bed nucleus of stria terminalis, hypothalamus). In addition, many structures in the emotional motor system contain populations of neurons involved in autonomic function[112,129,211,212]. By employing a PRV-mediated retrograde tract-tracing strategy, work by our laboratory group determined that several nodes of the emotional motor

system contain populations of neurons concurrently involved in somatomotor and sympathetic functions. These putative "dual-function" neurons are believed to engage in somatomotor-sympathetic integration, a process important for coordinating motor and autonomic outflows to drive behaviors requiring simultaneous engagement of the somatomotor and sympathetic nervous systems[112,156,157]. Emotionally motivated behaviors, including responses to stress, are one of many examples of behaviors requiring intricate coordination of the motor and autonomic systems[112,125–127]. Importantly, numerous aspects of emotional behavior regulation and stress reactivity are dysregulated in patients with clinical depression or anxiety-related disorders.

While significant attention has been devoted to delineating neural circuit disruptions involved in affective and cognitive disturbances in MDD[123,246], far fewer studies have explored neurobiological mechanisms governing motor or physiological impairments in this disorder. The experiments outlined in Chapters 2-3 of this dissertation sought to address this critical knowledge gap by investigating somatomotor and sympathetic circuit perturbations within the emotional motor circuitry of two distinct rat strains modeling genetic predisposition for depression- and anxiety-relevant behavioral phenotypes and altered stress reactivity. In both male WKY and bLR rats, there were no significant alterations in the quantity of presympathetic-premotor (dual-function) or presympathetic PVN efferents relative to their respective control strains. There were, however, marked reductions in the number of polysynaptic premotor efferents from PVN to skeletal muscle. Based on this observation, our follow-up analyses continued to probe disruptions in skeletal muscle-connected efferents in two additional areas, the LC and PAG. Echoing our findings in the PVN, male WKY and bLR rats also displayed fewer premotor connections from LC and PAG. However, there appeared to be differences between the WKY and bLR strains with respect to where PAG premotor circuit alterations are observed

across the dorsal-ventral axis. In WKY rats, significant attenuation of premotor efferents to skeletal muscle were observed in DMPAG, LPAG, and VLPAG, in addition to a trend for a decrease in DLPAG. In bLR rats, significant reductions in premotor efferents were observed only in the DLPAG (and trends for a reduction in these premotor circuits were also identified in LPAG and VLPAG). A more general observation amongst all of the rat strains, regardless of behavioral phenotype, was that far fewer premotor efferent neurons were located in the DLPAG compared to other subdivisions of this structure.

In summary, the initial experiments described in this dissertation add to our understanding of motor-related "wiring" perturbations in multiple nodes of the emotional motor system in two rat models with innate vulnerability to stress and emotional behavior dysregulation. The fact that these neuroanatomical alterations are observed in more than one model, each with distinct genetic backgrounds, could signal the possibility that certain features of emotional motor system dysregulation are conserved among various models of genetic vulnerability (in animals) or clinical depression subtypes (in human patients).

### 5.3 How could emotional motor circuit perturbations contribute to behavioral disturbances observed in clinical depression?

The neuroanatomical findings outlined in the previous section suggest several possibilities for how perturbations of premotor efferents to skeletal muscle in various nodes of the emotional motor system could contribute to psychomotor impairments underlying depressive symptoms. With respect to the PVN, this structure engages the hypothalamic-pituitary-adrenocortical (HPA) axis to exert neuroendocrine mechanisms preparing an animal to mount a response to stressful stimuli[142]. Decreases in the quantity of premotor neurons in the PVN with downstream

connections to skeletal muscle could facilitate reduced locomotor responses during stress in several ways. Firstly, a subset of CRH neurons in the PVN send direct projections to spinal cord[145]. If these CRH neurons are part of a population of neurons with premotor functions, it is possible that having fewer PVN premotor efferents, as was observed in our experiments using two distinct genetic rat models for emotional behavior dysregulation, diminishes an organism's ability to generate motor output following activation of the neuroendocrine stress axis via decreased functional connectivity between PVN and skeletal muscle.

It may also be possible that deficits in this more "direct" PVN-to-spinal cord projection are involved in mediating more general deficits in locomotor activity in clinically depressed patients. The HPA axis is central for regulating diurnal glucocorticoid (CORT) levels throughout the day, with CORT levels rising prior to waking to anticipate metabolic needs of the organism during the active phase of its diurnal cycle[139,247]. Considered alongside the observation that the diurnal CORT peak is altered in some depressed patients and in certain animal models relevant to depression[68,101,248], it is tempting to speculate that these neuroanatomical differences could somehow be implicated in mediating circadian-related motor or activity disturbances observed during the waking period in clinical depression or preclinical models for this disorder.

Projections from PVN to the LC[222] and/or to PAG[249] could also be implicated in reduction of stress-induced motor activity via indirect modulation of premotor efferents in the emotional motor circuit. Specifically, diminished premotor efferents from PVN may influence these downstream nodes by way of reduced functional connectivity to these structures and subsequent reductions in locomotor drive following stress. Alternatively, actions by neuronal populations of the PVN that lack premotor functions but do project to premotor efferents of the LC and PAG could also drive diminished locomotor activity. In response to stress, these nodes

could be less capable of driving motoric responses due to insufficient functional connectivity of these premotor circuits to skeletal muscle, as evidenced by decreases in the quantity of polysynaptic premotor projections from LC and PAG to skeletal muscle in both WKY and bLR rats (described in Chapter 3).

Perturbations of emotionally motivated motor responses could also potentially be explained by neural circuit mechanisms independent of (or in addition to) those involving the PVN. The role of PAG in driving active and passive coping responses to stress[223,250] seems especially salient when considering our finding that decreases in premotor efferents are observed in both dorsal/lateral and ventrolateral columns of this structure in two genetic rat models relevant to depression. Modulation of the PAG by limbic or other afferents provides information about the modality of a stressor (e.g., escapable versus inescapable; psychosensory versus homeostatic). Functional studies demonstrate that stress modality differentially modulates neural activity across the dorsal-ventral aspects of the PAG. Various prefrontal cortical structures project to specific subdivisions of the PAG to mediate their respective roles in active (dorsal/lateral) and passive (ventrolateral) coping[223,250]. Afferents from mPFC to dorsal PAG, for example, are implicated in mediating defensive reactions to social threat (i.e., social defeat in animal models relevant to depression)[224,225]. Our own observations within the WKY and bLR models suggest that premotor efferents in both the dorsal/lateral and ventrolateral PAG columns are diminished to some degree. Logically, the observation that fewer premotor efferents exist in the VLPAG, a structure thought to drive passive coping behaviors, seems more difficult to reconcile when considering the clear bias toward passive coping strategies in these rodent models relevant to depression. We propose an alternative way of considering this issue: perhaps behavioral abnormalities in these models reflect an inability to engage an active stress coping

strategy instead of a "preference" for passive coping, per se. This "inability to engage" could be mediated by reduced premotor functional connections in the dorsal/lateral PAG, which may weaken the ability of these rats to engage downstream motor targets for actively coping with stress. This deficit in active coping ability would therefore manifest as behaviors resembling a more "passive" phenotype. If this explanation is accurate, the role of VLPAG premotor efferents in these models is less clear. Premotor circuit abnormalities in this structure could implicated in other functional roles of the VLPAG that beyond the scope of this dissertation work.

### 5.4 Are emotional motor circuits modifiable by enriching experiences?

An extensive literature in animal models chronicles the beneficial impacts of environmental enrichment – characterized by its amplified sensory, cognitive, motor, and sometimes social features – on outcomes relevant to clinical anxiety and depression as well as other central nervous system disorders[237,241]. Earlier work in the bLR model is consistent with this observation, showing that exposure to environmentally complex conditions during adulthood reverses anxiety-like phenotypes and increases positive affect in bLR rats[54,240]. Extending on this work, the experiments outlined in Chapter 4 of this dissertation tested the hypothesis that exposure to increased environmental complexity from P25-P50 could improve emotional behavior outcomes in bLR rats by modifying ("re-wiring") emotional motor circuitry of the PVN. Ultimately, our behavioral and neuroanatomical findings showed that augmented environmental complexity at this earlier developmental stage (P25-P50) does not fully rescue behavioral or neuroanatomical endpoints in the bLR rat. In spite of this finding, however, the fact that outcomes are significantly improved when this manipulation is applied later in the lifespan does suggest that enriching experiences could still have the propensity to modify emotional

motor circuits. Although this earlier work did not examine the effects of greater environmental complexity on premotor circuitry of the PVN specifically, the study authors did correlational analyses between post-exposure corticosterone (CORT) levels and c-Fos activation (mRNA) in several areas implicated in reward processing: nucleus accumbens, infralimbic and prelimbic medial prefrontal cortex (mPFC), and dorsal and ventral PAG. These correlations were intended to evaluate phenotype-dependent "connectivity patterns" in reward-associated brain areas and only identified significant correlations in the nucleus accumbens for bLR rats[240]. Considered as a whole, it can be conjectured that enriching experiences have the capacity to modify emotional motor circuits in bLR rats. This effect likely depends on the age of the animal during the manipulation and the duration of exposure. However, further studies are needed to confirm this theory.

### 5.5 Future directions

The neuroanatomical and behavioral findings reported in this dissertation research reveal valuable insights into how premotor efferents of the emotional motor system may contribute to related functional impairments observed in clinical depression and relevant preclinical models. There are numerous future directions which could be explored in subsequent investigations. An obvious and biologically significant factor that should be explored in all aspects of this dissertation work is the consideration of sex differences. Clinical depression and anxiety disorders are more commonly reported in women than in men[1]. In addition, behavioral and symptomatic presentations of depression and anxiety, as well as treatment efficacy for these conditions, vary between sexes in animal models and in humans[251–254]. Work in animal models demonstrates that certain indices in various emotional behavioral assessments are more

appropriate for one sex versus the other (e.g., latency to immobility in females versus immobility duration in males in Porsolt's forced swim test) and that female and male rodents engage differential stress coping mechanisms[255,256]. Where or to what extent premotor circuit alterations are observed in male versus female rodents could play a significant role in modulating their disparate behavioral phenotypes. Furthermore, the observation that defensive coping strategies differ between sexes may be particularly salient with respect to our neuroanatomical findings in the PAG, as this structure modulates active and passive stress coping strategies[223,250].

Future studies should also expand upon the present neuroanatomical investigations by discerning the neurochemical composition of immunolabeled premotor efferents in the PVN, LC, and PAG. There are several candidate neurotransmitter and neuropeptide populations implicated in stress and/or emotional behavior regulation that could be explored in these studies, including corticotropin-releasing hormone (CRH), oxytocin (OXT), vasopressin (AVP), or markers of noradrenergic, serotonergic, glutamatergic, or GABAergic signaling. Insights from these neuroanatomical characterizations could facilitate future chemogenetic studies examining how mono-synaptic projections between nodes of the emotional motor circuitry contribute to discrete behavioral outcomes relevant to clinical psychopathology. Interpretation of the relevance of these potential mono-synaptic connections in the central emotional motor circuitry could be hampered, however, by the possibility that some but not cells expressing a certain marker may have descending connections to skeletal muscle. In addition, co-expression of several neuropeptides within one cell can be common for certain structures, as is the case for PVN[257,258]. Therefore, identifying that a population of premotor cells expresses one marker does not preclude the possibility that those cells also express another simultaneously. Finally, certain

molecules (e.g., CRH) are difficult to visualize by immunofluorescence, and alternative strategies may be needed to identify such populations.

Several lines of investigation could be proposed to follow up our study on the impacts of early life enrichment in the bLR model. Firstly, neuroanatomical investigations could be supplemented to include LC and PAG, other nodes of the emotional motor system that contain perturbations in muscle-projecting neuronal populations. Additional experiments in younger bLR rats could also evaluate whether alternative or combinatorial treatment strategies are more effective than enrichment at reversing emotional behavior and wiring abnormalities in this strain. Given that emotional behavior abnormalities in bLR rats are more significantly improved when enrichment occurs during adulthood[54,240], it may be prudent to either (1) supplement the experience of greater environmental complexity during early life in this model with an additional treatment approach (e.g., pharmacological interventions), or (2) determine whether other strategies in isolation are more effective for treating emotional behavior disturbances at earlier time points. With respect to the second approach, ketamine, a noncompetitive NMDA-receptor antagonist, has recently emerged as a promising candidate for remedying treatment-resistant depression[259] and may even be successful in remitting motor-related impairments in this disorder[260]. Like environmental enrichment, ketamine augments neural plasticity[259].

Lastly, future studies could implement the PRV tract-tracing approaches employed throughout this dissertation to determine whether negative experiences (e.g., chronic variable stress) or environmental conditions (e.g., early life adversity) lead to similar emotional motor circuit abnormalities as those observed in the genetic models studied here. Genetic and environmental influences are both implicated in vulnerability for depression and anxiety-related disorders[15]. Furthermore, experiences with negative emotional valence also have the propensity

to induce plastic changes in the brain, as exemplified by the fact that chronic stress is associated with dendritic atrophy and synapse loss[259].

### 5.6 Conclusions

The dissertation research presented here demonstrates that, in two rat models with innate differences in emotional behavior and stress reactivity, alterations in premotor efferents with polysynaptic projections to skeletal muscle exist in several nodes of the emotional motor system that are implicated in somatomotor-sympathetic integration. These structures – PVN, LC, and PAG – are all involved in various behavioral, autonomic, and/or neuroendocrine responses to stress and emotional stimuli. The observation that these neuroanatomical alterations exist across two models with distinct genetic backgrounds suggests the possibility that certain elements of emotional motor system dysregulation could be conserved in preclinical (genetic) models, which could have implications for understanding how these circuits are affected in human patients suffering from clinical depression. Our observation that enriching environmental experiences during early life in one of these models, the bLR rat, are partially but incompletely effective in rescuing behavioral and neuroanatomical abnormalities inherent to this strain suggests that further study is needed to identify the exact parameters necessary to promote antidepressant and anxiolytic outcomes and drive more robust experience-dependent plasticity in this model.

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