Stress-induced adaptations in neuropeptidergic and serotonergic circuits associated with distinct coping strategies

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Stress-induced adaptations in neuropeptidergic and serotonergic circuits associated with distinct coping strategies

Abstract

Adverse experiences, whether early in life or in adulthood, alter behavioral, neuroendocrine, and neurochemical responses to future stressors and confer vulnerability to developing stress-related psychiatric disorders including anxiety and mood disorders. One potential hypothesis for how prior stress modifies behavioral and physiologic responses to future stress is through stress-induced adaptations in serotonergic systems. To test this hypothesis, we investigated the effects of daily maternal separation, during a critical period of development, on behavioral coping strategies during social defeat stress in adulthood and serotonergic gene expression using in situ hybridization histochemistry. We also compared early life stress to a model of acute and repeated social defeat in adulthood to investigate whether prior stress in adulthood is sufficient to change behavior as well as functional cellular responses, as measured by the immunohistochemical staining of the neuronal activation marker, c-Fos, in brainstem serotonergic neurons and their afferent neuropeptidergic circuits originating in the forebrain. Maternal separation shifted the coping response during social defeat in adulthood to a more reactive coping strategy, characterized by increased anxiety- and fear-like behaviors, and away from proactive coping, characterized by decreased confrontational and escape behaviors. This behavioral shift was accompanied by stress-induced alterations in tryptophan hydroxylase 2
(tph2) mRNA, encoding the neuronal isoform of the rate-limiting enzyme of serotonin synthesis, specifically in serotonergic neurons in the ventrolateral dorsal raphe nucleus and ventrolateral periaqueductal gray, a subregion implicated in inhibiting fight-or-flight responses, and the dorsal part of the dorsal raphe nucleus (DRD), a subregion that facilitates conflict anxiety. Likewise, exposure to repeated, but not acute, social defeat in adulthood shifted the coping style towards reactive coping and away from proactive coping, and altered the cellular responses of tryptophan hydroxylase-expressing neurons specifically in the DRD. Social defeat also altered forebrain neuropeptidergic systems implicated in control of serotonergic neuronal activity, including orexin/hypocretin, melanin-concentrating hormone and corticotropin-releasing hormone systems. Together, these data identify functionally distinct subpopulations of serotonergic neurons and associated neuropeptidergic circuits that coordinate responses to stress. Dysfunction of these subpopulations and circuits may be involved in the adoption of reactive coping, which may be relevant for vulnerability to stress-related psychiatric disorders.
Dedication

I dedicate this work to my family for being so supportive and loving; to my friends for providing balance; to Anna for her love and patience.
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Chapter I

Introduction

1. Summary

Traumatic life experiences, either occurring earlier in life or in adulthood, can produce long-lasting alterations in neural circuits controlling behavioral and physiological responses to emotionally-salient events, and these alterations may increase the vulnerability to developing psychiatric disorders (Heim and Nemeroff, 2001; Nemeroff and Vale, 2005). Because adaptive behavioral coping mechanisms can potently moderate the neuroendocrine and neurochemical responses to stressful events (Coppens et al., 2010; Koolhaas et al., 2007; Koolhaas et al., 1999), considerable interest is directed at determining the underlying neurobiological substrates involved in mediating different coping strategies. An overwhelming body of literature supports the hypothesis that brainstem serotonergic systems modulate the activity of brain defense circuits that control the behavioral and physiologic responses to stress. What is less clear are the upstream mechanisms through which stressful experiences influence the activity of brain serotonergic neurons. In this dissertation, I test the overall hypothesis that specific subpopulations of serotonergic neurons are differentially influenced by adverse early life experience and social defeat in adulthood, and that altered afferent input originating from stress-sensitive forebrain neuropeptidergic systems may underlie the altered patterns of serotonergic activity and changes in behavioral coping strategies.
The first chapter of this dissertation briefly describes the rodent models of adverse experiences during early and adult life used to test the hypothesis that serotonergic circuits are involved in coping responses to stress. These models include neonatal maternal separation, which consists of daily separation of rat pups from their mothers during a critical period of development, and social defeat during adult life, an ethologically-relevant model of social stress consisting of an aggressive encounter between a subordinate male (called the intruder) and a dominant male (called the resident). Evidence that these stressors target subpopulations of serotonergic neurons involved in mediating distinct coping strategies will also be presented. The last part of the chapter will discuss afferent input to serotonergic systems originating from forebrain neuropeptides that are positioned to alter serotonergic activity in response to stress.

Several experiments designed to test the hypothesis that stress-induced changes in forebrain neuropeptides together with brainstem serotonergic systems are involved in distinct behavioral coping strategies are presented in Chapters II, III, and IV. Chapter II presents evidence that adverse early life experience programs serotonergic gene expression in specific subpopulations of serotonergic neurons and shifts the behavioral coping strategy during social defeat in adulthood towards a more reactive coping strategy and away from a proactive coping strategy. Chapter III suggests repeated social defeat in adulthood results in a similar shift to a more reactive coping style together with alterations in functional neuronal responses in specific subregions of the dorsal raphe nucleus (DR). The final study discussed in Chapter IV, raises the possibility that forebrain stress-sensitive neuropeptidergic systems may mediate the stress-induced changes in serotonergic activity and behavioral coping strategies.

The general discussion in Chapter V will integrate the findings from Chapters II-IV. Chapter V will highlight some of the key findings, including evidence that both neonatal maternal
separation and repeated defeat during adulthood, which both result in increased reactive coping, may share some underlying neural mechanisms.

2. Rodent models of stress, anxiety-, and depression-like states

Many of the rodent models of human anxiety and affective disorders revolve around observations that traumatic life events and chronic stressors are potent risk factors for the development of psychiatric illness (Heim and Nemeroff, 2001; Nemeroff and Vale, 2005). One such animal model of adverse early life experience, neonatal maternal separation, involves repeated separation of rodent pups from their mothers for 180 min each day during a critical period of development (Plotsky and Meaney, 1993). This model is based on observations in seminaturalistic settings that subordinate dams, relative to more dominate females, are forced to build their nests at further distances from food and water sources, resulting in longer durations (e.g., 120-180 min) of separation from their pups while foraging (Calhoun, 1962). Underlining the face validity of this model of adverse early life experience, maternally separated rodents display a number of behavioral and physiological abnormalities that correspond well with the symptomatology of anxiety and affective disorders as classified in the latest 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V; American Psychiatric Association, 2013). In adulthood, maternally separated rodents display increased anxiety-like behavior, anhedonia, ethanol preference, impaired sexual behavior, and hypersensitivity of the hypothalamic-pituitary-adrenal axis (HPA axis; Huot et al., 2001; Kalinichev et al., 2002a; Kalinichev et al., 2002b; Ladd et al., 2000; Ladd et al., 1996; Plotsky and Meaney, 1993; Rhees et al., 2001; Wigger and Neumann, 1999). Overall, animals subjected to neonatal maternal separation have increased emotional reactivity to stressors in adulthood. Later
experiments revealed that the effects of maternal separation are not due to the period of separation, *per se*, but rather the maternal behavior of the dams towards the pups upon returning from the separation period (Liu et al., 1997; Stern, 1997). The dams of pups separated for a prolonged period of time (e.g., 180 min) responded by engaging in less licking/grooming and arched-back nursing behaviors. On the other hand, dams of pups separated for brief durations (e.g., 15 min), an early life treatment called neonatal handling or maternal separation 15 min (MS15), engage in greater licking/grooming and arched-back nursing behaviors. In contrast to maternal care following prolonged periods of separation, this maternal care actually results in a resilient phenotype of neonatally handled pups, characterized by reduced stress reactivity (Meaney, 2001). Nevertheless, for our discussion whether the crucial “stress” factor of maternal separation is the separation period itself or the corresponding maternal care or a combination of both is irrelevant as we are only concerned with the resulting phenotype. The important point is that these early life manipulations involving brief or prolonged periods of maternal separation result in highly stabilized variations in stress reactivity with prolonged separation producing pups that as adults display increased stress reactivity, whereas brief separation results in pups that have reduced stress reactivity. This variation in reactivity towards stress produced by different early life experiences is thought to be important for vulnerability to adverse consequences following subsequent exposure to stressors and provides a useful rodent model to investigate the link between susceptibility to stress-related psychiatric disorders, including anxiety and affective disorders.

Rodents exposed to early life maternal separation have increased reactivity to acute stressors in adulthood, including altered patterns of aggressive behavior in confrontations with male conspecifics (Veenema et al., 2006). Aggressive confrontations between males (and females) occur in a number of species, including mammals, birds, fish, and some insects, and
typically serve to establish hierarchies in social species as well as territorial access to environmental resources and potential mates. In rodents, these encounters consist of stereotypical behaviors and posturing that are categorized into offensive (biting, threat, aggression, etc.) and defensive behaviors (upright defensive behavior, escape, submission, etc.) with one of the conspecifics ultimately establishing dominance and the other engaging in submission postures. In the laboratory, aggressive conspecific encounters are typically conducted using resident/intruder paradigms (i.e., social defeat), which generally consists of placing an intruder rodent into the home cage (i.e., territory) of a larger, more aggressive conspecific resident. At first, the intruder and resident are typically separated by a partition permitting visual, olfactory, and auditory cues, while restricting physical confrontation. Then, after a brief period of time (10 min), the partition is removed and the animals are allowed to freely interact at which point the resident quickly establishes dominance over the intruder through a series of offensive bouts resulting in the intruder engaging in submissive postures to signal defeat.

Studies in feral and laboratory rodents suggest there is individual variation in the behavioral responses to social defeat along a continuum between proactive coping behaviors, comprised of confrontational behaviors and behaviors with escape/flight or exploration components, and reactive (passive-submissive) coping behaviors, characterized by increased submissive behavior and anxiety- and fear-like behaviors. Rodents that have either proactive or reactive coping styles also display distinct neuroendocrine and autonomic profiles (De Boer and Koolhaas, 2003; Koolhaas et al., 1999). Proactive coping is typically associated with low HPA axis reactivity to stress (e.g., decreased plasma corticosterone), high testosterone, and increased sympathetic reactivity (e.g., elevated plasma catecholamines), whereas reactive coping is associated with a high HPA axis reactivity, low testosterone, and increased
parasympathetic reactivity (De Boer and Koolhaas, 2003; Koolhaas et al., 1999). The different behavioral and physiological profiles of proactive versus reactive coping suggest the two coping strategies may differentially increase vulnerability to specific types of stress, stress-related psychiatric diseases, and other medical conditions (e.g., cardiovascular disease). Overall, social defeat is an ethologically-relevant stressor and can be used in conjunction with early life manipulations to investigate the effects of adverse early life experience (or enrichment) on behavioral and physiological responses to stress in adulthood.

Our laboratory has reported previously that adverse early life experience interacts with social defeat in adulthood to alter patterns of serotonergic gene expression in specific subpopulations of DR serotonergic neurons and these changes in gene expression associate with distinct behavioral coping strategies (Gardner et al., 2005; Gardner et al., 2009a; Gardner et al., 2009b). This strongly suggests that adverse early life experiences, adult stress, or the interaction between life stressors may result in lasting changes in the function of specific subpopulations of serotonergic neurons.

3. Distinct serotonergic pathways control different behavioral coping strategies: the Deakin/Graeff hypothesis

The notion that different types of stress activate discrete serotonergic systems to coordinate adaptive defensive responses was originally proposed over 20 years ago by Bill Deakin and Frederico Graeff (Deakin and Graeff, 1991; Graeff et al., 1996). Based largely on studies involving site-specific administration of serotonergic receptor agonists and antagonists, they proposed the existence of three distinct serotonergic systems that were activated by
different types of threats and these systems, through diffuse projections to forebrain and
brainstem structures, would coordinate the appropriate behavioral response (Deakin and Graeff,
1991; Graeff et al., 1996). Considering the intimate connection between stress, the brain
defense circuitry, and psychiatric disease, they also postulated that dysfunction of specific
serotonergic systems would increase vulnerability to specific stress-related disorders (Deakin
and Graeff, 1991; Graeff et al., 1996). Deakin and Graeff hypothesized that serotonergic
projections from the DR to forebrain structures, including the amygdala and prefrontal cortex,
would be activated by distal or potential threats in order to trigger behavioral and cognitive
responses to facilitate escape and active avoidance with the goal of guiding the organism away
from potential danger. Dysfunction of this pathway would increase vulnerability to anxiety
disorders (e.g., GAD). In contrast, they hypothesized that serotonergic projections from the DR
to the dorsal periaqueductal gray (DPAG) would selectively respond to proximal or immediate
threats (e.g., predator exposure) and inhibit fight-or-flight behaviors. Since the behavioral and
physiologic responses evoked by DPAG stimulation resemble the symptoms of panic attacks in
humans, compromise of an inhibitory DR-DPAG circuit would increase risk for panic disorder.
Finally, they hypothesized that serotonergic projections from the median raphe nucleus (MnR)
to the hippocampal formation would be activated by inescapable or chronic stressors and would
promote resilience. Dysregulation of this pathway would increase susceptibility to depression.

Our laboratory and others have delineated the origins of these three serotonergic
pathways within the midbrain raphe complex and revealed important functional differences that
support the original arrangement outlined in the Deakin/Graeff hypothesis. One subpopulation of
serotonergic neurons, residing in the dorsal (DRD) portions of the DR, comprises a conflict-
anxiety circuit, which sends projections to forebrain structures involved in regulating anxiety-like
responses, including the basolateral and central nuclei of the amygdala, bed nucleus of the stria
terminalis, ventral hippocampus, and prefrontal cortex (Hale and Lowry, 2011; Lowry et al., 2008c; Paul and Lowry, 2013). Consistent with this, DRD serotonergic neurons are selectively activated by anxiogenic drugs with diverse pharmacological actions (Abrams et al., 2005), anxiety-related neuropeptides such as urocortin 2 (Hale et al., 2010; Staub et al., 2005; Staub et al., 2006) and substance P (Valentino et al., 2003), and behavioral tests and stressors known to provoke anxiety-related responses (Amat et al., 2005; Donner et al., 2012b; Evans et al., 2009; Rozeske et al., 2011; Spannuth et al., 2011; Spiacci, Jr. et al., 2012). Another subpopulation of serotonergic neurons residing in the ventrolateral DR (DRVL) and adjacent ventrolateral periaqueductal gray (VLPAG; i.e., the lateral wings of the DR) comprises a panic inhibition circuit that functions to inhibit panic-like or fight-or-flight responses through innervation of brainstem and medullary structures involved in behavioral and autonomic responses to stress, including the dorsal periaqueductal gray, caudal raphe nuclei (e.g., raphe magnus and obscurus nuclei, and the rostroventrolateral medulla; Hale and Lowry, 2011; Johnson et al., 2004; Paul and Lowry, 2013). These DRVL/VLPAG 5-HT neurons are sensitive to panicogenic agents including CO₂ (Johnson et al., 2005) and sodium lactate (Johnson et al., 2008) and are selectively targeted in two rodent models of panic disorder (Donner et al., 2012a; Johnson et al., 2008). The final subpopulation of serotonergic neurons that corresponds with the original Deakin/Graeff pathways is located in the interfascicular portion of the DR (DRI) and adjacent MnR. This functionally distinct subpopulation of DRI/MnR serotonergic neurons is hypothesized to form a stress resilience/antidepressant system through projections to the hippocampus and medial prefrontal cortex (Hale and Lowry, 2011; Paul and Lowry, 2013). Based on their respective neuroanatomical projections and functional responses to stress and anxiety-related stimuli, these three subpopulations of serotonergic neurons, located in the DRD, DRVL/DRVLPAG, and DRI/MnR, correspond well with the original functional systems identified in the Deakin/Graeff hypothesis.
Juxtaposing Deakin and Graeff’s hypothesis of serotonergic pathways with the concept of proactive and reactive coping in species engaged in intraspecific conflicts leads to several predictions about which serotonergic pathways may be involved in facilitating or inhibiting a specific coping style. The DRVL/VLPAG serotonergic neurons, which inhibit behavioral and sympathetic fight-or-flight responses generated by structures like the DPAG, would be predicted to inhibit the confrontation and escape/flight behaviors characteristic of proactive coping and in turn facilitate freezing, a reactive coping behavior. Similarly, DRD neurons that project to forebrain structures involved in anxiety-like responses may be predicted to facilitate reactive coping as rodents with a reactive coping strategy tend to display more anxiety-like behavioral responses. Alternatively, DRD neurons may facilitate active risk assessment and goal-oriented escape behaviors observed in proactive coping. This relationship illustrates the predictions derived from our underlying hypothesis of this dissertation, namely that adverse life experiences alter the activity of specific functionally distinct subpopulations of serotonergic neurons, and these alterations are important determinants of behavioral coping strategies. In the chapters that follow, I will present evidence that supports the above hypothesis as well as the original Deakin and Graeff hypothesis.
Chapter II

Adverse early life experience programs serotonergic gene expression and alters behavioral coping strategies during adult social defeat

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Abstract

Adverse early life experience produces long-term changes in neural circuits controlling autonomic, behavioral, and neuroendocrine responses to stress, and may confer vulnerability to developing stress-related psychiatric disorders. Previous evidence suggests early life stress alters a number of serotonergic neurotransmission markers. Most studies investigate changes in the dorsal raphe nucleus (DR) due to its dense forebrain projections, responsiveness to emotionally salient stimuli, and role in anxiety and affective disorders, rather than the median raphe nucleus (MnR), another midbrain serotonergic nucleus involved in chronic stress resiliency and anxiety and depression. Here we tested the hypothesis that adverse early life experience interacts with social defeat in adulthood to alter serotonergic gene expression in MnR, in addition to the DR, as well as behavioral coping strategies. Male Long Evans rat pups were exposed to either normal animal facility rearing (AFR) or daily maternal separation for 180 min (MS180) from post natal days 2-14 (PND2-14) followed by subsequent exposure to either a novel cage control condition or social defeat in adulthood (PND60). In situ hybridization histochemistry was used to measure the expression of the gene encoding the serotonin transporter (slc6a4) and tryptophan hydroxylase 2 (tph2) in subregions of the DR and MnR. Maternally separated rats displayed more reactive coping behaviors during social defeat in adulthood in association with altered serotonergic gene expression. Early life stress reduced basal tph2 mRNA expression in the caudal DRVL/VLPAG, a region involved in the inhibition of proactive coping; whereas stress increased tph2 in the rostral DRVL/VLPAG regardless of early life rearing condition. Rodents exposed to AFR, relative to MS180 rats, had elevated tph2 in the DRD, a region involved in anxiety-like behavior. There were relatively few changes in slc6a4 in the DR and MnR; whereas tph2 showed a trend for reduced expression in the MnR. These data
support the hypothesis that adverse early life experience together with social defeat in adulthood interact to alter neurotransmission in specific subpopulations of serotonergic neurons.
1. Introduction

Traumatic early life events can produce long-lasting changes in neural circuits involved in controlling the behavioral, neuroendocrine, and neurochemical responses to stress and can increase vulnerability to developing stress-related psychiatric disorders, including anxiety and affective disorders (Agid et al., 2000; Heim and Nemeroff, 2001). The underlying neurobiological mechanisms for how adverse early life events confer susceptibility to psychiatric illnesses are unclear, but likely involve alterations in brainstem serotonergic systems implicated in controlling adaptive coping mechanisms.

A well characterized rodent model of adverse early life experience involves daily maternal separation of pups for 180 min during the first two weeks of life. This model was originally shown to produce long-term changes in basal and stress-induced activity of the hypothalamic-pituitary-adrenal axis (HPA-axis; Plotsky and Meaney, 1993). These effects may be due to altered epigenetic modification of the glucocorticoid receptor (Weaver et al., 2004), an effect that has been replicated in humans with a history of child abuse (McGowan et al., 2009). In addition to altering HPA axis function, maternal separation modulates various aspects of serotonergic neurotransmission, including serotonin (5-HT) receptor expression and sensitivity (Gartside et al., 2003; Neumaier et al., 2002; van Riel et al., 2004), the responsiveness of serotonergic neurons (Arborelius et al., 2004), and tissue concentrations of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA; a major serotonin metabolite) within the dorsal raphe nucleus (DR) and forebrain projection regions (Arborelius and Eklund, 2007) implicated in behavioral and physiological responses to emotionally-salient events.
Consistent with these studies, we have previously reported that maternal separation interacts with social defeat in adulthood to alter expression patterns of tryptophan hydroxylase 2 (tph2) mRNA, encoding the neuronal isoform of the rate-limiting enzyme involved in 5-HT synthesis, and serotonin transporter mRNA (slc6a4) in specific subregions of the dorsal raphe nucleus such as the ventrolateral dorsal raphe nucleus/ventrolateral periaqueductal gray (Gardner et al., 2009a; DRVL/VLPAG; Gardner et al., 2009c). These alterations in serotonergic gene expression observed in maternally separated rats were also associated with a switch towards a more reactive coping style, characterized by increased anxiety- and fear-related behavior such as freezing, during social defeat in adulthood (Gardner et al., 2005). Although several studies have investigated the effects of adverse early life experience on serotonergic neurons located in the dorsal raphe nucleus, very little is known about the involvement of serotonergic neurons located in the median raphe nucleus, a brain region thought to promote resilience to chronic stress (Andrade et al., 2013; Graeff et al., 1996; Paul and Lowry, 2013). Studies investigating post-mortem brains of depressed suicide victims suggest dysregulated serotonergic function in the MnR (as well as the DR) with depressed suicides having increased TPH2 mRNA expression (Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008), reduced 5-HT$_{1A}$ receptor binding (Arango et al., 2001), and altered 5-HT turnover (Bach et al., 2014) in the MnR.

Here we test the hypothesis that adverse early life experience will interact with stress in adulthood to alter active versus reactive behavioral coping responses in association with altered tph2 and slc6a4 mRNA expression in specific subpopulations of the DR as well as the MnR. To test this hypothesis we exposed rat pups to either normal animal facility rearing control conditions or daily maternal separation for 180 min during a critical period of development followed by exposure to either a novel cage control condition or social defeat stress in
adulthood. Following social defeat, we assessed tph2 and slc6a4 mRNA expression using *in situ* hybridization histochemistry in discrete subregions of the DR and MnR.

2. Materials and methods

2.1. Subjects

Timed-pregnant (gestational day 12, GD12) Long Evans rats (Crl(LE)BR; Charles River, Portage, MI, USA) were individually housed in transparent polycarbonate cages (26 cm W x 47.6 cm L x 20.3 cm H; Cat. No., RC88D-PC, Alternative Designs, Siloam Springs, AR, USA) containing a layer of bedding (Cat. No., 7090, Teklad Sani-Chips, Harlan, Madison, WI, USA). The cages were covered with stainless steel wire lids (Cat. No. WL88R, Alternative Designs) with two compartments to accommodate food (Cat. No. 8640; Teklad 22/5 Rodent diet, Harlan Laboratories, Indianapolis, IN, USA) and tap water stored in 16 oz reduced height water bottles (Cat. No., WB16RH, Alternative Designs) with screw lids (Cat. No. FSPCST2.5, AnCare Corp., Bellmore, NY, USA), which allowed the animals free access to food and water. After birth during GD21 and GD22, females were culled and the dams along with litters of six cross-fostered males were housed in the same conditions as above. Male Long Evans retired breeder rats (Harlan Laboratories; 400-600 g) were used as residents for the social defeat procedure and were individually housed in the same conditions described above except in a separate colony room. The vivarium was maintained on a 12-h light/dark cycle (lights on at 07:00 h; light intensity = 115 lux) with the temperature kept at approximately 23 °C. All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Eighth edition, Institute for Laboratory Animal Research, The National Academies
Press, Washington, DC, 2011) and the Institutional Animal Care and Use Committee at the University of Colorado Boulder approved all procedures. All possible efforts were made to minimize the number of animals used and their suffering.

2.2. Maternal separation procedure

The maternal separation procedure was conducted according to a protocol that has been previously described in detail (Gardner et al., 2005; Gardner et al., 2009a; Gardner et al., 2009b; Huot et al., 2001; Plotsky and Meaney, 1993). The day of birth was designated as post-natal day 0 (PND0) and on the next day, PND1, the litters were sexed and the females were culled and removed from the experiment. The remaining male rats were pooled and randomly assigned to a foster mother with six male pups in each litter. After randomly cross-fostering the pups, the litters were randomly assigned to either one of two rearing conditions: 1) the maternal separation 180 (MS180) group received 180 min of maternal separation during PND2-14 or 2) the animal facility rearing (AFR) control group received normal animal facility rearing during PND2-14, which consisted of changing the dirty cages containing soiled bedding for new cages containing clean bedding twice per week. The daily separations for the MS180 group and twice per week cage cleaning for both AFR and MS180 groups took place starting one hour after lights on (0700 h) between 0800-1200 h. During the maternal separation period, the bedding in the home cages of the MS180 litters was not completely changed because the novelty of a new cage could be a potential early-life stressor; instead, twice per week while the animals were separated, half of the dirty bedding in the home cages was removed and replaced with clean bedding.
The procedure for maternal separation was performed as follows. First, each dam was removed from the home cage and placed into an identical cage containing bedding, food, and water. There was a separate cage for each dam that was used throughout PND2-14 and the bedding in these cages was never changed. Next, the pups were removed and placed into a smaller cage (18.4 cm W x 29.2 cm L x 12.7 cm H) containing bedding, which was not changed during the separation procedure (PND2-14), and then placed into an incubator (Cat. No. 47746-772, Incubator BD53-UL, Binder Inc., Great River, NY, USA) for 180 min. The temperature of the incubator was maintained at 32 ± 0.5 °C from PND2-5 and at 30 ± 0.5 °C from PND6-14. Following the 180 min of separation, pups were removed from the incubator and placed back into their respective home cages, and then the dams were returned to their respective home cages. After the maternal separation period (PND2-14), the animals were left completely undisturbed (no cage changes) until PND18 when twice per week cage changes resumed. Then on PND21 the animals were weaned from their respective mothers and housed as complete litters until PND30 when they were randomly pair-housed with littermates from the same litter and consequently the same early life treatment group until adulthood.

2.3. Social defeat and novel cage control procedures

In adulthood, designated as PND60, half of the rats in both the MS180 and AFR groups were randomly assigned to either acute social defeat (SD) or a novel cage control condition (Control). The novel cage control animals were placed into a clean novel cage with new bedding and transported to an experimental room, adjacent but separate from the room where the social defeats were conducted, where they were weighed and then remained for 20 min before being returned to their home cages. The previously described social defeat procedure (Gardner et al.,
2005; Martinez et al., 1998; Paul et al., 2011) lasted for 20 min and consisted of both a 10 min pre-defeat phase and a 10 min defeat phase. During the pre-defeat phase the rats were separated by a transparent 0.3 cm-wide poly(methyl methacrylate) (PMMA) partition with 9 (0.3 cm diameter) holes drilled 5 cm apart (3 x 3 grid), which allowed auditory, olfactory, and visual cues, but no physical contact. The partition was removed for the defeat phase, thus allowing the resident and intruder to freely interact. After exposure to either social defeat or a novel cage, the rats were placed back into their home cages and brought back to the colony room.

2.4. Analysis of social defeat behavior

Behavior of the intruder and resident during both the pre-defeat and defeat phases was recorded with a video camera mounted on a tripod to allow a slightly elevated, horizontal view of the social defeat arena and later quantified by an experimenter blind to treatment using Noldus The Observer (Noldus Information Technology, Leesburg, VA, USA), a software program for the analysis of observational data. During the pre-defeat phase the following behaviors were quantified and are defined in Table 1: defensive burying, inactivity, freezing, locomotion, rearing, self-grooming, and sniffing bedding. Behaviors during the defeat phase were divided into three categories including reactive (i.e., passive-submissive) coping, proactive coping, and neutral behaviors (Koolhaas et al., 1999). Reactive (passive-submissive) coping behaviors included freezing, full submission, sideways submission, passive genital sniff, genital sniff, and sniffing bedding; whereas, proactive behaviors included rearing, upright defensive behavior, aggression, defensive burying, social interaction and escape. Locomotion, inactivity, and self-grooming were representative of neutral coping behaviors (Table 1). These categories are based on observations that populations of both feral and laboratory rodents (as well as other
species) display marked individual variations in their behavioral coping responses to conspecific aggression and that these differences have profound implications for vulnerability to the deleterious effects of stress (Coppens et al., 2010; Ebner et al., 2005; Frank et al., 2006; Koolhaas et al., 1999; Koolhaas et al., 2007; Koolhaas et al., 2013; Miczek et al., 2004; Veenema and Neumann, 2007; Walker et al., 2009).

Table 1. Definitions of behavioral categories and individual behaviors quantified during the pre-defeat and defeat phases of social defeat.

<table>
<thead>
<tr>
<th>Pre-defeat behaviors</th>
<th>Defeat behaviors</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reactive coping</strong></td>
<td>Sniffing bedding</td>
<td>Behaviors with vigilance, anxiety-related, fear-related, or risk assessment components as well as submissive behaviors</td>
</tr>
<tr>
<td></td>
<td>Freezing</td>
<td>Sniffing the substrate without locomotion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intruder is crouching with his back arched, occasionally exhibiting piloerection, and is motionless except for movement associated with respiration and scanning of the environment with the head</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The intruder lies on its back with its full belly exposed to the resident</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The intruder crouches below the resident and turns to expose part of its belly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Being sniffed by the resident</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sniffing the resident’s genitals</td>
</tr>
<tr>
<td><strong>Proactive coping</strong></td>
<td>Rearing*</td>
<td>Confrontational behaviors and behaviors with exploration or escape components</td>
</tr>
<tr>
<td></td>
<td>Defensive burying</td>
<td>Bipedal posture</td>
</tr>
<tr>
<td></td>
<td>Aggression</td>
<td>Shoveling bedding towards the resident</td>
</tr>
<tr>
<td></td>
<td>Escape</td>
<td>Biting, kicking, boxing, wrestling and fighting the resident</td>
</tr>
<tr>
<td></td>
<td>Upright defensive behavior</td>
<td>Fleeing from the resident</td>
</tr>
<tr>
<td></td>
<td>Social interaction</td>
<td>Rearing while facing the resident</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grooming and sniffing the resident outside the ano-genital region, nosing and crawling over/under the resident</td>
</tr>
<tr>
<td><strong>Neutral</strong></td>
<td>Locomotion</td>
<td>Non-vigilant behaviors not directed towards or in response to the resident male</td>
</tr>
<tr>
<td></td>
<td>Self-grooming</td>
<td>Walking around the cage</td>
</tr>
<tr>
<td></td>
<td>Inactivity</td>
<td>Licking or scratching coat</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lying or sitting motionless</td>
</tr>
</tbody>
</table>

*(e.g. Explorative escape as defined by De Boer and Koolhaas, 2003).

2.5. Blood collection and corticosterone assay

Following a period of 4 hrs from the onset of exposure to social defeat or a novel cage, the rats were removed from the vivarium and taken to a separate experimental room where they were rapidly decapitated and trunk blood was collected into sterile 10 mL Falcon tubes
containing 200 µL of 2% ethylene-diamine-tetraacetic acid (EDTA; Cat. No. EDS, Sigma-Aldrich, St. Louis, MO, USA) and 5% heparin (Cat. No. H6279, Sigma-Aldrich), both prepared in 0.05 M phosphate buffered saline (PBS). The Falcon tubes containing trunk blood were kept on wet ice and subsequently centrifuged at 10,000 rpm at 4 °C for 15 min. After centrifugation, the tubes were returned to wet ice and the plasma was aliquoted and stored at –80 °C until later assay of corticosterone. Plasma total corticosterone concentrations were measured in duplicate using an enzyme-linked immunosorbent assay kit (Cat. No. 900-097, Assay Designs, Ann Arbor, MI, USA) according to the manufacturer’s instructions.

2.6. Brain extraction and sectioning

After collecting trunk blood (i.e., 4 hrs following the onset of social defeat or novel cage exposure), the brains were quickly extracted, fast-frozen in liquid isopentane, and stored in a freezer at –80 °C until later sectioning. We selected a 4 hr sacrifice time point because previous studies revealed stress-induced increases in brain mRNA at this time point (Gardner et al., 2009a; Gardner et al., 2009b; Harbuz et al., 1993; Harbuz and Lightman, 1989). After freezing and just before sectioning, each brain was blocked just caudal to the mammillary bodies using a rat brain matrix (RB-4000C, ASI Instruments, Warren, MI, USA), resulting in forebrain and hindbrain sections. The flat plane of the hindbrain was mounted on a specimen holder using optimum cutting temperature (OCT) compound (Tissue-Tek®, Sakura®, Finetek, Cat. No. 25608-930, VWR Scientific, West Chester, PA, USA) and coronal brain sections (12 µm) of the DR and MnR (e.g., –7.016 mm to –8.744 mm) were collected using a precision cryostat (Leica CM1900). Alternate brain sections were mounted directly onto eight sets of electrochemically treated glass slides (VistaVision HistoBond® adhesive slides, Cat. No. 16004-406, VWR
Scientific) and stored at –80 °C until further analysis. The alternate section approach ensured that each set of slides contained a representation of the rostrocaudal extent of the DR and MnR with a 96 µm interval between each section. A separate set of adjacent slides from each subject was used for detection of each gene (tph2 and slc6a4) using in situ hybridization histochemistry. To ensure consistency across dorsal-ventral, rostral-caudal, and medial-lateral planes between subjects’ brains, each brain was blocked, mounted, sectioned, and coronal brain sections were placed on the glass slides using the same routine.

2.7. Tph2 riboprobe preparation and semi-quantitative in situ hybridization histochemistry

A previously described protocol (Donner and Handa, 2009; Donner et al., 2012b; Donner et al., 2012a) was used to generate a 583 base-pair fragment of rat tph2 cRNA radiolabeled with $[^{35}S]$-uridine-5′-triphosphate (UTP, Cat. No. 5610301, MP Biomedicals, Santa Ana, CA, USA) that was complementary to bases 761–1343 of rat tph2 mRNA. Coronal brain sections of the DR and MnR from one of the eight alternate sets of slides from each rat were thawed at room temperature, post-fixed in 4% paraformaldehyde made in 0.05 M PBS, rinsed three times for 10 min each in 2X saline sodium-citrate (SSC) buffer, and then 90 µL of hybridization buffer (pH 7.4), which consisted of $1 \times 10^6$ cpm $[^{35}S]$-UTP labeled tph2 cRNA, 25 mM Tris, 40% deionized formamide, 500 µg/ml single-stranded salmon sperm DNA, 250 µg/ml transfer RNA, 1X Denhardt’s solution, 4 mM EDTA, 5 mM sodium chloride, and 10% dextran sulfate (w/v), was dispensed on each glass slide. Glass slides were coverslipped with parafilm cut into rectangular strips to fit each slide and incubated overnight in a humidified chamber set at 55 °C. On the next day, the parafilm coverslips were removed and the hybridization buffer was washed off using 2X SSC buffer followed by three 10 min washes with 1X SSC. Then slides were incubated in
ribonuclease A solution, which consisted of 0.05 M Tris–Cl, 0.025 M EDTA, 0.5 M NaCl, and 20 µg/ml RNase A, for 1 hr at 37 °C. After incubation, the slides were washed in 1X SSC buffer for 30 min at room temperature and then again in 1X SSC buffer for 30 min at 60 °C. Finally, slides were desalted in 0.5X SSC and 0.1X SSC for 10 min each, dehydrated in an ascending series of ethanol solutions (i.e., 50, 70, 90, and then 100%) containing 0.3 M ammonium acetate, air-dried for 20 min, and apposed to a single Kodak BioMax autoradiography film (PerkinElmer, Waltham, MA, USA) for 7 days.

2.8. Slc6a4 oligonucleotide probe preparation and semi-quantitative in situ hybridization histochemistry

To detect slc6a4 mRNA we generated a synthetic 50-base antisense oligonucleotide probe complementary to bases 207–256 of rat slc6a4 mRNA and radiolabeled at the 3' end with $[^{35}\text{S}]$-labeled deoxyadenosine 5'-triphosphate (dATP, Cat.No. 5620001, MP Biomedicals) using a protocol that has been previously described in detail (Donner et al., 2012a; Gardner et al., 2009a). Glass slides containing coronal brain sections of the DR and MnR from one of the eight alternate sets of slides from each rat were thawed and equilibrated to room temperature, then they were post-fixed for 10 min in 4% paraformaldehyde in 0.05 M PBS, washed twice in 0.05 M PBS, and immersed for 10 min into freshly prepared 0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine. Next, brain sections were dehydrated in an ascending series of ethanol solutions, immersed in chloroform for delipidation, rehydrated through a series of descending ethanol solutions, air-dried, and then each slide was coated with 90 µL of hybridization buffer containing $1 \times 10^5$ cpm $[^{35}\text{S}]$-labeled dATP, 50% deionized formamide, 20X SSC, 10 mg/ml sheared salmon sperm DNA, 25 mg/ml yeast transfer RNA, 50X Denhardt's
solution, 10 mM dithiothreitol (DTT), and 50% dextran sulfate. Next, glass slides were coverslipped with parafilm and incubated overnight in a humidified chamber maintained at 37 °C. On the next day, the parafilm coverslips were removed in 1X SSC and slides were washed with agitation four times for 15 sec each in 1X SSC. Slides were subsequently washed four times for 15 min each in 1X SSC in a shaking water bath at 55 °C, followed by two 30 min washes each in 1X SSC at room temperature, and one brief (1–2 sec) wash in distilled H₂O at room temperature. Finally, slides were air-dried for 20 min and apposed to a single Kodak BioMax autoradiography film for 7 days.

2.9. Imaging and densitometry of in situ hybridization autoradiograms

Autoradiographic images of the probe bound to tph2 mRNA or to slc6a4 mRNA together with ¹⁴C-labeled standards were quantified by an experimenter blind to treatment using the public domain NIH Image program, ImageJ (developed at the U.S. National Institutes of Health and available for free download at http://rsb.info.nih.gov/nih-image/). Since all slides for a particular gene were apposed to a single film, we were able to use a single ¹⁴C-labeled standard for reference. Analyses atlases of tph2 and slc6a4 gene expression in subdivisions of the DR (Figure 1a and 1b) and MnR (Figure 2a and 2b) were created from autoradiographic images of individual subjects in the current study and were used during the densitometry analysis to determine rostrocaudal levels and boundaries of subdivisions of the DR and MnR. These analyses atlases were created by comparing anatomical features of the autoradiographic images with a standardized stereotaxic rat brain atlas (Paxinos and Watson, 1998) an atlas of tryptophan hydroxylase immunostaining (Abrams et al., 2004) and tph2 mRNA expression within the DR (Gardner et al., 2009b), and an atlas of slc6a4 mRNA expression within the DR.
(Gardner et al., 2009a; Lowry et al., 2008a). For the DR, 9 rostrocaudal levels were analyzed for $tph2$ (designated +6 to –2 from –7.592 to –8.360 mm bregma) and $slc6a4$ (designated +7 to –1 from –7.496 to –8.264 mm bregma) containing seven subdivisions, including the rostral dorsal raphe nucleus, dorsal part (rDRD; +6 to +3 for $tph2$, +7 to +3 for $slc6a4$), caudal dorsal raphe nucleus, dorsal part (cDRD; +2 to –2 for $tph2$, +2 to –1 for $slc6a4$), rostral dorsal raphe nucleus, ventral part (rDRV; +6 to +3 for $tph2$, +7 to +3 for $slc6a4$), caudal dorsal raphe nucleus, ventral part (cDRV; +2 to –2 for $tph2$, +2 to –1 for $slc6a4$), rostral dorsal raphe nucleus, ventrolateral part (rDRVL)/ventrolateral periaqueductal gray (VLPAG; +5 to +3 for $tph2$, +5 to +3 for $slc6a4$), caudal dorsal raphe nucleus, ventrolateral part (cDRVL)/ventrolateral periaqueductal gray (VLPAG, +2 to –2 for $tph2$, +2 to –1 for $slc6a4$), and dorsal raphe nucleus, interfascicular part (DRI), although $slc6a4$ mRNA expression was not quantified in the DRI. A total of 7 rostrocaudal levels were analyzed for $tph2$ (designated +5 to –1 from –7.688 to –8.360 mm bregma) and $slc6a4$ (designated +6 to 0 from –7.592 to –8.264 mm bregma) in the MnR, which was also partitioned into the rostral MnR (rMnR; +5 to +3 for $tph2$, +6 to +3 for $slc6a4$) and caudal MnR (cMnR; +2 to –1 for $tph2$, +2 to 0 for $slc6a4$). For the densitometry analysis, templates outlining the subdivisions of the DR and the MnR were created based on the analyses atlases for $tph2$ and $slc6a4$, overlaid on the image, and the optical density x area within each template was obtained using a constant threshold function throughout the analysis. The background optical density for each image was measured and subtracted from each value.
Figure 1. Analyses atlases illustrating autoradiographic images of coronal brain sections (96 µm intervals) of tph2 (a) and slc6a4 (b) mRNA expression in rostrocaudal levels and subregions of the DR. The rostrocaudal levels selected for analysis of tph2 mRNA expression (a) ranged from –7.592 mm (designated level +6) to –8.360 mm from bregma (designated level –2) and the analysis of slc6a4 mRNA expression (b) covered levels –7.496 mm (designated level +7) to –8.264 mm from bregma (designated level –1). Dotted lines outline the boundaries of DR subregions analyzed at each rostrocaudal level. The scale bar (1 mm) is represented as a black line in the lower left corner of the top left panel in each figure. Abbreviations: dorsal raphe nucleus, caudal part (DRC), dorsal raphe nucleus, dorsal part (DRD), dorsal raphe nucleus, interfascicular part (DRI), dorsal raphe nucleus, ventral part (DRV), dorsal raphe nucleus, ventrolateral part (DRVL), ventrolateral periaqueductal gray (VLPAG).
Figure 2. Analyses atlases illustrating autoradiographic images of coronal brain sections (96 µm intervals) of tph2 (a) and slc6a4 (b) mRNA expression in rostrocaudal levels and subregions of the MnR. The rostrocaudal levels selected for analysis of tph2 mRNA expression (a) ranged from –7.688 mm (designated level +5) to –8.264 mm from bregma (designated level –1) and the analysis of slc6a4 mRNA expression (b) covered levels –7.592 mm (designated level +6) to –8.168 mm from bregma (designated level 0). Dotted lines outline the boundaries of the MnR analyzed at each rostrocaudal level. The scale bar (1 mm) is represented as a black line in the lower left corner of the top left panel in each figure.

2.10. Statistical analysis

Prior to statistical analysis, outliers were identified using statistical tests to determine single and multiple outliers (Grubbs, 1969), excluded from further statistical analysis, and are not depicted in the tables and graphical representation of the data. Statistical significance was
accepted at $P \leq 0.05$ for all statistical analyses, which were performed using PASW statistics (Version 21 for Macintosh, SPSS Inc., Chicago, IL, USA). In the instances where the statistical analysis of a main effect or interaction between main effects either did not reach significance or approached significance, post hoc individual and multiple comparisons among means were nevertheless conducted in the following circumstances: 1) if the a priori hypothesis or specific comparisons built into the experimental design suggest the appropriate comparisons should be made; and 2) if deliberate inspection of the data identified changes warranting a posteriori comparisons (Wilcox, 1987; Winer et al., 1991).

2.10.1. Statistical analysis of behavior

The behavior during the pre-defeat and defeat phases was analyzed separately. For both the pre-defeat and defeat phases, separate independent Student's $t$-tests were performed on the duration and frequency of each behavior in order to determine if rats exposed to either MS180 or AFR conditions differed in their behavioral responses during social defeat. Furthermore, the behaviors quantified during the defeat phase were grouped into distinct behavioral categories, including proactive coping, reactive (passive-submissive) coping, neutral behavior, and the ratio of proactive to reactive coping behaviors (Coppens et al., 2010; Ebner et al., 2005; Frank et al., 2006; Gardner et al., 2005; Koolhaas et al., 1999; Koolhaas et al., 2007; Koolhaas et al., 2013; Miczek et al., 2004; Paul et al., 2011; Veenema and Neumann, 2007; Walker et al., 2009). The individual behaviors that comprised reactive, proactive and neutral categories (Table 1) were summed together to determine the total amount of behavior for each respective category and separate independent Student's $t$-tests were conducted on the duration and frequency of each behavioral category. In all analyses, Levene's Test for Equality
of Variances was conducted to determine if the data met the independent Student's $t$-test assumption of homogeneity of variance and the degrees of freedom were adjusted accordingly in the event that the data violated this assumption.

2.10.2. Statistical analysis of tph2 and slc6a4 mRNA expression

To determine the effects of early life experience, adult stress, and their interaction on gene expression within the DR and MnR, separate linear mixed models were conducted on the expression of either *tph2* or *slc6a4* within rostral and caudal subdivisions of the DR and MnR, complete subdivisions of the DR (i.e., rostral and caudal parts combined), and the whole DR or MnR. The linear mixed model analyses used *early life experience* (e.g., AFR or MS180) and *adult treatment* (e.g., Control or SD) as fixed factors (i.e., between-subjects factors) and, where appropriate, *subregion* (e.g., DR subregion or rostral versus caudal MnR) as a repeated measure. The analyses incorporated an unstructured covariance structure for repeated measures and used the maximum likelihood method to estimate parameters in the statistical model. The linear mixed model analysis is preferable to the repeated measures ANOVA under circumstances where data sets contain missing data, unbalanced designs, complex covariate structures, and situations where multiple measurements (e.g., brain region) are derived from the same unit of observation (Cnaan et al., 1997; Krueger and Tian, 2004; Spannuth et al., 2011). In the event of significant main effects or interactions, differences between groups were determined using Fisher's Protected Least Significant Difference (PLSD) post hoc tests.

2.10.3. Statistical analysis of plasma corticosterone
Comparison of the plasma corticosterone concentrations in animals exposed to either normal animal facility rearing or maternal separation followed by either novel cage control or social defeat in adulthood were analyzed using a linear mixed model with early life experience and adult treatment as fixed factors.

2.10.4. Statistical analysis of correlations between behavior and gene expression

Pearson product-moment correlation tests were conducted to determine the association between gene expression in subdivisions of the midbrain raphe complex (i.e., DR and MnR) and the frequency and duration of individual behaviors during both the pre-defeat and defeat phases of social defeat as well as reactive, proactive, and neutral coping strategies.

3. Results

3.1. Behavioral analysis

Statistical analysis of the pre-defeat and defeat behavior revealed that animals exposed to different early life rearing conditions responded with different patterns of behavior. Individual Student’s t-tests on each behavior during the pre-defeat phase revealed a robust decrease in the frequency ($t_{(15)} = 4.064$, $P \leq 0.001$) and duration ($t_{(14.715)} = 4.536$, $P \leq 0.001$) of rearing behavior (Table 2) as well as a decrease in the duration of self-grooming behavior ($t_{(14)} = 2.47$, $P \leq 0.05$; Table 2) in animals exposed to MS180 ($n = 10$) early in life, compared to AFR animals.
(n = 7). Analysis of the defeat-phase of social defeat using individual Student’s t-tests on each behavior revealed a significant increase in the frequency and duration of freezing (t_{13.814} = -2.17, P ≤ 0.05 for frequency; t_{9.225} = -3.558, P ≤ 0.01 for duration) and inactivity (t_{10.827} = -2.858, P ≤ 0.05 for frequency; t_{9.207} = -2.611, P ≤ 0.05 for duration Table 3) in maternally separated rats (n = 10), compared to AFR rats (n = 7). Maternally separated rats also displayed greater frequency of defensive burying (t_{9} = -2.623, P ≤ 0.05; Table 3) and a decrease in the frequency of rearing behavior (t_{14} = 2.535, P ≤ 0.05), while the decrease in duration of rearing approached significance (t_{14} = 2.051, P = 0.059; Table 3).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Duration AFR</th>
<th>MS180</th>
<th>Frequency AFR</th>
<th>MS180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniffing bedding</td>
<td>111.4±19.3</td>
<td>120.1±14.4</td>
<td>24.6±3.9</td>
<td>25.1±3.0</td>
</tr>
<tr>
<td>Freezing</td>
<td>0.0±0.0</td>
<td>3.4±2.3</td>
<td>0.0±0.0</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Rearing</td>
<td>193.3±10.3</td>
<td>112.6±14.6</td>
<td>31.0±1.6</td>
<td>19.5±2.1**</td>
</tr>
<tr>
<td>Defensive burying</td>
<td>8.5±5.7</td>
<td>4.5±2.8</td>
<td>3.6±2.3</td>
<td>1.4±0.8</td>
</tr>
<tr>
<td>Locomotion</td>
<td>48.6±5.1</td>
<td>43.4±4.5</td>
<td>29.6±3.2</td>
<td>24.0±2.4</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>35.7±10.3</td>
<td>11.1±3.7*</td>
<td>4.0±1.2</td>
<td>3.4±0.9</td>
</tr>
<tr>
<td>Inactivity</td>
<td>14.4±6.7</td>
<td>22.8±7.9</td>
<td>2.7±1.1</td>
<td>2.4±0.9</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001. Values are presented as mean±S.E.M.

Because individual coping strategies are thought to fall into two distinct behavioral spectrums, proactive and reactive (passive-submissive) coping, individual behaviors were
grouped into categories and analyzed with separate independent Student’s t-tests. Analysis of coping strategies revealed that MS180 rats, relative to AFR rats, responded during social defeat with increased duration of reactive coping behaviors ($t_{(12.029)} = –3.639, P ≤ 0.01$; **Figure 3a**) and decreased duration ($t_{(15)} = 2.634, P ≤ 0.05$; **Figure 3b**) and frequency ($t_{(15)} = 3.086, P ≤ 0.01$; **Figure 3f**) of proactive behaviors. Consistent with this altered coping strategy, MS180 rats had an increase in the ratio of duration ($t_{(15)} = –2.489, P ≤ 0.05$) and frequency ($t_{(15)} = –2.679, P ≤ 0.05$) of reactive versus proactive coping behaviors (**Figure 3d, h**). Maternally separated rats also showed a reduction in the frequency of neutral behaviors ($t_{(15)} = 2.895, P ≤ 0.05$) during social defeat (**Figure 3g**).

**Figure 3.** Maternally separated animals have altered coping strategies during adult social defeat. Bar graphs depict the comparison of early life experience on the duration (a, b, c and d) and frequency (e, f, g and h) of behavioral coping strategies during the defeat phase of social defeat. Behavioral categories include reactive coping behaviors, (a, e), proactive coping behaviors and (b, f), neutral behaviors (c, g), and the ratio of reactive to proactive coping behaviors. Abbreviations: AFR, normal animal facility rearing; MS180, daily maternal separation for 180 min (PND2-14). Frequency and duration of coping behavior categories were analyzed using independent Student’s t-tests comparing AFR (n = 7) with MS180 (n = 10) rats and data are presented as means ± S.E.M. *$P ≤ 0.05$, **$P ≤ 0.01$.

3.2. tph2 and slc6a4 mRNA expression within subdivisions of the DR
The linear mixed model analysis of tph2 mRNA expression in rostral and caudal subregions of the DR revealed a significant interaction between early life experience and subregion \((F(6, 37.828) = 2.68, P \leq 0.05)\), adult treatment and subregion \((F(6, 37.828) = 2.387, P \leq 0.05)\), and the interaction between early life experience, adult treatment, and subregion approached statistical significance \((F(6, 37.828) = 2.269, P = 0.057)\). There was a trend towards a significant main effect of early life experience on tph2 mRNA expression in rostral and caudal DR subregions \((F(1, 39.002) = 3.704, P = 0.062)\). Analysis of tph2 mRNA expression in the combined rostral and caudal portions of each DR subregion largely paralleled these findings, showing a significant interaction between early life experience, adult treatment, and subregion \((F(3, 37.562) = 3.144, P \leq 0.05)\) and a significant main effect of early life experience \((F(1, 38.959) = 4.16, P \leq 0.05)\). In contrast, analysis of tph2 mRNA expression in the whole DR only identified a significant main effect of early life experience \((F(1, 39) = 5.048, P \leq 0.05)\) and no main effect of adult treatment \((F(1, 39) = 1.846, P = 0.182)\) or interaction between early life experience and adult treatment \((F(1, 39) = 2.469, P = 0.124)\), highlighting the subregion-specific effects of an interaction between early life experience and adult stress.

An analysis of the effects of early life experience, without taking into consideration adult treatment, on tph2 mRNA expression in rostral and caudal portions of each subregion revealed that MS180 rodents, relative to AFR rodents, had decreased tph2 expression in the caudal, but not rostral, DRD \((t_{(37)} = 2.082, P \leq 0.05; \text{Figure 4b})\), DRV \((t_{(37)} = 2.038, P \leq 0.05; \text{Figure 4e})\), and DRVL/VLPAG \((t_{(37)} = 3.603, P \leq 0.001; \text{Figure 4h})\). Likewise, after combining rostral and caudal portions of each DR subregion, MS180 rats had reduced tph2 expression in the DRD \((t_{(37)} = 2.314, P \leq 0.05; \text{Figure 4c})\) and DRVL/VLPAG \((t_{(37)} = 3.383, P \leq 0.01; \text{Figure 4i})\).
Figure 4. Maternal separation reduces *tph2* expression in subregions of the dorsal raphe nucleus. Bar graphs depict mean density x area of *tph2* mRNA expression in the rostral (a, d, and g), caudal (b, e, and h), and the combined rostral and caudal (c, f, and i) portions of the DRD (a, b, and c), DRV (d, e, and f), and DRVL/VLPAG (g, h, and i) in animals exposed to either normal animal facility rearing or maternal separation, regardless of their adult treatment. Abbreviations: AFR, normal animal facility rearing; MS180, daily maternal separation for 180 min (PND2-14); rDRD, rostral dorsal raphe nucleus, dorsal part; cDRD, caudal dorsal raphe nucleus, dorsal part; rDRV, rostral dorsal raphe nucleus, ventral part; cDRV, caudal dorsal raphe nucleus, ventral part; DRD, dorsal raphe nucleus, ventral part; rDRVL/VLPAG, rostral dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; cDRVL/VLPAG, caudal dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray. Data were analyzed using
independent Student’s t-tests comparing AFR (n = 14) with MS180 (n = 25) rats and presented as means ± S.E.M. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Analysis of the effects of adult treatment, without incorporating early life experience into the model, identified a robust social defeat-induced increase of tph2 mRNA in the rostral, but not caudal, DRVL/VLPAG (t_{37} = –3.685, P ≤ 0.001; Figure 5a). After combining the rostral and caudal portions of the DRVL/VLPAG, this stress-induced increase in tph2 mRNA only approached significance (t_{37} = –1.748, P = 0.089; Figure 5c).

**Figure 5. Social defeat elevates tph2 mRNA expression in the rostral ventrolateral dorsal raphe nucleus/ventrolateral periaqueductal gray. Bar graphs illustrate mean density x area of tph2 mRNA expression in the rostral (a), caudal (b), and the combined rostral and caudal (c) portions of the DRVL/VLPAG (c) in animals exposed to either social defeat stress or novel cage control in adulthood, irrespective of early life rearing conditions. Abbreviations: Control, novel cage control; SD, social defeat; see Figure 4 for remaining abbreviations. Data were analyzed using independent Student’s t-tests comparing Control (n = 23) with SD (n = 16) rats and presented as means ± S.E.M. ***P ≤ 0.001, ~P ≤ 0.10.**

Post hoc analyses using Fisher’s PLSD on the rostral and caudal portions of each subregion as well as the combined rostral and caudal portions of each subregion revealed a stress-induced increase in tph2 mRNA expression in a subregion-specific manner and largely in animals that were raised in normal animal facility rearing environments. Social defeat increased tph2 mRNA expression within the DRI (Figure 6j), caudal DRD (Figure 6b), and this increase approached significance in the whole DRD (P = 0.84; Figure 6c) and rostral DRV (P = 0.75; Figure 6d), but only in animals that were exposed to AFR early in life. Additionally, social defeat
increased *tph2* mRNA expression in the rostral DRVL/VLPAG in both AFR and MS180 animals (Figure 6g), although this effect was not evident in the caudal or whole DRVL/VLPAG, suggesting functional differences between the rostral and caudal DRVL/VLPAG. Animals exposed to maternal separation, compared to normal animal facility rearing rats, showed reduced *tph2* expression in the caudal DRVL/VLPAG (Figure 6h) and this effect approached significance in the whole DRVL/VLPAG (*P* = 0.052; Figure 6i). Among animals exposed to social defeat, AFR rodents, compared to MS180 rodents, had elevated *tph2* mRNA expression in the caudal (Figure 6b) and whole DRD (Figure 6c), and caudal (Figure 6h) and whole DRVL/VLPAG (Figure 6i), and the DRI (Figure 6j).
Figure 6. Interaction between early life experience and adult social defeat on tph2 mRNA expression in subregions of the dorsal raphe nucleus. Bar graphs illustrate mean density x area of tph2 mRNA expression in the rostral (a, d, and g), caudal (b, e, and h), and the combined rostral and caudal (c, f, and i) portions of the DRD (a, b, and c), DRV (d, e, and f), DRVL/VLPAG (g, h, and i), and DRI (j) in animals exposed to either maternal separation or normal animal facility rearing early in life and either social defeat stress or novel cage control condition in adulthood. Abbreviations: AFR, normal animal facility rearing; MS180, daily maternal separation for 180 min; Control, novel cage control condition; SD, social defeat; DRI, dorsal raphe nucleus, interfascicular part; see Figure 4 for remaining subregion abbreviations. Data were analyzed using Fisher’s Protected Least Significant Difference (PLSD) post hoc tests comparing AFR/Control (n = 7), AFR/SD (n = 7), MS180/Control (n = 16), and MS180/SD (n = 9) groups and presented as means ± S.E.M. *P ≤ 0.01, **P ≤ 0.01, ~P ≤ 0.10.

In contrast to an interaction between early life experience and adult treatment on tph2 mRNA expression in specific subdivisions of the DR, the linear mixed model analysis of slc6a4 mRNA expression in rostral and caudal parts of each subdivision revealed only an adult treatment main effect (F(1,36.522) = 7.081, P ≤ 0.05) and a trend towards a significant interaction between adult treatment and subregion (F(5,37.558) = 2.283, P = 0.066). There was no significant interaction between early life experience, adult treatment and subregion (F(5,37.558) = 1.085, P = 0.384; Figure 7). At the level of individual subregions (i.e., combined rostral and caudal portions of each subregion), the linear mixed model provided similar results with a strong adult treatment main effect (F(1,38.82) = 8.042, P ≤ 0.01), an interaction between adult treatment and subregion (F(2,38.821) = 4.546, P ≤ 0.05), and a trend towards a significant interaction between early life experience and subregion (F(2,38.821) = 2.516, P = 0.094). Again, the interaction between early life experience, adult treatment and subregion failed to meet significance (F(2,38.821) = 1.663, P = 0.203; Figure 7). In agreement, statistical analysis of the whole DR with all three subregions (e.g., DRD, DRV, and DRVL/VLPAG) averaged together revealed a strong main effect of adult treatment on slc6a4 mRNA expression (F(1,39) = 7.95, P ≤ 0.01).
Figure 7. Interaction between early life experience and adult social defeat on \textit{slc6a4} mRNA expression in subregions of the dorsal raphe nucleus. Bar graphs illustrate mean density x area of \textit{slc6a4} mRNA expression in the rostral (a, d, and g), caudal (b, e, and h), and the combined rostral and caudal (c, f, and i) portions of the DRD (a, b, and c), DRV (d, e, and f), and DRVL/VLPAG (g, h, and i) in animals exposed to either maternal separation or normal animal facility rearing early in life and followed by either social defeat stress or novel cage control condition in adulthood. Abbreviations: AFR, normal animal facility rearing; MS180, daily maternal separation for 180 min; Control, novel cage control condition; SD, social defeat; see Figure 4 for subregion abbreviations. Data were analyzed using Fisher's Protected Least Significant Difference (PLSD) post hoc tests comparing AFR/Control (n = 7), AFR/SD (n = 7), MS180/Control (n = 16), and MS180/SD (n = 9) groups and presented as means ± S.E.M.
Even though there was only a trend towards a significant interaction between adult treatment and subregion \( F_{(5, 37,558)} = 2.283, P = 0.066 \) on \( slc6a4 \) expression in rostral and caudal portions of each subregion, post hoc comparisons were conducted because the a priori hypothesis was that adult treatment effects would be limited to specific DR subregions or rostrocaudal portions of each subregion and a posteriori inspection of the data revealed possible differences among means in rostral and caudal portions of specific DR subregions (Wilcox, 1987; Winer et al., 1991). A Fisher’s PLSD post hoc analysis of the effects of adult treatment only (i.e., without regard to early life experience) on \( slc6a4 \) expression in DR subregions revealed a social defeat stress-induced increase in \( slc6a4 \) mRNA within the rostral DRV \( (t_{37} = -2.36, P \leq 0.05; \text{Figure 8d}) \), whole DRV \( (t_{37} = -2.273, P \leq 0.05; \text{Figure 8f}) \), and a trend towards significance in the whole DRD \( (t_{37} = -1.728, P = 0.092; \text{Figure 8c}) \).

Figure 8. Social defeat stress, relative to novel cage control, increases \( slc6a4 \) mRNA expression in select subregions of the dorsal raphe nucleus. Bar graphs demonstrate mean density \( \times \) area of \( slc6a4 \) mRNA expression in the rostral (a and d), caudal (b and e), and the combined rostral and caudal (c and f) portions of specific DR subregions.
caudal (c and f) portions of the DRD (a, b, and c) and DRV (d, e, and f) in animals exposed to either social defeat stress or novel cage control conditions in adulthood, without considering early life rearing conditions. Abbreviations: Control, novel cage control condition; SD, social defeat; see Figure 4 for subregion abbreviations. Data were analyzed using independent Student’s t-tests comparing Control (n = 23) with SD (n = 16) rats and presented as means ± S.E.M. *P ≤ 0.01, ~P ≤ 0.10.

3.3. tph2 and slc6a4 mRNA expression within the MnR

Unlike the DR, adverse early life experience followed by social defeat in adulthood had little effect on gene expression in the MnR. The linear mixed model analysis of the rostral and caudal portions of the MnR revealed a trend towards reduced tph2 mRNA expression in maternally separated animals (i.e., main effect of early life experience, $F_{(1, 39)} = 3.675$, $P = 0.063$). Consistently, analysis of the combined rostral and caudal levels of the MnR showed a similar trend towards a significant main effect of early life experience ($F_{(1, 39)} = 3.935$, $P = 0.054$; Figure 9a) and no effect of adult treatment ($F_{(1, 39)} = 0.203$, $P = 0.654$; Figure 9b) or the interaction between early life experience and adult treatment ($F_{(1, 39)} = 0.196$, $P = 0.66$; Figure 9c) on tph2 mRNA expression. The were no significant main effects of early life experience ($F_{(1, 38)} = 0.896$, $P = 0.35$; Figure 9d), adult treatment ($F_{(1, 38)} = 0.522$, $P = 0.475$; Figure 9e) or interactions between early life experience and adult treatment ($F_{(1, 38)} = 1.161$, $P = 0.212$; Figure 9f) on slc6a4 mRNA expression in the whole MnR or rostral and caudal components of the MnR (data not shown).
Figure 9. Gene expression in the MnR is relatively unaltered by early life experience, adult treatment, and their interaction. Bar graphs depict the effects of early life experience (a and d), adult treatment (b and e), and the interaction between early life stress and adult treatment (c and f) on tph2 mRNA (a, b, and c) and slc6a4 mRNA (d, e, and f) expression in the entire median raphe nucleus. See Figure 6 for treatment abbreviations. Data were analyzed using independent Student’s t-tests comparing AFR (n = 14 for tph2; n = 13 for slc6a4) with MS180 (n = 25) rats or comparing Control (n = 23 for tph2; n = 22 for slc6a4) with SD (n = 16) rats and Fisher’s Protected Least Significant Difference (PLSD) post hoc tests comparing AFR/Control (n = 7 for tph2; n = 6 for slc6a4), AFR/SD (n = 7 for both tph2 and slc6a4), MS180/Control (n = 16 for both tph2 and slc6a4), and MS180/SD (n = 9 for both tph2 and slc6a4) groups. Data are presented as means ± S.E.M. ∼P ≤ 0.10.

3.4. Plasma corticosterone concentrations following social defeat or novel cage control

The linear mixed model analysis found no significant effects of early life experience (F(1, 39) = 1.711, P = 0.199; data not shown), adult treatment (F(1, 39) = 0.042, P = 0.838; data not shown) or interactions between early life experience and adult treatment (F(1, 39) = 0.022, P = 0.884; Figure 9).
on plasma corticosterone levels in either AFR or MS180 rats exposed to either novel cage control or social defeat in adulthood.

Figure 10. Bar graphs illustrate plasma corticosterone concentrations (ng/mL) following exposure to either novel cage control or social defeat in adulthood in animals that previously received either normal animal facility rearing or 180 min maternal separation early in life. See Figure 6 for abbreviations. Data were analyzed with a linear mixed model comparing AFR/Control (n = 7), AFR/SD (n = 7), MS180/Control (n = 16), and MS180/SD (n = 9) groups and presented as means ± S.E.M.

3.5. Correlations between tph2 mRNA expression and social defeat behavior

Pearson product-moment correlation tests conducted between tph2 mRNA expression in specific rostrocaudal subregions of the DR and behavior during social defeat revealed that tph2 expression in the caudal DRD was correlated with the frequency (Figure 11a) and duration (Figure 11b) of rearing (e.g., explorative escape) during the pre-defeat phase. Similarly, tph2 expression in the caudal DRVL/VLPAG was correlated with the frequency and duration of rearing behavior during both the pre-defeat (Figure 11c,d) and defeat phases (Figure 11e,f) of social defeat.
4. Discussion

Maternal separation shifted behavioral coping strategies during social defeat in adulthood toward a more reactive coping style, in association with an altered pattern of stress-induced increases in \textit{tph2} mRNA within subregions of the DR. During social defeat MS180 rats responded with a more reactive (passive-submissive) coping strategy, characterized by increased anxiety- and fear-like behavior such as freezing, and decreased proactive coping behaviors such as rearing. Consistent with previous studies of adverse early life experience (Lukkes et al., 2013), maternal separation decreased \textit{tph2} mRNA expression selectively in the caudal DRVL/VLPAG under baseline conditions, suggesting long-term effects of adverse early life experience on baseline \textit{tph2} mRNA expression in this subset of DR serotonergic neurons. Also consistent with previous studies, social defeat increased \textit{tph2} mRNA expression within the rostral DRVL/VLPAG, a region thought to promote reactive emotional coping responses; this effect was observed in both AFR control rats and MS180 rats, whereas in previous studies this effect was only observed in MS180 rats (Gardner et al., 2009b). In addition, AFR control rats, but not MS180 rats, responded to social defeat with increased \textit{tph2} mRNA expression in the caudal DRD, a region associated with conflict anxiety These data, taken together, suggest that adverse early life experience programs \textit{tph2} mRNA expression in subregions that mediate adaptive coping responses such as the DRVL/VLPAG and DRD, and these altered patterns of gene expression may limit maternally separated rats from mounting adaptive behavioral (e.g., proactive coping) responses to social defeat in adulthood.
Similar to a previous study in our laboratory using the same maternal separation model (Gardner et al., 2005), rodents exposed to maternal separation, relative to rodents that received normal animal facility rearing conditions, responded during social defeat in adulthood with more reactive coping, characterized by increased anxiety- and fear-related behaviors, and less proactive coping, characterized by confrontation, escape, and exploration behaviors. Indeed, both studies observed that rodents exposed to 180 min daily maternal separation during a critical period of development (PND2-14), showed a decrease in the duration of proactive coping as well as an increase in the ratio of both the frequency and duration of reactive (passive-submissive) to proactive coping (Gardner et al., 2005). In the current study, the shift towards a more reactive coping style during the defeat phase of social defeat observed in MS180 rats was primarily due to increased freezing and decreased rearing, behavioral effects that showed similar trends in the study conducted by Gardner et al., (2005). Also consistent with Gardner and colleagues (2005), MS180 rats, compared with AFR rats, showed a robust decrease in rearing behavior (also known as explorative escape, De Boer and Koolhaas, 2003), a proactive behavior with both exploration and flight/escape components, during the pre-defeat phase of social defeat. Together, these studies confirm that maternally separated rodents respond with altered patterns of behavior that result in more reactive coping and less proactive coping during social defeat in adulthood.

The shift towards a more reactive (passive-submissive) coping strategy observed during social defeat in adulthood in rodents with a history of maternal separation appears to generalize to other stressors such as the forced swim test. Rodents exposed to the forced swim test, a mild stressor classically used to test for the efficacy of putative antidepressants, cope with this stressor by engaging in proactive coping behaviors such as swimming, climbing, and diving as
well as reactive behaviors like immobility (i.e., floating). Interestingly, rodents exposed to 180 min of daily maternal separation during the first two weeks of life, relative to normal animal facility reared rats, respond to this task in adulthood with greater immobility (Dimatelas et al., 2012; Lambas-Senas et al., 2009; Lee et al., 2007; Veenema et al., 2006), indicating a switch towards a reactive coping strategy, and reduced swimming (Veenema et al., 2006), suggesting a shift away from proactive coping. Maternally separated rodents also display increased anxiety-related behaviors in the elevated plus-maze (Benekareddy et al., 2011; Daniels et al., 2004; Lee et al., 2007; Li et al., 2013; Wigger and Neumann, 1999), open-field (Benekareddy et al., 2011; Lambas-Senas et al., 2009; Ogawa et al., 1994; Rentesi et al., 2010), and novelty-induced suppression of feeding (Caldji et al., 2000), all consistent with the increased anxiety- and fear-related behavior observed in rodents engaged in reactive coping. Taken together, maternal separation appears to make rodents more susceptible to developing anxiety- and depression-like coping responses later in life and these changes in behavior may be related to early life stress-induced changes in serotonergic gene expression.

Maternal separation resulted in decreased basal tph2 mRNA expression in a subset of serotonergic neurons, particularly in the caudal DRVL/VLPAG. This programming of tph2 mRNA expression in a specific subregion of the DR by adverse early life experience is consistent with evidence that another form of adverse early life experience, social isolation during early adolescence. Specifically, social isolation during early adolescence decreases tph2 mRNA expression in the DRVL/VLPAG and caudal DRV (Lukkes et al., 2013), a subregion that showed a similar trend in the current study. Similarly, rats exposed to postnatal dual immune challenges on PND 3 and 5 with lipopolysaccharide have altered tph2 mRNA expression selectively in the DRVL/VLPAG on PND14 (Sidor et al., 2010) and this early life dual immune challenge increases emotional reactivity and anxiety-like behavior (Walker et al., 2004; Walker et al.,
2008). These disparate forms of adverse early life experience that all alter \( tph2 \) mRNA expression selectively in DRVL/VLPAG serotonergic neurons and increase anxiety-like behavioral responses, suggests adverse early life experience in general may produce long lasting changes in DRVL/VLPAG serotonergic neurons, which may lead to increased behavioral reactivity and emotionality. Our results have suggested in the case of maternal separation this adverse early life experience-induced alteration in \( tph2 \) mRNA expression localizes to the caudal portion of the DRVL/VLPAG. The caudal portion of the DRVL and the adjacent VLPAG contain a subpopulation of serotonergic neurons implicated in inhibiting proactive coping responses and panic-like behaviors, and instead facilitating behavioral quiescence, hyporeactivity, bradycardia, hypotension, and opioid-induced analgesia in response to stress (Johnson et al., 2004;Keay and Bandler, 2001;Paul and Lowry, 2013).

Neuroanatomical tracing studies reveal caudal DRVL/VLPAG serotonergic neurons send multisynaptic projections to both the sympathectomized gastrocnemius skeletal muscle as well as the adrenal gland, suggesting these serotonergic neurons are ideally positioned to control both motor and sympathetic output in response to threatening events (Kerman et al., 2006). Serotonergic neurons originating in the caudal DRVL/VLPAG can also inhibit fight-or-flight behaviors and panic-like responses through projections to the dorsal periaqueductal gray (DPAG; Beitz, 1982;Cassell et al., 1986a;Cassell et al., 1986b;Jansen et al., 1998;Stezhka and Lovick, 1997;Stezhka and Lovick, 1994;Vertes, 1991). The DPAG is a longitudinal structure divided into functionally distinct rostral and caudal portions, both of which produce different patterns of proactive coping behavior, with the former eliciting a confrontation defense reaction (e.g., upright defensive behavior) and the latter an escape or flight reaction (e.g., rearing). The accompanied sympathetic response consists of hypertension and tachycardia with changes in blood flow to match the requirements of the particular behavioral response. The rostral DPAG
diverts blood flow to the skin and skeletal muscles of the face (extracranial vasodilation) and away from the hindlimb skeletal muscle and viscera (hindlimb and renal vasoconstriction), and initiates non-opioid analgesia in preparation to confront the threat; conversely, the caudal DPAG evokes extracranial and renal vasoconstriction, hindlimb vasodilation, and non-opioid analgesia in order to prepare for escape/flight behaviors (Keay and Bandler, 2001). This arrangement has led to the hypothesis that the DRVL/VLPAG and DPAG have opposing roles in mediating reactive (passive-submissive) and proactive coping responses, respectively. Considering the role of the DRVL/VLPAG in mediating reactive coping, the altered baseline tph2 mRNA expression in caudal DRVL/VLPAG serotonergic neurons may be important for the switch to a reactive coping strategy.

Because the behavioral and sympathetic responses evoked by DPAG stimulation are markedly similar to the symptoms of a panic attack (Deakin and Graeff, 1991; Graeff, 2004; Jenck et al., 1995; Schenberg et al., 2001), dysfunction of a serotonergic DRVL/VLPAG-DPAG panic inhibition circuit may be involved in the etiology and pathophysiology of panic disorder (Deakin and Graeff, 1991; Graeff et al., 1996; Graeff, 2004; Johnson et al., 2004; Keay and Bandler, 2001; Paul and Lowry, 2013). Support for this hypothesis comes from studies reporting that DRVL/VLPAG serotonergic neurons respond to panicogenic stimuli and that dysfunction of these neurons may increase vulnerability to panic-like responses. Serotonergic neurons in the DRVL/VLPAG are sensitive to acidosis (i.e., increased H+) due to elevated arteriole pCO₂ (Guyenet et al., 2013; Hodges and Richerson, 2010; Richerson, 2004) and are selectively activated by the panicogenic agents, sodium lactate and CO₂ (Johnson et al., 2008; Johnson et al., 2005; Johnson et al., 2004), which can induce panic attacks in humans with panic disorder (Amaral et al., 2013; Gorman et al., 1989; Griez and Schruers, 1998; Guttmacher et al., 1983; Kellner, 2011; Klein, 1993; Pitts, Jr. and McClure, Jr., 1967) and panic-like behavioral
and physiological responses in panic-prone rodents (Johnson et al., 2004; Johnson et al., 2008). Using a rodent model of panic disorder in which chronic disinhibition of GABAergic neurons in the dorsomedial hypothalamus produces panic-prone rodents that are vulnerable to panic-like behavioral and cardiorespiratory responses following exposure to panicogenic agents, we have reported that sodium lactate activates DRVL/VLPAG serotonergic neurons in naïve rats who do not panic, but fails to activate the DRVL/VLPAG in panic-prone rats who display panic-like responses. This attenuated functional cellular response of DRVL/VLPAG serotonergic neurons to sodium lactate in panic-prone rats suggests that this subset of serotonergic neurons normally inhibits panic-like behavioral and physiological responses. Likewise, in another rodent model of chronic anxiety and panic vulnerability, priming of the basolateral amygdala by repeated injections of the stress-related neuropeptide, urocortin 1, results in increased basal anxiety, and heightened sensitivity to panicogenic agents (Rainnie et al., 2004; Sajdyk et al., 1999), and selectively alters tph2 expression in the DRVL/VLPAG (Donner et al., 2012a).

The reduced basal tph2 mRNA expression observed in maternally separated rats in the current study could represent an overall reduction in serotonin turnover in this subset of caudal DRVL/VLPAG serotonergic neurons and consequently projection regions such as the caudal DPAG, which would be predicted to increase vulnerability to panicogenic agents and consequently panic-like behavioral and physiological responses. Several lines of evidence in both rodents and humans suggest that adverse early life experiences result in respiratory abnormalities, which are risk factors for panic disorder and panic attacks (Abelson et al., 2001; Goodwin and Pine, 2002; Martinez et al., 2001; Simon and Fischmann, 2005). For example, adverse early life experience alters the development of respiratory control as measured by greater hypoxic ventilatory responses in maternally separated male rats (Genest et al., 2007). Female rats exposed to maternal separation display a 63% greater ventilatory response to
hypercapnia (i.e., 5% CO$_2$; Dumont et al., 2011), which is consistent with epidemiological evidence showing increased prevalence of panic disorder in females (Gater et al., 1998; Kessler et al., 2005). An unstable maternal environment produced by cross-fostering mouse pups following birth produces a number of respiratory abnormalities indicative of heightened CO$_2$ sensitivity, including increased hypercapnic (i.e., 6% CO$_2$) ventilatory responses and avoidance of CO$_2$-enriched environments (D’Amato et al., 2011). Importantly, in all of these studies none of these respiratory abnormalities are observed in control rodents raised in a normal animal facility rearing environment. Likewise, in humans, there are strong associations between childhood separation anxiety, adverse early life experiences resulting from parental separation (e.g., parental loss) or unstable environments, and respiratory abnormalities (e.g., heightened CO$_2$ sensitivity), all of which increase susceptibility to developing panic disorder or panic attacks (Battaglia et al., 1995; Battaglia et al., 2009; Roberson-Nay et al., 2010; Spatola et al., 2011). An important direction for future research is to determine if the effects of adverse early life experience on CO$_2$ hypersensitivity may be mediated by long-lasting changes in chemosensitive serotonergic neurons located in the DRVL/VLPAG.

Both AFR and MS180 rats displayed increased tph2 mRNA expression within the rostral DRVL/VLPAG following social defeat in adulthood, a pattern consistent with the stress-induced increase in tph2 mRNA observed in a previous study, although this effect was only apparent in MS180 rats (Gardner et al., 2009b). The rostral DRVL/VLPAG, similar to the caudal DRVL/VLPAG, sends projections to other longitudinal columns of the central gray, including the DPAG (Beitz, 1982; Cassell et al., 1986a; Cassell et al., 1986b; Jansen et al., 1998; Stezhka and Lovick, 1997; Stezhka and Lovick, 1994; Vertes, 1991). It is unclear whether the rostral DRVL/VLPAG preferentially innervates the rostral DPAG, however, under the assumption that it does, a stress-induced increase in tph2 mRNA expression in the rostral DRVL/VLPAG would be
predicted to preferentially increase serotonergic activity in the rostral portion of the DPAG and consequently inhibit behavioral and sympathetic responses evoked by the rostral DPAG. The rostral DPAG, as mentioned above, facilitates proactive coping responses that are directed towards confronting and facing the threat, rather than the caudal DPAG-evoked escape/fleeing (Keay and Bandler, 2001). Consistent with this arrangement, both AFR and MS180 rats exposed to social defeat had increased \textit{tph2} mRNA within the rostral DRVL/VLPAG and consequently no differences in upright defensive behavior and aggression, confrontational behaviors thought to be mediated by the rostral DPAG. Taken together with the observation that MS180 rats, relative to AFR rats, had decreased basal \textit{tph2} expression in the caudal DRVL/VLPAG as well as altered rearing behavior, an escape/flight behavior presumably dependent on the caudal DPAG, these data suggest a functional difference between the rostral and caudal portions of the DRVL/VLPAG. Depending on the nature of the threat, the rostral DRVL/VLPAG would be recruited and preferentially inhibit behavior and sympathetic responses generated by the rostral DPAG (e.g., confrontational defense reaction), whereas if a stressor activated the caudal DRVL/VLPAG, then caudal DPAG-elicited responses (e.g., escape/flight) would be inhibited.

In addition to social defeat altering patterns of \textit{tph2} mRNA expression in the DRVL/VLPAG, social defeat elevated \textit{tph2} mRNA expression in the DRD, a subregion involved in facilitating conflict anxiety, but, unexpectedly, this effect was only observed in AFR rats. This increased \textit{tph2} mRNA expression in the DRD might be due to altered behavior during the pre-defeat phase when conflict anxiety would be most evident. Rats raised in AFR conditions, relative to MS180 rats, displayed more self-grooming behavior during the pre-defeat phase of social defeat, a behavior that is associated with anxiety-like behavior and may serve as a behavioral index for stress and anxiety levels (Ferre et al., 1995; Kalueff and Tuohimaa,
Consistent with self-grooming being associated with stress and anxiety, self-grooming behaviors are dependent on levels of the stress-related neuropeptide, corticotropin-releasing hormone (Aubry et al., 1995; Castanon and Mormede, 1994), and are elicited by activation of the HPA axis (Gispen et al., 1975; Gispen and Isaacson, 1981) and a variety of stressors, including social defeat (Spruijt et al., 1992; van Erp et al., 1994). There is evidence that serotonergic systems are involved in the expression of grooming behaviors as stimulation of 5-HT$_{2C}$ receptors evokes excessive self-grooming behavior (Bagdy et al., 1992) and blockade of 5-HT$_{2C}$ receptors attenuates the excessive self-grooming behavior elicited by central administration of the stress- and arousal-related neuropeptide, orexin-A/hypocretin-1 (Duxon et al., 2001). Likewise, mice with a genetic deficiency in the serotonin transporter (heterozygous SERT+/-), which increases anxiety-like behaviors and emotional reactivity to stress (Murphy and Lesch, 2008), also display increased grooming behaviors (Kyzar et al., 2012). Together, these data suggest that the increased tph2 mRNA expression in DRD serotonergic neurons may be associated with the increased self-grooming observed in AFR rats during the pre-defeat phase of social defeat.

Early life stress failed to alter the basal expression of slc6a4 mRNA expression in subregions of the DR; however, social defeat, independent of early life experience, increased slc6a4 mRNA expression in the rostral DRV. The lack of interaction between maternal separation and social defeat on slc6a4 mRNA expression was surprising because previous studies have revealed that social defeat alters slc6a4 mRNA expression selectively in the DRVL/VLPAG subregion, but only in maternally separated rats (Gardner et al., 2009a). Likewise, in a sample of human depressed subjects, decreased binding potential of the serotonin transporter is associated with a history of childhood abuse (Miller et al., 2009). Genetic polymorphisms in the serotonin transporter are associated with vulnerability to stress,
including adverse early life experiences, and the later development of stress-related disorders such as depression (Brown et al., 2013; Caspi et al., 2003; Grabe et al., 2005), although a meta review suggests many studies have failed to replicate these findings or have only partially replicated them (Risch et al., 2009). However, our findings are consistent with evidence that adolescent social isolation, another model of adverse early life experience, fails to alter \textit{slc6a4} mRNA expression in DR subregions (Lukkes et al., 2013). Likewise, priming of the basolateral amygdala results in a chronic anxiety-like phenotype with susceptibility to panicogenic agents (Rainnie et al., 2004; Sajdyk et al., 1999), but fails to change \textit{slc6a4} mRNA levels in DR subpopulations of serotonergic neurons (Donner et al., 2012a). Together, these preclinical rodent models of stress- and anxiety-related disorders and genetic association studies in humans have provided mixed evidence in regards to the association of stressful life events and serotonin transporter function, highlighting an important area of research that needs to be resolved with future research.

**Adverse early life experience together with social defeat in adulthood failed to alter \textit{slc6a4} or \textit{tph2} expression in the MnR.** There was a nonsignificant trend for reduced \textit{tph2} mRNA expression in maternally separated rats due to lower basal levels. In contrary to evidence suggesting the MnR is involved in stress resilience (Andrade et al., 2013; Graeff et al., 1996; Paul and Lowry, 2013), these data suggest adverse early life experience and social defeat in adulthood selectively alter subpopulations of serotonergic neurons in the DR, but not the MnR. It is also possible that adverse early life experience or social defeat or both interacting together could perturb MnR serotonergic function through a different molecular target, for example the gene encoding the 5-HT$_{1A}$ receptor. In support of this, the post-mortem brains of depressed suicide victims show reduced 5-HT$_{1A}$ receptor binding (Arango et al., 2001).
Lastly, plasma corticosterone levels were unaltered in maternally separated animals, which is in contrast to other studies that have shown hyperactive HPA-axis responses to stress in maternally separated rodents (Plotsky and Meaney, 1993). This is most likely due to timing in the experimental design as rats were sacrificed 4 hrs after the onset of stress, a time point that is optimal for stress-induced alterations in serotonergic gene expression, but likely too late to detect stress-induced increases in plasma corticosterone.

5. Conclusions

Adverse early life experience results in long-lasting changes in *tph2* mRNA expression in subregions of the raphe important for behavioral and physiological responses to stress. These changes may underlie altered coping strategies observed during social defeat. Specifically, reduced basal *tph2* mRNA in the caudal DRVL/VLPAG, a region implicated in the inhibition of fight-or-flight responses, may result in increased reactive coping, although the underlying mechanism is unclear. This may also increase susceptibility to respiratory abnormalities associated with panic disorder. Overall, these data are consistent with the hypothesis that specific subpopulations of DR serotonergic neurons are targeted by adverse early life experience and social defeat in adulthood, and identify subregions potentially involved in different coping strategies. Altered basal and stress-induced patterns of serotonergic gene expression may limit maternally separated rats from mounting adaptive behavioral (e.g., proactive coping) responses to social defeat in adulthood.
Repeated social defeat increases reactive emotional coping behavior and alters functional responses in serotonergic neurons in the rat dorsal raphe nucleus

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Abstract

Chronic stress is a vulnerability factor for a number of psychiatric disorders, including anxiety and affective disorders. Social defeat in rats has proven to be a useful paradigm to investigate the neural mechanisms underlying physiologic and behavioral adaptation to acute and chronic stress. Previous studies suggest that serotonergic systems may contribute to the physiologic and behavioral adaptation to chronic stress, including social defeat in rodent models. In order to test the hypothesis that repeated social defeat alters the emotional behavior and the excitability of brainstem serotonergic systems implicated in control of emotional behavior, we exposed adult male rats either to home cage control conditions, acute social defeat, or social defeat followed 24 h later by a second social defeat encounter. We then assessed behavioral responses during social defeat as well as the excitability of serotonergic neurons within the dorsal raphe nucleus using immunohistochemical staining of tryptophan hydroxylase, a marker of serotonergic neurons, and the protein product of the immediate-early gene, c-fos. Repeated social defeat resulted in a shift away from proactive emotional coping behaviors, such as rearing (explorative escape behavior), and toward reactive emotional coping behaviors such as freezing. Both acute and repeated defeat led to widespread increases in c-Fos expression in serotonergic neurons in the dorsal raphe nucleus. Changes in behavior following a second exposure to social defeat, relative to acute defeat, were associated with decreased c-Fos expression in serotonergic neurons within the dorsal and ventral parts of the mid-rostrocaudal dorsal raphe nucleus, regions that have been implicated in 1) serotonergic modulation of fear- and anxiety-related behavior and 2) defensive behavior in conspecific aggressive encounters, respectively. These data support the hypothesis that serotonergic systems play a role in physiologic and behavioral responses to both acute and repeated social defeat.
1. Introduction

Chronic stress has been identified as an important vulnerability factor for a number of psychiatric disorders, including anxiety disorders such as panic disorder and post-traumatic stress disorder, and affective disorders such as major depressive disorder. The mechanisms through which chronic stress increases vulnerability to anxiety and affective disorders are unclear.

Social defeat, which is the result of intraspecific confrontation between male rats, is an ethologically relevant paradigm that can be used to understand the physiologic and behavioral adaptations to repeated stress. Rats and Syrian hamsters that have experienced a single social defeat display changes in neuroendocrine, autonomic, and behavioral responses (Herbert, 1987; Raab et al., 1986), including increases in fear- and anxiety-like behaviors (Markham et al., 2010a; Markham et al., 2009a; Razzoli et al., 2006a). Rats exposed to repeated social defeat (i.e., 2 exposures to social defeat 24 h apart) also respond with increases in anxiety-like behaviors (Kinn et al., 2008), but also hippocampal dendritic reorganization (Kole et al., 2004), decreased food intake and body weight gain (Meerlo et al., 1996) and altered sleep patterns (Kinn et al., 2008). Rats exposed to chronic social defeat, when compared to controls, (i.e., 4-7 daily exposures to social defeat) respond with long-lasting depressive-like behaviors (Hollis et al., 2010), changes in defensive behaviors (Tornatzky and Miczek, 1993), and long-term impairment of autonomic circadian rhythms (Tornatzky and Miczek, 1993). One mechanism through which repeated or chronic social defeat may elicit these physiologic and behavioral adaptations is through altered activity of brainstem neuromodulatory systems, such as serotonergic systems.
Previous studies support a role for serotonergic systems in physiologic and behavioral adaptations following social defeat. A single exposure to social defeat increases serotonergic neuronal activity, as evidenced by increases in expression of the protein product of the immediate-early gene, c-fos (Cooper et al., 2008b; Gardner et al., 2005), and increases in extracellular serotonin within the dorsal raphe nucleus (DR) (Amat et al., 2010). Studies by Herbert and colleagues suggest that both acute and chronic social defeat equally increase c-Fos expression within the DR; however, it is unknown whether these increases are in serotonergic neurons and if they are specific to subregions of the DR (Martinez et al., 1998; Martinez et al., 2002).

A single exposure to social defeat has been found to selectively activate serotonergic neurons in the dorsal part of the mid-rostrocaudal and caudal dorsal raphe nucleus (mid-rostrocaudal DRD and DRC) (Gardner et al., 2005). Serotonergic neurons in the DRD and DRC have been shown to be activated following exposure to a number of fear- and anxiety-related stimuli, including anxiogenic drugs such as the adenosine receptor antagonist caffeine, the serotonin 5-HT\textsubscript{2A/2C} receptor agonist m-chlorophenyl piperazine (mCPP), and the partial inverse agonist at the benzodiazepine allosteric site on the \(\gamma\)-aminobutyric acid A (GABA\textsubscript{A}) receptor, \(N\)-methyl-beta-carboline-3-carboxamide (FG-7142) (Abrams et al., 2005), the anxiety-related neuropeptide urocortin 2 (Ucn2; Staub et al., 2005; Staub et al., 2006), and inescapable stress (Grahn et al., 1999). The responses of topographically organized subpopulations of serotonergic neurons following repeated exposure to social defeat have not been tested.

In order to test the hypothesis that behavioral adaptations following exposure to social defeat are associated with changes in the excitability of topographically organized populations of serotonergic neurons, we exposed rats to either a single social defeat, or social defeat
followed, 24 h later, by a second social defeat encounter. We then assessed, using immunohistochemical detection of the protein product of the immediate-early gene, c-fos, the functional excitability of serotonergic neurons within topographically organized subregions of the DR, the source of the majority of serotonergic projections to forebrain limbic structures regulating fear and anxiety states.

2. Materials and methods

2.1. Subjects

Male Long Evans rats (Harlan Laboratories, Indianapolis, IN, USA; 238-298 g, mean ± S.E.M., 271 ± 1.28 g) were housed in groups of 3 in cages (38 cm W x 48 cm L x 21 cm H; Techniplast cages, Techniplast, Kettering, UK) containing a thin layer of bedding (Cat. No. 7090; Teklad Sani-Chips; Harlan Laboratories). Rats were maintained on a 12 h light/12 h dark cycle (lights on at 0700 h) with free access to food (Cat. No. 8640; Teklad 22/5 Rodent diet, Harlan Laboratories) and tap water stored in 16 oz reduced-height water bottles (Cat. No. WB16RH; Alternative Designs, Siloam Springs, AR, USA) with screw lids (Cat. No. FSPCST2.5; AnCare Corp., Bellmore, NY, USA). Male Long Evans retired breeders (Harlan Laboratories, 411-598 g, mean ± S.E.M., 511 ± 18.2 g) were used as resident males for the social defeat. Residents were singly housed in transparent polycarbonate cages (26 cm W x 47.6 cm L x 20.3 cm H; Cat. No., RC88D-PC, Alternative Designs) and maintained as described above. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC).
2.2. Social defeat procedure

Following two days of acclimation, rats were exposed to either no defeat (home cage control, HCC) on days 1 and 2, a social defeat encounter on day 1 (acute defeat, AD) or social defeat encounters, separated by 24 h, on both day 1 and day 2 (repeated defeat, RD). Social defeat occurred during the light phase (~450 lux) at 0800 hr, one hour following lights on. It is common for social defeat experiments to be conducted during either the light phase (Berton et al., 2006; Gardner et al., 2005; Nikulina et al., 1998) or the dark phase on a reversed light/dark cycle, usually under a dim red light (Chung et al., 1999; Cooper et al., 2009b; Kollack-Walker et al., 1997; Kollack-Walker et al., 1999; Martinez et al., 1998). Social defeat encounters lasted for 20 min and consisted of both a pre-defeat phase (10 min) and defeat phase (10 min). The social defeat encounters occurred in the resident male’s home cage. During the pre-defeat phase the resident and intruder were separated by a transparent 0.3 cm-wide Plexiglas® partition with 9 (0.3 cm diameter) holes drilled 5 cm apart in it so that physical contact was prevented, but visual, auditory and olfactory cues remained. For the defeat phase, the partition was removed allowing the rats to freely interact. The behavior of the intruder and resident was recorded with a digital video camera (Sony Handycam DCR-HC52 and DCR-HC35E, Sony Corporation of America, New York, NY, USA) mounted on a tripod and later quantified “off-line” using Noldus, The Observer (Version 5, Noldus Information Technology, Wageningen, The Netherlands) by an experimenter blind to treatment group. Home cage control rats consisted of two groups (home cage control 1 and home cage control 2), which were time matched for perfusion with fixative in preparation for immunohistochemical procedures (see below) with AD rats on day 1 and RD rats on day 2, respectively. No differences in cell counts were observed
between home cage control 1 and home cage control 2 groups (see results below), therefore both groups were merged into one group called home cage control (HCC). Home cage control rats were transferred to an adjacent room 1 hr prior to the social defeat period (3 hrs prior to transcardial perfusion), weighed, and returned to their home cages where they remained during the social defeat period for time-matched rats exposed to social defeat.

2.3. Behavioral analysis

The specific behaviors that were scored during both the pre-defeat and defeat phases were based on previous work by Gardner et al. 2005 (Gardner et al., 2005) (Table 1). During the defeat phase the style of behavioral coping was further divided into reactive coping, proactive coping and neutral behaviors (Table 1). Reactive coping includes sniffing bedding, freezing, full submission, sideways submission passive genital sniff and genital sniff. Proactive coping includes rearing, defensive burying, aggression, escape, upright defensive behavior and social interaction. Finally, neutral behaviors consisted of locomotion, self-grooming and inactivity.
2.4. Tissue preparation

Two hours following the onset of social defeat, rats were deeply anesthetized with sodium pentobarbital (Fatal-Plus, MWI Veterinary Supply, Meridian, ID, USA; 200 mg/kg, intraperitoneal (i.p.)) and transcardially perfused with ice-cold 0.05 M phosphate-buffered saline (PBS, pH 7.4) followed by ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) containing 1.5% sucrose. The brains were dissected out and post-fixed overnight in the same fixative, followed by two 12 h washes in 0.1 M PB and then switched to 0.1 M PB containing 30% sucrose for 2-3 days until saturated. Brains were blocked into forebrain and hindbrain sections by placing the brain into a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA) and bisecting the brain, in the coronal plane, with a razor blade directly caudal to the mammillary bodies (approximately –5.60 mm from bregma). Next, the forebrain
and hindbrain sections were flash-frozen with isopentane (cooled between –30 and –40 °C with dry ice) and stored at –80 °C until sectioning. Coronal tissue slices (30 µm) were prepared using a precision cryostat (Leica CM1900) and stored as six alternate sets of sections in 24-well tissue culture plates containing cryoprotectant (30% ethylene glycol, 20% glycerol, 0.05 M PB, pH 7.4) at –20 °C until further immunohistochemical staining.

2.5. Immunohistochemistry

Double-immunohistochemical staining for c-Fos and tryptophan hydroxylase (TPH) is described below. One set of sections, representing every sixth section throughout the DR from each rat was removed from cryoprotectant and washed twice in 0.05 M PBS for 15 min. Endogenous peroxidase activity was then neutralized in 0.05 M PBS containing 1% H₂O₂ (15 min), then sections were rinsed twice with 0.05 M PBS (15 min each time), rinsed with 0.05 M PBS containing 0.3% Triton X-100 (15 min) and subsequently incubated overnight at room temperature (RT) in rabbit anti-c-Fos 1° polyclonal antibody (Cat. No. PC38; Lot No. D00080180; Calbiochem (EMD Chemicals), Gibbstown, NJ, USA) diluted to 1:3000 with 0.05 M PBS containing 0.1% Triton X-100 and 0.01% sodium azide. The next day, the tissue was rinsed twice with 0.05 M PBS (15 min each time), then incubated for 90 min in biotinylated donkey anti-rabbit 2° antibody (Cat. No. 711-065-152; Lot No. 86689; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted to 1:3000 with 0.05 M PBS containing 0.1% Triton X-100 and 0.01% sodium azide. The next day, the tissue was rinsed twice with 0.05 M PBS (15 min each time), then incubated for 90 min in biotinylated donkey anti-rabbit 2° antibody (Cat. No. 711-065-152; Lot No. 86689; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted to 1:500 with 0.05 M PBS, washed twice with 0.05 M PBS (15 min each time), incubated for 90 min in an avidin-biotin-peroxidase complex (Elite ABC reagent, Cat. No. PK-6100; Vector Laboratories, Burlingame, CA, USA) diluted to 1:200 in 0.05 M PBS, washed twice with 0.05 M PBS (15 min) and finally reacted in a peroxidase substrate (Cat. No. SK4700; Vector SG chromogen kit; Vector
Laboratories, diluted as recommended by the vendor). Following the reaction, the tissue was washed twice in 0.05 M PBS (15 min each time), then placed in 0.05 M PBS containing 1% H$_2$O$_2$ (15 min) followed by two additional 0.05 M PBS washes (15 min each time). Next, the tissue was incubated overnight at RT in sheep anti-TPH 1° antibody (Cat. No. T8575; Lot No. 047K1223; Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:12,000 in 0.05 M PBS containing 0.1% Triton X-100 and 0.01% sodium azide. The next day, tissue was rinsed twice with 0.05 M PBS (15 min each time) and then incubated for 90 min in biotinylated rabbit anti-sheep 2° polyclonal antibody (Cat No. PK-6106; Vector Elite kit; Vector Laboratories) diluted to 1:200 with 0.05 M PBS. Following incubation in 2° antibody, tissue was rinsed twice in 0.05 M PBS (15 min each time), then incubated for 90 min in the avidin-biotin-peroxidase complex reagent diluted to 1:200 in 0.05 M PBS, rinsed twice in 0.05 M PBS (15 min each time), reacted with 0.01% 3-3’-diaminobenzidine tetrahydrochloride (DAB; Cat. No. D9015, Sigma-Aldrich) in 0.05 M PBS containing 0.005% H$_2$O$_2$, rinsed twice in 0.05 M PBS (15 min) and finally stored at 4 °C in 0.1 M PB containing 0.01% sodium azide. Tissue was then floated onto glass slides; after the tissue was air dried cover slips were mounted using Entellen mounting medium (Electron Microscopy Science, Hatfield, PA, USA) and cell counts were conducted as specified below.

2.6. Cell counts

The anatomical regions of the DR were identified using a stereotaxic rat brain atlas (Paxinos and Watson, 1998) and an atlas illustrating the distribution of TPH immunostaining throughout the rat DR (Abrams et al., 2004). Four rostrocaudal levels (−7.46 mm, −8.00 mm, −8.18 mm and −8.54 mm bregma; **Figure 1**) of the DR along with the corresponding subdivisions were selected for analysis. The subdivisions analyzed included: the dorsal raphe
nucleus, dorsal part (DRD) and dorsal raphe nucleus, ventral part (DRV) at −7.46 mm bregma; the DRD, DRV and dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG) at −8.00 mm bregma; the DRD, DRV, DRVL/VLPAG and dorsal raphe nucleus, interfascicular part (DRI) at −8.18 mm bregma; and the DRI and dorsal raphe nucleus, caudal part (DRC) at −8.54 mm bregma. Cell counts included the numbers of c-Fos-immunoreactive (c-Fos-ir) serotonergic neurons (c-Fos-ir/TPH-ir neurons; distinguished by a dark blue/black-stained nucleus located entirely within a light brown-stained cytoplasm), the numbers of c-Fos-ir non-serotonergic cells (c-Fos-ir/TPH-immunonegative cells; distinguished by a dark blue/black-stained nucleus) and total numbers of TPH-ir neurons (c-Fos-ir/TPH-ir neurons and c-Fos-immunonegative/TPH-ir neurons; identified by a light brown-stained cytoplasm). An experimenter blind to treatment group conducted the cell counts using bright-field microscopy with a 10× objective lens; c-Fos-ir/TPH-ir neurons were confirmed with a 40× objective lens.

Figure 1. Photomicrographs illustrating the rostrocaudal levels and subdivisions of the dorsal raphe nucleus sampled for analysis of immunohistochemical staining. Dashed lines delineate each subdivision. Numbers in the lower left of each panel indicate the distance from bregma, based on a standard stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). (A) −7.46 mm bregma, (B) −8.00 mm bregma, (C) −8.18 mm bregma, and (D) −8.54 mm bregma. Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part;
2.7.1. Statistical analysis of behavior

The behavioral data were separated into pre-defeat and defeat phases, and the frequency and duration of each behavior in acute and repeated defeat groups were compared using independent Student’s *t*-tests (PASW Statistics 17.0.2 for Macintosh, SPSS Inc., Chicago, IL, USA). Additionally, the behaviors during the defeat phase were grouped into behaviorally-related categories, including reactive coping behaviors, proactive coping behaviors and neutral behaviors, that were analyzed separately using independent Student’s *t*-tests.

Prior to statistical analysis, outliers were identified by Grubb’s test (Grubbs, 1969); outliers were excluded from further analysis and were not included in the tables and graphical representation of the data. For the pre-defeat phase data, 1 out of 161 data points for frequency were excluded (0.6% of total data) and 5 out of 161 data points for duration were excluded (3.1% of total data); for the defeat phase data, 13 out of 345 data points for frequency were excluded (3.8% of total data) and 13 out of 345 data points for duration were excluded (3.8% of total data).

2.7.2. Statistical analysis of cell counts for home cage groups
Prior to the analysis of cell counts, a two-factor analysis of variance (ANOVA) with repeated measures (PASW statistics), using day (two levels: home cage control 1 and home cage control 2) as the between-subjects factor and region (11 levels) as the within-subjects factor, was used to determine if home cage control 1 and home cage control 2 rats differed in the numbers of c-Fos-ir/TPH-ir neurons, c-Fos-ir/TPH-immunonegative cells and the total number of serotonergic neurons sampled. A Greenhouse-Geisser correction epsilon (ε) was used to correct for potential violation of the sphericity assumption.

Outliers were identified using the Grubb’s test (Grubbs, 1969) and excluded from further analysis. Any missing values were replaced using the Peterson method in order to run the repeated measures ANOVA (Petersen, 1985), but these values were not included in the post hoc analyses or the graphical representation of the data. The Grubb’s test analysis identified 9 outliers out of 308 data points (2.9%) for c-Fos-ir/TPH-ir cell counts, 10 outliers out of 308 data points (3.2%) for c-Fos-ir/TPH-immunonegative cell counts and 2 outliers out of 308 data points (0.6%) for the total number of serotonergic neurons sampled.

### 2.7.3. Statistical analysis of treatment effects for cell counts

Cell counts were analyzed using separate two-factor analysis of variance (ANOVA) with repeated measures (PASW statistics) on the numbers of 1) c-Fos-ir/TPH-ir neurons, 2) c-Fos-ir/TPH-immunonegative cells and 3) the total number of serotonergic neurons with treatment (three levels: acute social defeat, repeated social defeat and home cage control) as a between-subjects factor and region (11 levels) as a within-subjects factor. A Greenhouse-Geisser correction epsilon (ε) was used to correct for potential violation of the sphericity assumption.
When appropriate, post hoc analyses using Fisher’s Protected LSD tests (PASW statistics) were conducted.

Outliers were identified using the Grubb’s test (Grubbs, 1969) and excluded from further analysis. Any missing values were replaced using the Peterson method in order to run the repeated measures ANOVA (Petersen, 1985), but these values were not included in the post hoc analyses or the graphical representation of the data. The Grubb’s test analysis identified 11 outliers out of 539 data points (2.0%) for c-Fos-ir/TPH-ir cell counts, 9 outliers out of 539 data points (1.7%) for c-Fos-ir/TPH-immunonegative cell counts and 2 outliers out of 539 data points (0.4%) for the total number of serotonergic neurons sampled.

2.7.4 Statistical analysis of correlations

Both the frequency and duration of individual behaviors that were different between acute and repeated social defeat subjects were correlated with numbers of c-Fos-ir/TPH-ir neurons in specific subregions of the DR, where treatment effects were observed, using the Pearson Product Moment correlation test.

3. Results

3.1. Pre-defeat behavior
Rats exposed to social defeat 24 h prior to testing, relative to naive rats, responded with altered duration and frequency of a number of behaviors during the pre-defeat phase (Table 2). Analysis of the duration of pre-defeat behaviors revealed that repeated defeat subjects, compared to acute defeat subjects, responded with a greater duration of freezing ($p = 0.028$). Repeated defeat subjects displayed significantly less rearing ($p = 0.009$) and locomotion ($p = 0.010$). Additionally, rats exposed to repeated defeat tended to respond with a greater duration of inactivity, although this comparison only approached statistical significance ($p = 0.056$). Results from analysis of the frequency of pre-defeat behaviors paralleled the results from analysis of the duration of pre-defeat behaviors. Rats exposed to repeated defeat, compared to rats exposed to acute defeat, demonstrated more freezing ($p = 0.018$) and inactivity ($p = 0.009$), but less rearing ($p = 0.035$) and locomotion ($p = 0.007$). No additional differences were found for either the duration or frequency of any other pre-defeat behaviors.

![Table 2](image)

**Table 2:** Duration and frequency of the intruders' behavior during the pre-defeat phase of social defeat.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Duration</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Repeated</td>
</tr>
<tr>
<td>Sniffing bedding</td>
<td>68.7±9.6</td>
<td>62.9±8.0</td>
</tr>
<tr>
<td>Freezing</td>
<td>0.3±0.3</td>
<td>36.9±11.7*</td>
</tr>
<tr>
<td>Rearing</td>
<td>173.1±14.8</td>
<td>97.6±23.2**</td>
</tr>
<tr>
<td>Defensive burying</td>
<td>6.6±2.0</td>
<td>2.0±1.1</td>
</tr>
<tr>
<td>Locomotion</td>
<td>73.0±14.3</td>
<td>25.2±4.3*</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>70.5±8.8</td>
<td>70.9±9.0</td>
</tr>
<tr>
<td>Inactivity</td>
<td>12.0±3.7</td>
<td>38.6±9.6</td>
</tr>
</tbody>
</table>

* P<0.05; **P<0.01. Values are presented as mean±S.E.M.

3.2. Defeat behavior

Rats exposed to social defeat 24 h prior to testing, relative to naive rats, responded with altered duration and frequency of a number of behaviors during the defeat phase (Table 3).
Analysis of the duration of behaviors during the defeat phase revealed that repeated defeat, when compared with acute defeat, resulted in increased freezing ($p = 0.035$) and reductions in rearing ($p = 0.039$), social interaction ($p = 0.024$) and locomotion ($p < 0.001$). The analysis of the frequency of defeat behaviors was largely consistent with the analysis of the duration of defeat behaviors. Repeated defeat rats, compared to acute defeat rats, respond with a higher frequency of freezing ($p = 0.012$) and inactivity ($p = 0.008$). In contrast, repeated defeat rats displayed less frequent genital sniffing ($p = 0.048$), rearing ($p = 0.035$), social interaction ($p = 0.011$) and locomotion ($p = 0.001$).

### Table 3: Duration and frequency of the intruders' behavior during the defeat phase of social defeat.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Duration</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Repeated</td>
</tr>
<tr>
<td><strong>Reactive coping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sniffing bedding</td>
<td>5.8±2.9</td>
<td>6.7±5.3</td>
</tr>
<tr>
<td>Freezing</td>
<td>42.6±16.1</td>
<td>115.7±30.7*</td>
</tr>
<tr>
<td>Full submission</td>
<td>10.2±2.8</td>
<td>12.1±2.8</td>
</tr>
<tr>
<td>Sideways submission</td>
<td>2.8±1.4</td>
<td>5.3±1.6</td>
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<tr>
<td>Passive genital sniff</td>
<td>25.0±6.7</td>
<td>32.5±7.3</td>
</tr>
<tr>
<td>Genital sniff</td>
<td>2.1±0.9</td>
<td>0.0±0.0</td>
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<tr>
<td><strong>Proactive coping</strong></td>
<td></td>
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<tr>
<td>Rearing</td>
<td>43.8±10.4</td>
<td>16.3±4.8*</td>
</tr>
<tr>
<td>Defensive burying</td>
<td>0.2±0.1</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.3±0.2</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>Escape</td>
<td>0.7±0.3</td>
<td>1.8±1.2</td>
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<tr>
<td>Upright defensive behavior</td>
<td>153.4±37.9</td>
<td>182.3±53.7</td>
</tr>
<tr>
<td>Social interaction</td>
<td>13.7±3.0</td>
<td>4.2±2.1*</td>
</tr>
<tr>
<td><strong>Neutral</strong></td>
<td></td>
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<tr>
<td>Locomotion</td>
<td>30.5±5.3</td>
<td>4.5±1.0***</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>20.0±6.7</td>
<td>12.7±5.6</td>
</tr>
<tr>
<td>Inactivity</td>
<td>34.2±12.7</td>
<td>55.2±17.1</td>
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* $P<0.05$; **$P<0.01$; ***$P<0.001$. Values are presented as mean±S.E.M.

Rats exposed to social defeat 24 h prior to testing, relative to naive rats, responded with altered duration and frequency of reactive and proactive behavioral strategies during the defeat phase (Figure 2). Rats exposed to repeated defeat responded with a greater duration of reactive
coping behavior relative to rats exposed to a single defeat ($p = 0.026$; Figure 2A). In addition, the comparison of the ratio of the duration of reactive versus proactive coping behavior approached statistical significance ($p = 0.054$; Figure 2D). Rats exposed to repeated defeat responded with a lower frequency of proactive behaviors ($p = 0.018$; Figure 2F) and a greater ratio of the frequency of reactive versus proactive behaviors relative to rats exposed to a single defeat ($p = 0.005$; Figure 2H). No statistically significant differences were observed in either the duration (Figure 2C) or frequency (Figure 2H) of neutral behaviors.

3.3. Immunohistochemistry
3.3.1. Cell counts in home cage control rats

Statistical analysis using two-factor repeated measures ANOVA revealed that there were no differences between home cage control 1 and home cage control 2 rats on the numbers of c-Fos-ir/TPH-ir neurons (day x region interaction, $F_{(10, 260)} = 1.30, p = 0.273, \varepsilon = 0.44$; day main effect, $F_{(1, 26)} = 1.13, p = 0.297, \varepsilon = 0.44$; region main effect, $F_{(10, 260)} = 13.58, p < 0.001, \varepsilon = 0.44$; data not shown), c-Fos-ir/TPH-immunonegative cells (day x region interaction effect, $F_{(10, 260)} = 0.94, p = 0.410, \varepsilon = 0.24$; day main effect, $F_{(1, 26)} = 0.47, p = 0.500, \varepsilon = 0.24$; region main effect, $F_{(10, 260)} = 70.26, p < 0.001, \varepsilon = 0.24$; data not shown) or TPH-ir neurons (day x region interaction effect, $F_{(10, 260)} = 0.38, p = 0.819, \varepsilon = 0.39$; day main effect, $F_{(1, 26)} < 0.001, \varepsilon = 0.49$; region main effect, $F_{(10, 260)} = 46.78, p < 0.001, \varepsilon = 0.39$; data not shown). Consequently, cell counts from the two control groups were combined for analysis of treatment effects.

3.3.2. c-Fos-ir/TPH-ir neurons

Acute and repeated defeat differentially increased c-Fos expression in DR serotonergic neurons. Statistical analysis using multifactor ANOVA with repeated measures revealed that social defeat altered c-Fos expression within serotonergic neurons (i.e., altered the numbers of c-Fos-ir/TPH-ir neurons) in the DR (social defeat x region interaction effect, $F_{(20, 460)} = 4.89, p < 0.001, \varepsilon = 0.49$; treatment main effect, $F_{(2, 46)} = 28.10, p < 0.001, \varepsilon = 0.49$; region main effect, $F_{(10, 460)} = 27.08, p < 0.001, \varepsilon = 0.49$; Figures 3 and 4). Post hoc Fisher’s Protected LSD tests revealed that both acute and repeated social defeat, compared to home cage control conditions, increased c-Fos-ir/TPH-ir staining in several subdivisions of the DR. These included the DRD and DRV at −7.46 mm bregma, the DRD at −8.00 mm bregma, the DRD, DRV and
DRVL/VLPAG at −8.18 mm bregma and the DRC at −8.54 mm bregma. The comparison of c-Fos-ir/TPH-ir staining within the DRVL/VLPAG at −8.00 mm bregma approached statistical significance for rats exposed to either acute ($p = 0.073$) or repeated ($p = 0.051$) defeat (Figure 3). In addition, subjects exposed to acute social defeat displayed increased c-Fos-ir/TPH-ir staining in the DRV at −8.00 mm bregma and the DRI at −8.54 mm bregma; subjects exposed to repeated social defeat exhibited increased c-Fos-ir/TPH-ir staining in the DRI at −8.18 mm bregma while staining in the DRI at −8.54 mm bregma approached statistical significance ($p = 0.062$). When comparing acute to repeated social defeat subjects, repeated social defeat rats showed decreased c-Fos-ir/TPH-ir staining within the DRD ($p = 0.018$) and DRV ($p = 0.015$) at −8.00 mm bregma; this pattern of c-Fos-ir/TPH-ir staining approached statistical significance within the DRD ($p = 0.061$) and DRV ($p = 0.066$) at −8.18 mm bregma, suggesting a consistent pattern of responses in the mid-rostrocaudal DRD and DRV. Finally, repeated social defeat subjects, when compared with acute defeat, exhibited increased c-Fos-ir/TPH-ir staining in the DRI ($p = 0.004$) at −8.18 mm bregma.
Figure 3. Graphs illustrating the effects of acute defeat, repeated defeat or home cage control conditions on serotonergic neurons in the dorsal raphe nucleus. Graphs illustrate the total numbers of tryptophan hydroxylase (TPH)-positive neurons (open bars) and the numbers of c-Fos-positive/TPH-positive neurons (filled bars). *p < 0.05; **p < 0.01; ***p < 0.001 compared with home cage controls; a p < 0.05 versus acute defeat group; b p < 0.01 versus acute defeat group, Fisher’s Protected Least Significant Difference (LSD) tests. Bar graphs represent the means ± S.E.M. (n = 28 for home cage control; n = 11 for acute defeat; n = 10 for repeated defeat). For abbreviations, see Figure 1 legend.
Figure 4. Photomicrographs illustrate tryptophan hydroxylase/c-Fos immunostaining in the mid-rostrocaudal dorsal raphe nucleus (~8.00 mm bregma) in representative rats from each treatment group. Photomicrographs illustrate immunostaining in rats exposed to (A-C) home cage control conditions, (D-F) acute social defeat and (G-I) repeated social defeat. Black boxes in A, D and G indicate regions displayed at higher magnification in B, C, E, F, H and I. Black boxes in B, C, E, F, H and I indicate regions shown at higher magnification within insets located in the lower-right hand corner of these respective panels. Black arrows indicate c-Fos-immunoreactive non-serotonergic cells (blue/black nuclear staining); white arrowheads indicate TPH-immunoreactive/c-Fos-immunonegative neurons (brown/orange cytoplasmic staining); black arrowheads represent c-Fos-immunoreactive/TPH-immunoreactive neurons (brown/orange cytoplasmic staining with blue/black nuclear staining). Abbreviations: DRD, dorsal raphe nucleus, dorsal part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray. Scale bar in panel I applies as follows: 250 μm for panels A, D, G; 100 μm for panels B, C, E, F, H, I; 50 μm for insets.

3.3.3. c-Fos-ir/TPH-immunonegative cells
Acute and repeated defeat increased c-Fos expression in non-serotonergic cells within the DR. A separate two-factor ANOVA with repeated measures showed that social defeat altered the expression of c-Fos within non-serotonergic cells (i.e., c-Fos-ir/TPH-immunonegative cells) in subregions of the DR (social defeat x region interaction effect, $F_{(20, 460)} = 4.90, p < 0.001, \varepsilon = 0.23$; treatment main effect, $F_{(2, 46)} = 16.62, p < 0.001, \varepsilon = 0.23$; region main effect, $F_{(10, 460)} = 121.01, p < 0.001, \varepsilon = 0.23$; Figures 4 and 5). Further analysis using post hoc Fisher’s Protected LSD tests revealed that rats exposed to either acute or repeated social defeat, compared to rats exposed to home cage control conditions, exhibited increased numbers of c-Fos-ir/TPH-immunonegative cells in all regions studied, excluding the DRI at $-8.18$ mm bregma where only rats exposed to repeated social defeat responded with increased c-Fos expression.
3.3.4. Total numbers of TPH-ir neurons

Acute and repeated defeat had no effect on the numbers of serotonergic neurons within the DR. A separate two-factor ANOVA with repeated measures showed that social defeat did not alter the total numbers of serotonergic neurons (i.e., the sum of c-Fos-
immunonegative/TPH-ir neurons and c-Fos-ir/TPH-ir neurons) within subregions of the DR (social defeat x region interaction effect, $F_{(20, 460)} = 1.43, p = 0.103, \varepsilon = 0.48$; treatment main effect, $F_{(2, 46)} = 0.012, p = 0.988, \varepsilon = 0.48$; region main effect, $F_{(10, 460)} = 75.69, p < 0.001, \varepsilon = 0.48$; Figures 3 and 4).

3.4. Correlations

Correlation analysis was conducted for the duration of freezing behavior and subregions of the DR that displayed statistically significant increases in the numbers of c-Fos-ir/TPH-ir neurons and that are known to be involved in regulating behavioral coping strategies (i.e., DRD and DRVL/VLPAG). A Pearson's Product Moment Correlation test showed that the numbers of c-Fos-ir/TPH-ir neurons within the DRVL/VLPAG at $−8.00$ mm bregma were positively correlated with the duration of freezing behavior ($r^2 = 0.361, P = 0.018$, Figure 6). No behaviors during the pre-defeat phase were significantly correlated with numbers of c-Fos-ir/TPH-ir neurons in these regions.

![Figure 6. Graph illustrating the correlation between the number of c-Fos-immunoreactive serotonergic neurons within the dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG; $−8.00$ mm bregma) and the duration of freezing behavior during the defeat phase of social defeat. Open circles, acute defeat subjects; closed circles, repeated defeat subjects.](image-url)
4. Discussion

Repeated defeat, relative to acute defeat, resulted in a shift away from a proactive emotional coping style during the defeat phase of the test and toward a reactive emotional coping style. Rats exposed to either acute defeat or repeated defeat responded with increased c-Fos expression in serotonergic neurons in multiple subdivisions of the DR, relative to rats exposed to home cage control conditions. In most cases, increases in c-Fos expression in serotonergic neurons were similar in rats exposed to acute or repeated defeat. However, rats exposed to repeated defeat responded with less c-Fos expression in serotonergic neurons, relative to rats exposed to acute defeat, within the dorsal (DRD) and ventral (DRV) parts of the mid-rostrocaudal DR.

Rats exposed to acute and repeated social defeat displayed different behavioral coping strategies. Rats exposed to repeated defeat responded with a decrease in proactive emotional coping behavior (e.g. decreased duration and frequency of rearing and social interaction, and decreased frequency of proactive coping) and an increase in reactive emotional coping behavior (e.g. increased duration and frequency of freezing, and increased duration of reactive coping) relative to rats exposed to a single defeat. Overall, rats exposed to a second social defeat encounter, relative to rats exposed to a single social defeat, responded with an increase in the ratio of the frequency of reactive to proactive emotional coping behaviors. Behavior during the pre-defeat phase paralleled these findings. During the pre-defeat phase, rats exposed to repeated social defeat, compared to acute social defeat, responded with a reduction in the duration and frequency of rearing and locomotion, and with an increase in the duration and frequency of freezing. A reactive emotional coping strategy or development of a subordinate status has been proposed to be a more adaptive, flexible behavioral strategy (Koolhaas et al.,
1999) during periods of unstable social structure that may serve to avoid danger, limit injury and conserve energy (Korte et al., 2005). Previous studies have suggested that serotonergic systems may play a role in the inhibition of proactive coping responses including aggression (Bannai et al., 2007; Miczek et al., 1989), escape behaviors (Graeff, 2004; Graeff and Zangrossi, Jr., 2010), and in the facilitation of passive-submissive behaviors (Chung et al., 1999; Cooper et al., 2008b; Lowry and Hale, 2010), including fear- and anxiety-like behaviors (Lowry and Hale, 2010).

Rats exposed to acute defeat responded with increased c-Fos expression in serotonergic neurons in multiple subdivisions of the DR, relative to rats exposed to home cage control conditions. These findings are consistent with other studies showing that social defeat increases 5-HT neurotransmission within the DR (Amat et al., 2010; Cooper et al., 2009b; Martinez et al., 1998). Widespread activation of DR serotonergic systems has been described with other stressors including restraint stress (Takase et al., 2005) and inescapable tail shock (Takase et al., 2005). Thus, serotonergic responses to acute social defeat resemble those observed following exposure to relatively intense uncontrollable stressors. This widespread activation of serotonergic systems may be due to activation of multiple excitatory afferents to the DR, including those arising from the central nucleus of the amygdala, bed nucleus of the stria terminalis, lateral habenula, locus coeruleus, and lateral parabrachial nucleus (Baraban and Aghajanian, 1981; Kalen et al., 1989; Lee et al., 2003; Peyron et al., 1998a). Indeed, these structures that provide excitatory input to the DR are also activated by uncontrollable stressors (Day et al., 2004; Jasnow et al., 2004a; Kollack-Walker et al., 1997; Martinez et al., 2002; Wirtshafter et al., 1994). Our own previous studies found more anatomically restricted increases in c-Fos expression following exposure to social defeat (Gardner et al., 2005) compared to those in the current study. However, rats in the control group
in the previous study by Gardner and colleagues (Gardner et al., 2005) were exposed to a novel cage and moved to a novel environment for the duration of the social defeat period for time-matched rats exposed to social defeat, whereas rats in the control group in our study were removed from their cage 1 hr prior to social defeat, moved to a novel environment to be weighed, and then returned to their home cage environment where they remained during the social defeat period for time-matched rats exposed to social defeat. Consistent with these differences in experimental design, the levels of c-Fos expression in the control group from the study by Gardner and colleagues (Gardner et al., 2005) were considerably greater than in our study. Also, a recent study by Hinwood et al., 2010 (Hinwood et al., 2010) reported that repeated social defeat, when compared to a home cage control condition (like the one used in the current study), increased ΔFosB expression in the infralimbic and prelimbic regions of the medial prefrontal cortex and both the core and shell of the nucleus accumbens; however, when comparing repeated social defeat subjects to a sham-stress control group (i.e. a control animal that is exposed to the resident’s cage in the absence of the resident) the increase in ΔFosB expression was limited to only the infralimbic region of the prefrontal cortex. Studies investigating the effects of social defeat stress on immediate-early gene expression often differ in the type of home cage controls utilized and either use a home cage control condition (Anstrom et al., 2009; Nikulina et al., 1998), exposure to a novel cage (Chung et al., 1999; Cooper et al., 2009b; Gardner et al., 2005; Martinez et al., 1998), or exposure to the resident’s cage in the absence of the resident (Hinwood et al., 2010; Kollack-Walker et al., 1999; Kollack-Walker et al., 1997), but, as the aforementioned studies illustrate, the type of control group can be critical for interpreting the results. Future studies should include a sham-stress control group to identify the specific contributions of psychosocial stress per se (as opposed to those of handling, novelty, olfactory stimuli, etc.) on the activation of DR serotonergic systems. The current study, however, is consistent with prior studies showing
increased c-Fos expression in the DR following social defeat (Kollack-Walker et al., 1997; Kollack-Walker et al., 1999; Martinez et al., 1998; Martinez et al., 2002), although these previous studies did not distinguish between serotonergic and non-serotonergic neurons in the DR.

In the majority of subdivisions analyzed, there were no differences between responses of DR serotonergic neurons in rats exposed to acute defeat or repeated defeat. However, in the mid-rostrocaudal DR, including both the dorsal (DRD) and ventral (DRV) parts, c-Fos expression in serotonergic neurons was lower in rats exposed to repeated defeat, compared to rats exposed to acute defeat. These regions of the DR receive a unique set of afferents and give rise to topographically organized projections to forebrain targets. The mid-rostrocaudal DRD gives rise to extensive projections to subcortical limbic sites involved in fear and anxiety, whereas the mid-rostrocaudal DRV gives rise to sensorimotor and motor structures, including extensive cortical projections (Hale and Lowry, 2010; Lowry et al., 2008a). Together, these structures may modulate fear and anxiety states and sensorimotor and motor function.

The mechanisms underlying the anatomically selective adaptations to repeated defeat in the mid-rostrocaudal DRD and DRV are not clear. However, functional anatomical studies suggest that the mid-rostrocaudal DRD and/or DRV are selectively activated by a number of anxiety- and stress-related stimuli (Gardner et al., 2005; Lowry et al., 2005; Lowry et al., 2008b; Lowry and Hale, 2010; Staub et al., 2005; Staub et al., 2006). Interestingly, maternal separation, an adverse early life experience that results in a prolonged increase in anxiety state and a shift toward a more reactive emotional behavioral strategy during social defeat results in a pronounced increase in tph2 mRNA expression throughout the mid-rostrocaudal DRV (Gardner et al., 2009b). Although previous studies have not shown selective activation of the mid-
rostrocaudal DRV by social defeat, activation of the lateral orbital cortex, which gives rise to a dense projection to the mid-rostrocaudal DRV, has been associated with negative reward anticipation, losing outcome, and evaluation of wrong choices (Holland and Gallagher, 2004; Liu et al., 2007). It would be interesting to investigate whether a more chronic social defeat paradigm would amplify the site-specific effects of repeated social defeat on c-Fos expression within the mid-rostrocaudal DRD and DRV, as well as the functional consequences.

There are a number of mechanisms that could account for the decrease in c-Fos expression within serotonergic neurons in the mid-rostrocaudal DRD and DRV seen in animals exposed to repeated social defeat, compared to acute social defeat. It is possible that a selective increase in 5-HT$_{1A}$ receptor autoinhibition of serotonergic neurons in the mid-rostrocaudal DRD and DRV could explain a decrease in activation of this region during a second exposure to social defeat. Social defeat increases 5-HT$_{1A}$ mRNA expression throughout the DR (Cooper et al., 2009b)[43], which could decrease activity in the DRD and DRV, but this is unlikely since these mRNA expression changes are not selective to subregions of the DR. It is also possible that excitatory input to the mid-rostrocaudal DRD and DRV is diminished, that serotonergic autoinhibition is increased, or that non-serotonergic inhibitory input is increased, during a second exposure to social defeat. Alternatively, the reduction in c-Fos expression within the mid-rostrocaudal DRD and DRV observed in repeated social defeat subjects could be due to autoinhibiton of gene expression via transrepression of the c-fos promotor by a c-Fos/c-Jun heterodimer complex (Lucibello et al., 1989; Sassone-Corsi et al., 1988). Future experiments should investigate whether the reductions in c-Fos expression in serotonergic neurons observed in subjects exposed to repeated social defeat are due to altered afferent input to the DR or autoinhibitory mechanisms.
The amount of c-Fos expression in the mid-rostral DRVL/VLPAG was positively correlated with the duration of freezing behavior. This is consistent with a number of studies demonstrating that the VLPAG plays an important role in regulating a type of freezing behavior related to defensive responses and conditioned fear (Brandao et al., 2008; Vianna et al., 2001); indeed, lesions of the VLPAG prevent freezing associated with conditioned fear (Amorapanth et al., 2000; LeDoux et al., 1988). In our study, a greater level of activation of the DRVL/VLPAG region (as indicated by increased c-Fos expression in serotonergic neurons) was correlated with increased freezing behavior. As neurons in the DRVL/VLPAG region are known to project to the dorsal periaqueductal gray (Stezhka and Lovick, 1997), this effect may be due to serotonergic inhibition of escape behaviors, resulting in a shift toward a more reactive emotional coping style. Numerous studies have shown that activation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors within the dorsal periaqueductal gray inhibits escape behavior (Beckett and Marsden, 1997; Beckett et al., 1992; de Bortoli et al., 2008; Jacob et al., 2002). In contrast, c-Fos expression in non-serotonergic neurons within the DRVL/VLPAG region was not correlated with freezing behavior, suggesting that the effects of DRVL/VLPAG stimulation on freezing may be mediated by serotonergic mechanisms. Consistent with this hypothesis, intra-DR injections of corticotropin-releasing factor (CRF) increase freezing behavior that is temporally correlated with increased 5-HT release in the central amygdaloid nucleus (Forster et al., 2006), which, like the dorsal periaqueductal gray, is innervated by serotonergic neurons in the DRVL/VLPAG (Halberstadt and Balaban, 2006).

Acute and repeated defeat resulted in increased c-Fos expression in non-serotonergic neurons throughout all subregions of the DR relative to home cage control conditions. The DR contains a variety of neurotransmitters, including aspartate, dopamine, GABA, glutamate, glycine, nitric oxide, norepinephrine, and the peptide transmitters calbindin, calretinin,
cholecystokinin, corticotropin-releasing factor, leu- and met-enkephalin, galanin, neuropeptide Y, neurotensin, somatostatin, substance P and vasoactive intestinal polypeptide (Lowry et al., 2008a; Michelsen et al., 2007). Social defeat is known to produce changes in other neurotransmitter systems such as dopamine (Nikulina et al., 2008) and a variety of neuropeptides (Panksepp et al., 2007), however, to our knowledge, the effects of social defeat on other neurotransmitters specifically within the DR has yet to be investigated. There were no differences in the level of c-Fos activation in non-serotonergic neurons in rats exposed to acute and repeated defeat. This is consistent with previous studies that did not distinguish between serotonergic and non-serotonergic neurons reporting that there were no differences in the patterns of c-Fos expression within the DR following exposure to either acute or repeated (chronic) social defeat (Kollack-Walker et al., 1999; Martinez et al., 1998; Martinez et al., 2002). Identifying other cell types that are activated following social defeat should be an important direction for future research.

5. Conclusions

In summary, rats exposed to repeated social defeat, when compared with rats exposed to acute social defeat, responded with an increase in reactive coping behavior that was associated with a decrease in c-Fos expression within serotonergic neurons in the mid-rostrocaudal DRD and DRV. Furthermore, c-Fos expression within serotonergic neurons in the mid-rostrocaudal DRVL/VLPAG was positively correlated with the duration of freezing. These data support the hypothesis that the DR serotonergic neurons have a functional topographical organization and that novel therapeutic strategies for stress-related neuropsychiatric disorders could target topographically organized subpopulations of serotonergic neurons.
Acute and repeated social defeat differentially alter functional cellular responses in forebrain neuropeptidergic systems controlling stress and anxiety-like responses

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Abstract

Stressful life events increase vulnerability to subsequent stressors and psychiatric diseases. In rodents, repeated exposure to social defeat results in behavioral, neuroendocrine, and sympathetic alterations that mimic some of the symptoms observed in anxiety and affective disorders. One potential mechanism through which chronic stress may control behavioral and physiological responses is through stress-induced activation of forebrain neuropeptidergic systems controlling stress and anxiety-related behavioral responses, including orexin-A/hypocretin-1 (Orx-A/Hcrt-1) and melanin-concentrating hormone (MCH) systems located in the hypothalamus and extrahypothalamic corticotropin-releasing hormone (CRH) systems in the extended amygdala. In order to test the hypothesis that these forebrain neuropeptidergic systems are activated by repeated social defeat, we exposed male Long Evans rats to either home cage control (HCC) conditions, acute social defeat (AD), or repeated social defeat (RD; a double exposure of social defeat separated by 24 hrs) and then assessed functional neuronal responses by measuring the immunohistochemical expression of the neuronal activity marker, c-Fos, in forebrain neuropeptidergic neurons. Repeated social defeat activated CRH neurons in both the bed nucleus of the stria terminalis (BnST) and central nucleus of the amygdala (CeA), related to anxiety- and fear-like behaviors, respectively. Both AD and RD increased c-Fos expression in Orx-A/Hcrt-1 neurons, although AD rats had significantly more activation. Similarly both AD and RD increased the cellular activation marker in MCH neurons. These data are consistent with the hypothesis that these forebrain neuropeptidergic systems respond differentially to acute versus repeated exposure to stressful stimuli. Together with previous studies, these data suggest that these neuropeptidergic systems act in concert with serotonergic systems to modify behavioral coping strategies.
1. Introduction

Conspecific aggressive encounters in various socially-oriented species serve to establish hierarchies that determine access to environmental resources and potential mates. These encounters typically involve a more dominant male establishing dominance over a less aggressive subordinate male through a series of species-specific behavioral interactions. The ability to cope with conspecific aggression potently moderates the behavioral, neuroendocrine, and neurochemical consequences of the aggressive social encounter and subsequent stressors (Koolhaas et al., 2007; Koolhaas et al., 1999; Olff et al., 1995; Walker et al., 2009). Rodent species display individual variations in their coping responses to social defeat and typically engage in either proactive coping, characterized by the expression of behaviors with components of confrontation, escape, and exploration, or reactive (passive-submissive) coping, characterized by submissiveness and anxiety- and fear-related behaviors (Coppens et al., 2010; De Boer and Koolhaas, 2003; Gardner et al., 2005; Koolhaas et al., 1999; Paul et al., 2011; Veenema and Neumann, 2007). Because stressful life events can increase vulnerability to developing stress-related psychiatric disorders (Agid et al., 2000; Nemeroff and Vale, 2005) and the ability to cope can moderate the deleterious consequences of stress (Koolhaas et al., 1999; Koolhaas et al., 2007; Olff et al., 1995; Walker et al., 2009), there is considerable interest in delineating the underlying neurobiological mechanisms of distinct coping strategies (Coppens et al., 2010).

We previously reported (Paul et al., 2011) that exposure to repeated social defeat (RD; two acute social defeat encounters separated by 24 hrs), relative to acute social defeat (AD), shifts the behavioral coping response towards a reactive coping style and away from a proactive coping style. This behavioral shift in coping was also associated with altered patterns of c-Fos
expression in specific subpopulations of serotonergic neurons in the dorsal raphe nucleus (DR), including the dorsal part of the DR (DRD), a subregion implicated in facilitating conflict-anxiety (Hale and Lowry, 2011; Lowry et al., 2008c; Paul and Lowry, 2013), and the ventral part of the DR (DRV), a subregion that controls motor and cognitive function (Hale and Lowry, 2011; Paul and Lowry, 2013). It is unclear, however, how prior social defeat alters functional cellular responses in DR serotonergic neurons and how these alterations may ultimately change behavior. One potential mechanism underlying these responses is that social defeat stress alters the afferent input to DR serotonergic neurons originating from stress-related neuropeptidergic systems in forebrain limbic structures.

Extrahypothalamic corticotropin-releasing hormone (CRH) neurons located in the bed nucleus of the stria terminalis (BnST) and central nucleus of the amygdala (CeA) are potential candidates as these neurons are involved in mediating the behavioral and physiological responses to stress and anxiety/fear-provoking stimuli, including responses to social defeat (Cooper and Huhman, 2005; Cooper and Huhman, 2010; Wood et al., 2010; Wood et al., 2012). The BnST and CeA each send dense topographically organized projections to midbrain serotonergic systems (Oka et al., 2008; Petrovich and Swanson, 1997; Peyron et al., 1998a), including CRH projections to the serotonergic neurons located in the dorsal part of the dorsal raphe nucleus (DRD), implicated in facilitation of anxiety and conditioned fear, and serotonergic neurons in the lateral wings, comprising the ventrolateral dorsal raphe nucleus (DRVL) and adjacent ventrolateral periaqueductal gray (VLPAG; ) (Gray and Magnuson, 1992), a subregion associated with inhibiting proactive coping and facilitating reactive coping (Hale and Lowry, 2011; Johnson et al., 2004; Keay and Bandler, 2001; Paul and Lowry, 2013).
Although hypothalamic melanin-concentrating hormone (MCH) and orexin/hypocretin (Orx-A/Hcrt-1) neurons are classically involved in controlling sleep/wake states, ingestive behaviors, and energy homeostasis (de Lecea, 2010; Hervieu, 2006; Sakurai, 2007), more recent anatomical tracing studies reveal that these neuropeptides send multisynaptic projections to the sympathectomized gastrocnemius muscle and the adrenal gland, positioning these neuropeptides to control multiple aspects of the motor and sympathetic responses to a variety of environmental and psychological stressors (Kerman et al., 2007; Kerman, 2008). The hypothesis that MCH and Orx/Hcrt neurons may facilitate reactive and proactive coping strategies (Kerman et al., 2007), respectively, taken together with anatomical evidence revealing these neuropeptides send relatively dense topographically organized projections to midbrain serotonergic systems (Elias and Bittencourt, 1997; Lee et al., 2005; Nambu et al., 1999; Peyron et al., 1998b; Wang et al., 2005; Yoon and Lee, 2013), suggest these neuropeptides may be involved in previously observed stress-induced changes in serotonergic activity and behavioral responses towards social defeat (Paul et al., 2011).

Here we test the hypothesis that acute and repeated social defeat will differentially modulate patterns of activity in forebrain neuropeptidergic systems, and that these changes in activity will be associated with alterations in behavioral coping strategies. To test this hypothesis, we used brain tissue from rats previously exposed to either home cage control (HCC), AD, or RD to immunohistochemically stain for the presence of the neuronal activation marker, c-Fos, in forebrain neuropeptidergic systems (e.g., CRH, MCH, and Orx-A/Hcrt-1). We also correlated the functional neuronal responses of these neuropeptides with the neuronal responses of serotonergic neurons and the behavior observed in the previous study to determine if neuropeptidergic activity is associated with serotonergic activity or discrete behaviors or behavioral categories.
2. Materials and methods

2.1. Subjects

The rodents described here were used in a previously published experiment (Paul et al., 2011); in the current study we used their forebrain tissue for immunohistochemical staining of c-Fos expression in orexin-A/hypocretin-1 (Orx-A/Hcrt-1) and melanin-concentrating hormone (MCH) neurons located in the hypothalamus as well as in CRH neurons located in the BnST and CeA. Male Long Evans rats (Harlan Laboratories, Indianapolis, IN, USA; 238–298 g, mean ± S.E.M., 271 ± 1.28 g) were housed in groups of 3 in cages (38 cm W × 48 cm L × 21 cm H; Techniplast cages, Techniplast, Kettering, UK) containing a thin layer of bedding (Cat. No. 7090; Teklad Sani-Chips; Harlan Laboratories) with free access to food (Cat. No. 8640; Teklad 22/5 Rodent diet, Harlan Laboratories) and tap water stored in 16 oz reduced-height water bottles (Cat. No. WB16RH; Alternative Designs, Siloam Springs, AR, USA) with screw lids (Cat. No. FSPCST2.5; AnCare Corp., Bellmore, NY, USA). Male Long Evans retired breeders (Harlan Laboratories, 411–598 g, mean ± S.E.M., 511 ± 18.2 g) were used as resident males for the social defeat procedure and were individually housed in transparent polycarbonate cages (26 cm W × 47.6 cm L × 20.3 cm H; Cat. No., RC88D-PC, Alternative Designs) with stainless steel wire lids (Cat. No. WL88R, Alternative Designs) containing two compartments to accommodate free access to food and water. Resident males were housed in a separate room and vivarium away from the experimental animals. All rodents were maintained on a 12 h light/12 h dark cycle (lights on at 0700 h) with the temperature held at approximately 23 °C. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (Institute
2.2. Social defeat procedure

The social defeat procedure has been described in detail previously (Gardner et al., 2005; Koolhaas et al., 1999; Koolhaas et al., 2007; Koolhaas et al., 2013; Paul et al., 2011). Briefly, after two days of acclimation, rats were exposed to either home cage control (HCC), acute social defeat (AD) or repeated social defeat (RD), consisting of two social defeat encounters, each separated by 24 hours. Social defeat encounters were conducted during the light phase (~450 lux) beginning at 0800 h, one hour following lights on at 0700 h, and finished approximately at 1300 h. The social defeat encounter occurred in the home cage of the resident male and lasted for 20 minutes, consisting of a 10 min pre-defeat phase and 10 min defeat phase. During the pre-defeat phase the resident and intruder were separated by a transparent 0.3 cm-wide poly(methyl methacrylate) partition with nine 0.3 cm diameter holes drilled 5 cm apart in a grid pattern (3 x 3 grid) so that physical contact was prevented, but visual, auditory, and olfactory cues remained. At the onset of the defeat phase the partition was removed allowing the rats to freely interact. Home cage control rats consisted of two groups (home cage control 1 and home cage control 2), which were time matched with AD rats on day 1 and RD rats on day 2, respectively, for perfusion with paraformaldehyde fixative in preparation for immunohistochemical staining. Time-matched home cage control rats were transferred to an adjacent room during the social defeat period (approximately 2 h prior to transcardial perfusion), weighed, and following 20 min (e.g., the duration of social defeat) returned to the vivarium
where they remained until being anesthetized and transcardially perfused with their time-matched AD and RD counterparts.

2.3. Tissue preparation

Two hours following the onset of social defeat, rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (Fatal-Plus, MWI Veterinary Supply, Meridian, ID, USA; 200 mg/kg) and transcardially perfused with about 150 mL ice-cold 0.05 M phosphate-buffered saline (PBS, 67 g/L sodium phosphate dibasic heptahydrate, Cat. No.S2429-3KG, Sigma-Aldrich, St. Louis, MO, USA; 45 g/L sodium chloride, Cat. No. S7653-1KG, Sigma-Aldrich; 1 L distilled water; pH 7.4) followed by approximately 250 mL ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, 53.6 g/L sodium phosphate dibasic heptahydrate, Cat. No. S9390, Sigma-Aldrich; 27.6 g/L sodium phosphate monobasic monohydrate, Cat. No. S9638-1KG, Sigma-Aldrich; 500 mL distilled water) containing 1.5% sucrose. The rats were then decapitated using a guillotine and the brains were extracted and post-fixed overnight in 4% paraformaldehyde fixative, followed by two overnight washes in 0.1 M PB, and then placed in a 30% sucrose solution freshly prepared in 0.1 M PB for 2–3 days until saturated. Brains were blocked into forebrain and hindbrain sections by placing the brain into a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA) and bisecting the brain, in the coronal plane, with a razorblade directly caudal to the mammillary bodies (approximately −5.60 mm bregma). Next, the forebrain and hindbrain sections were rapidly froze by immersion in chilled isopentane (cooled between −30 and −40 °C with dry ice) for approximately 30 sec and stored at −80 °C until sectioning. The brains were mounted onto a specimen holder with optimum cutting temperature (OCT) compound (Tissue-Tek®, Sakura®, Finetek, Cat. No.
25608-930, VWR Scientific, West Chester, PA, USA) and a precision cryostat (Leica CM1900, North Central Instruments, Plymouth, MN, USA) was used to section the brains into 30 µm coronal slices that were subsequently stored in 6 alternate wells of a 24-well (6 wells per row) tissue culture plate with each well containing cryoprotectant (30% ethylene glycol and 20% glycerol prepared in 0.05 M PB, pH 7.4). Brain sections from forebrains and hindbrains were placed in the first two rows and last two rows of the well plate, respectively; therefore each well contained a rostrocaudal distribution of forebrain or hindbrain sections separated by 120 µm. After sectioning, well plates containing brain sections were wrapped in parafilm, covered with a well-plate lid, and stored at −20 °C until further immunohistochemical staining.

2.4. Immunohistochemical staining for c-Fos and orexin-A/hypocretin-1

For the double-immunohistochemical staining of c-Fos and orexin-A/hypocretin-1 (Orx-A/Hcrt-1), one well containing sections of the hypothalamus from each rat was removed from cryoprotectant and placed into separate wells in a different 24-well plate. To remove the cryoprotectant the brain tissue was washed twice for 15 min per wash in 0.05 M PBS (0.5 mL per well) and then immersed for 15 min in 1% H$_2$O$_2$ freshly prepared in 0.05 M PBS (800 µl of 30% H$_2$O$_2$ into 23.2 mL 0.05 M PBS) to neutralize endogenous peroxidase activity. Next, the brain tissue was rinsed twice for 15 min each time in 0.05 M PBS, then submerged for 15 min in 0.3% Triton X-100 freshly made in 0.05 M PBS to increase antibody penetration, and followed by an overnight incubation at room temperature (RT) in rabbit anti-c-Fos polyclonal antibody (Cat. No. PC-38; Lot No. D00080180; Calbiochem (EMD Chemicals), Gibbstown, NJ, USA) diluted to 1:3000 with 0.05 M PBS containing 0.1% Triton X-100 and 0.01% sodium azide. The next day, the tissue was rinsed twice for 15 min per rinse with 0.05 M PBS, incubated at RT for
90 min in biotinylated donkey anti-rabbit antibody (Cat. No. 711-065-152; Lot No. 96652; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted to 1:500 with 0.05 M PBS, then washed twice for 15 min each wash with 0.05 M PBS. Next, the tissue was incubated at RT for 90 min in an avidin–biotin–peroxidase complex (Vector Elite ABC kit; Cat. No. PK-6106; Vector Laboratories, Burlingame, CA, USA) diluted to 1:200 in 0.05 M PBS and prepared 30 min prior to use, then washed twice with 0.05 M PBS for 15 min each time, and reacted at RT in a peroxidase substrate (Vector SG peroxidase substrate kit; Cat. No. SK-4700; Vector Laboratories; diluted as recommended by the vendor). After verifying the presence of c-Fos-immunoreactivity, the reaction was stopped by two 15 min washes in 0.05 M PBS, then placed for 15 min in 1% H$_2$O$_2$ freshly made in 0.05 M PBS, followed by two additional 15 min washes in 0.05 M PBS. Next, the tissue was incubated overnight at RT in rabbit anti-pan orexin polyclonal antibody (Cat. No. ab6214; Lot No. 629941; Abcam, Cambridge, MA, USA), which is specific to Orx-A/Hcrt-1 (generated using a peptide antigen corresponding to amino acids 14-33 of cow Orx-A/Hcrt-1), diluted to 1:5,000 in 0.05 M PBS containing 0.1% Triton X-100 and 0.01% sodium azide. The next day, brain sections were rinsed twice for 15 min each time with 0.05 M PBS and then incubated at RT for 90 min in biotinylated donkey anti-rabbit polyclonal antibody (Cat No. 711-065-152; Lot No. 96652; Jackson ImmunoResearch Laboratories) diluted to 1:500 with 0.05 M PBS. Next, tissue was rinsed twice in 0.05 M PBS for 15 min per rinse, then incubated at RT for 90 min in the avidin–biotin–peroxidase complex reagent (Vector Elite ABC kit; Vector Laboratories) diluted to 1:200 in 0.05 M PBS prepared 30 min prior to use, and subsequently rinsed twice for 15 min each time in 0.05 M PBS. Lastly, coronal brain sections were immersed in 0.01% 3-3′-diaminobenzidine tetrahydrochloride (DAB; Cat. No. D9015, Sigma-Aldrich) freshly prepared in 0.05 M PBS and activated with 0.005% H$_2$O$_2$. Following verification of the presence of Orx-A/Hcrt-1-immunoreactivity, the reaction was neutralized by two 15 min rinses in 0.05 M PBS, then the brain sections were transferred into 0.1 M PB
containing 0.01% sodium azide, and stored at 4 °C. At a later time, brain sections were briefly immersed in a 0.15% gelatin solution, floated onto single-end frosted glass slides (VistaVision™ microscope slides; Cat. No. 16005-106; VWR Scientific), and allowed to air dry. After drying, coronal sections were dehydrated with increasing concentrations of ethanol (70%, 95%, and 100% for 2 min each), cleared for 5 min in xylenes (ACS reagent grade; Cat. No. X5-500; Fisher Scientific, Pittsburgh, PA, USA), and cover slips were mounted on the glass slides using Entellen mounting medium (Electron Microscopy Science, Hatfield, PA, USA) in preparation for bright-field microscopy.

2.5. Immunohistochemical staining for c-Fos and melanin-concentrating hormone

For the double-immunohistochemical staining of c-Fos and melanin-concentrating hormone (MCH), hypothalamic coronal brain sections from each rat from a well adjacent to the well containing sections used for c-Fos/orexin-A/hypocretin-1 immunohistochemistry were transferred to a new 24-well plate. The immunohistochemical procedure was conducted exactly as described above in section 2.4, but with the use of a rabbit anti-MCH antibody (Cat. No. H-047-47; Lot No. 00606; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) diluted 1:12,000 in 0.05 M PBS containing 0.1% Triton X-100 and 0.01 % sodium azide.

2.6. Immunohistochemical staining for c-Fos and corticotropin-releasing hormone

To immunohistochemically stain for the presence of c-Fos and corticotropin-releasing hormone (CRH), coronal brain sections of the bed nucleus of the stria terminalis and central
nucleus of the amygdala were collected from wells of each rat and placed into separate wells in new 24-well plates. The immunohistochemical procedure was conducted as described above in section 2.4, but with several modifications outlined below. After the peroxidase substrate reaction to immunohistochemically stain for the presence of c-Fos, the tissue was rinsed twice for 15 min per rinse in 0.05 M PBS, then immersed for 15 min in 1% H$_2$O$_2$ freshly prepared in 0.05 M PBS, followed by a 15 min rinse in 0.05 M PBS containing 0.3% Triton X-100 and 0.04% bovine serum albumin, and then a 48 h incubation at RT with agitation in rabbit anti-CRH antibody (Cat. No. IHC-8561; Peninsula Labs Europe, Merseyside, England; now available at Bachem, Cat. No. T-4037.0050; Torrance, CA, USA) diluted to 1:8,000 in 0.05 M PBS containing 0.3% Triton X-100, 0.04% bovine serum albumin, and 0.01% sodium azide. After the incubation period, brain sections were washed twice for 15 min each in 0.05 M PBS containing 0.3% Triton X-100 and 0.04% bovine serum albumin, incubated for 90 min at RT with agitation in biotinylated donkey anti-rabbit polyclonal antibody (Cat No. 711-065-152; Lot No. 96652; Jackson ImmunoResearch Laboratories) diluted to 1:500 in 0.05 M PBS containing 0.3% Triton X-100, 0.04% bovine serum albumin, and 0.01% sodium azide. The tissue was then rinsed twice for 15 min each in 0.05 M PBS containing 0.3% Triton X-100 and 0.04% bovine serum albumin and incubated for 90 min at RT in avidin–biotin–peroxidase complex reagent (Vector Elite ABC kit; Vector Laboratories) diluted to 1:200 in 0.05 M PBS and prepared 30 min prior to use. Next, the tissue was rinsed once for 15 min in 0.05 M PBS containing 0.3% Triton X-100 and 0.04% bovine serum albumin, followed by another rinse in 0.05 M PBS, and then reacted in 0.01% 3-3′-diaminobenzidine tetrahydrochloride (DAB; Cat. No. D9015, Sigma-Aldrich) freshly prepared in 0.05 M PBS and activated with 0.005% H$_2$O$_2$. After the reaction was completed, as determined by the visual inspection of CRH-immunoreactivity, the brain sections were rinsed once for 15 min in 0.05 M PBS, then rinsed once for 15 min in 0.01 M PB, and then stored in
0.01 M PB containing 0.01% sodium azide at 4 °C. Brain sections were mounted on glass slides, air dried, dehydrated, cleared, and coverslipped exactly as described in section 2.4.

2.7. Quantitative analysis of c-Fos-, MCH-, and orexin-A/hypocretin-1-immunoreactivity in the hypothalamus

Two standardized stereotaxic rat brain atlases (Paxinos and Watson, 1998; Swanson et al., 2005) were used to identify the anatomical regions of the rat hypothalamus. Studies examining the distribution of MCH- and Orx-A/Hcrt-1-expressing neurons in the hypothalamus (Bittencourt, 2011; Kerman et al., 2007; Knigge et al., 1996; Nambu et al., 1999; Peyron et al., 1998b; Swanson et al., 2005) and their projections to structures involved in coordinating the behavioral and physiologic response to stress (Bittencourt, 2011; Bittencourt et al., 1992; Elias and Bittencourt, 1997; Harthoorn, 2007; Kerman et al., 2007; Kilduff and de Lecea, 2001; Nambu et al., 1999; Peyron et al., 1998b) such as subpopulations of midbrain raphe serotonergic neurons (Wang et al., 2005; Yoon and Lee, 2013) were used to select the appropriate rostrocaudal level for quantifying stress-induced changes in these neuropeptides. One rostrocaudal level (≈3.14 mm bregma, approximately panels 29–30 in Swanson et al., 2005) of the hypothalamus was selected for analysis, based on evidence that this rostrocaudal level contains high densities of MCH and orexin/hypocretin neurons that send polysynaptic projections to both the sympathectomized hindlimb skeletal muscle (e.g., gastrocnemius) and adrenal gland (Kerman et al., 2007; Kerman, 2008). These so called presympathetic-premotor neurons (PSPMs), together with serotonergic neurons located in the DRVL/VLPAG that also send multisynaptic projections to the hindlimb and adrenal gland, comprise a distributed sympathomotor command center capable of controlling multiple components of the fight-or-flight
response, including proactive and reactive coping responses (Hale and Lowry, 2011; Johnson et al., 2004; Kerman et al., 2006; Paul and Lowry, 2013).

Cell counts included the total number of Orx-A/Hcrt-1-immunoreactive (ir) neurons (e.g., c-Fos-ir/Orx-A/Hcrt-1-ir neurons and c-Fos-immunonegative/Orx-A/Hcrt-1-ir neurons) and total number of MCH-ir neurons (e.g., c-Fos-ir/MCH-ir neurons and c-Fos-immunonegative/MCH-ir neurons), both identified by a light brown-stained cytoplasm as well as the number of double-immunostained c-Fos-ir/Orx-A/Hcrt-1-ir neurons and the number of double-immunostained c-Fos-ir/MCH-ir neurons, both distinguished by a dark blue/black-stained nucleus located entirely within a light brown-stained cytoplasm. An experimenter blind to treatment group conducted the cell counts using bright-field microscopy with a 20x objective lens and eyepiece with an overlaid grid; double-immunostained c-Fos-ir/Orx-A/Hcrt-1-ir neurons and double-immunostained c-Fos-ir/MCH-ir neurons were verified using a 40x objective lens.

2.8. Quantitative analysis of c-Fos- and CRH-immunoreactivity in the bed nucleus of the stria terminalis and central nucleus of the amygdala

Three rostrocaudal levels of the BnST (−0.16 mm, −0.28 mm, −0.40 mm relative to bregma) and CeA (−2.30 mm, −2.56 mm, and −2.80 mm relative to bregma) were selected for analysis of CRH-immunoreactivity. These levels were selected based on neuroanatomical studies outlining the distribution of CRH neurons in the BnST and CeA (Cassell et al., 1986a; Gray and Magnuson, 1992; Sakanaka et al., 1986; Veening et al., 1984; Wang et al., 2011), neuroanatomical tracing studies documenting projections from the BnST and CeA to the DRVL/VLPAG subregion (Gray and Magnuson, 1992; Oka et al., 2008; Petrovich and Swanson, 2011).
1997), and evidence suggesting that lentiviral overexpression of the *crh* gene in the BnST (e.g., approximately –0.30 mm relative to bregma) decreases *crh* type 2 receptor mRNA specifically within the DRD subregion (Sink et al., 2013). Cell counts of the total number of CRH-ir neurons (e.g., c-Fos-ir/CRH-ir neurons and c-Fos-immunonegative/CRH-ir neurons) and the numbers of double-immunostained CRH-ir neurons (e.g., c-Fos-ir/CRH-ir neurons) were conducted as described in section 2.7.

2.9. Statistical analysis

Outliers were identified using statistical tests for single and multiple outliers (Grubbs 1969), and were subsequently excluded from further statistical analysis and are not represented in the graphs. Microsoft® Excel® (Version 14.3.9 for Macintosh, Microsoft Corporation, Redmond, WA, USA) was used to analyze the raw data and conduct the statistical tests for outliers; PASW statistics (Version 21 for Macintosh, SPSS Inc., Chicago, IL, USA) was used to perform all other statistical analyses (e.g., independent Student’s *t*-tests, linear mixed models, and Fisher’s Protected Least Significance Difference tests). The level of statistical significance was set at *P* ≤ 0.05 for all analyses.

2.9.1. Statistical analysis of immunohistochemical data

Prior to statistical analysis, subjects in the HCC1 group were compared to subjects in the HCC2 group using separate independent Student’s *t*-tests on the total numbers of Orx-A/Hcrt-1-immunoreactive neurons, MCH-ir neurons, double-immunostained c-Fos-ir/Orx-A/Hcrt-1-ir
neurons, and double-immunostained c-Fos-ir/MCH-ir neurons expressed throughout the hypothalamus. Levene’s Test for Equality of Variances was performed in conjunction with all independent Student’s t-tests and the degrees of freedom were adjusted in the event that the test was significant (e.g., \( P \leq 0.05 \)). In regards to comparing CRH-ir neurons and double-labeled c-Fos-ir/CRH-ir neurons in the BnST and CeA between home cage groups, a linear mixed model analysis was conducted on the BNST and CeA with treatment (2 levels: HCC1 and HCC2) as a fixed factor and region (6 levels: BnST –0.16 mm, BnST –0.28 mm, BnST –0.40 mm and CeA –2.30 mm, CeA –2.56 mm, and CeA –2.80 mm) as a repeated measure. The model incorporated an unstructured covariance structure for the repeated measure and used the method of maximum likelihood to estimate parameters in the model. The linear mixed model analysis is an appropriate statistical model under circumstances where data sets contain missing data, unbalanced designs, complex covariate structures, and situations where repeated measurements (e.g., brain region) are derived from the same unit of observation (e.g., rat; Cnaan et al., 1997; Krueger and Tian, 2004; Spannuth et al., 2011). In the absence of significant differences in cell counts between HCC1 and HCC2 groups, these groups were pooled into one group called home cage control (HCC), which was used for further statistical analysis with AD and RD groups incorporated into the statistical models.

After comparing home cage groups, the effects of social defeat on neuropeptidergic neurons were analyzed with separate linear mixed model analyses performed on the total number of Orx-A/Hcrt-1-ir neurons, total number of MCH-ir neurons, the number of double-immunostained c-Fos-ir/Orx-A/Hcrt-1-ir neurons, and the number of double-immunostained c-Fos-ir/MCH-ir neurons using treatment (3 levels: HCC, AD, and RD) as a fixed factor and the method of maximum likelihood to estimate parameters in the model. For the analysis of treatment effects on CRH in the BnST and CeA, separate linear mixed models were used on the
total number of CRH-ir neurons and the number of double-immunostained c-Fos-ir/CRH-ir neurons with *treatment* (3 levels: HCC, AD, and RD) as a fixed factor and *region* (6 levels: BnST –0.16 mm, BnST –0.28 mm, BnST –0.40 mm and CeA –2.30 mm, CeA –2.56 mm, and CeA –2.80 mm) as a repeated measure. The model incorporated an unstructured covariance structure for the repeated measure and used the maximum likelihood function to estimate parameters in the model. Post hoc comparisons were conducted where appropriate using Fisher’s Protected Least Significant Difference (LSD) tests.

Since the total numbers of Orx-A/Hcrt-1-ir neurons, MCH-ir neurons, or CRH-ir neurons varied between groups, the percentage of Orx-A/Hcrt-1 neurons, MCH-ir neurons, or CRH-ir neurons that stained for c-Fos was derived to control for this variation and analyzed by separate linear mixed models as described above. If the overall omnibus test revealed a significant main effect or interaction, post hoc comparisons were conducted using Fisher’s Protected Least Significant Difference (LSD) tests.

2.9.2. Correlations with dorsal raphe nucleus c-Fos-ir/TPH-ir neurons or behavior during social defeat

In a previous study with the same rodents, the rats exposed to repeated defeat, when compared with rats exposed to acute defeat, had a reduction in neuronal activation (as measured by a decrease in the expression of c-Fos in tryptophan hydroxylase-immunoreactive neurons (c-Fos-ir/TPH-ir) that was restricted to the dorsal (DRD) and ventral (DRV) parts of the dorsal raphe nucleus (Paul et al., 2011). These neuronal adaptations were also associated with a shift towards a more reactive (passive-submissive) coping style, characterized by anxiety- and
fear-like behaviors such as freezing, and away from a proactive coping style, comprised of confrontational and escape oriented behaviors like rearing (Paul et al., 2011). Because subregions of the DR (and serotonergic neurons) receive projections from forebrain neuropeptidergic neurons such as Orx-A/Hcrt-1 neurons (Lee et al., 2005; Muraki et al., 2004; Nambu et al., 1999; Peyron et al., 1998b; Sakurai et al., 2005; Wang et al., 2005; Yamanaka et al., 2003), MCH neurons (Bittencourt et al., 1992; Bittencourt, 2011; Elias and Bittencourt, 1997; Kilduff and de Lecea, 2001; Yoon and Lee, 2013), and CRH neurons (Gray and Magnuson, 1992; Oka et al., 2008; Petrovich and Swanson, 1997), and these circuits are thought to be important for controlling the behavioral and physiologic response to stressors, we used Pearson Product Moment correlation tests to correlate the total amount of hypothalamic c-Fos-ir/Orx-A/Hcrt-1-ir neurons, c-Fos-ir/MCH-ir neurons, or both Bnst and CeA c-Fos-ir/CRH-ir neurons observed in the current study with the total amount of c-Fos-ir/TPH-ir neurons in subregions of the dorsal raphe nucleus as well as behavior during social defeat that were observed in the previous study.

3. Results

3.1. Comparison of Orx-A/Hcrt-1-ir and c-Fos-ir/Orx-A/Hcrt-1-ir neurons in home cage control rodents

Independent Student’s t-tests revealed that there were no differences in the total number of Orx-A/Hcrt-1-ir neurons (e.g., c-Fos immunonegative/Orx-A/Hcrt-1-ir neurons and c-Fos-ir/Orx-A/Hcrt-1-ir neurons; t(24) = 0.692, P = 0.496) or the number of double-labeled Orx-A/Hcrt-1 (e.g., c-Fos-ir/Orx-A/Hcrt-1-ir neurons; t(9.549) = –1.419, P = 0.188) between home cage control 1
and home cage control 2 groups (data not shown). Since there were no statistically significant differences between home cage groups, the two groups were combined for further statistical analysis of treatment (i.e., acute or repeated defeat) effects.

3.2. Effects of acute and repeated social defeat on Orx-A/Hcrt-1-ir and c-Fos-ir/Orx-A/Hcrt-1-ir neurons

The statistical test for outliers identified one outlier in the c-Fos-ir/Orx-A/Hcrt-1-ir (1/49 data points) data; in addition, one subject was excluded (1/49 data points for single-labeled Orx-A/Hcrt-1-ir data and 1/49 data points for c-Fos-ir/Orx-A/Hcrt-1-ir data) from the statistical analysis due to technical issues with the immunostaining. The overall linear mixed model analysis revealed there was no main effect of treatment on the total number of Orx-A/Hcrt-1-ir neurons ($F_{(2, 43)} = 1.854; p = 0.169$; open bars, Figure 1a); however, there was a significant main effect of treatment on the number of double-immunostained c-Fos-ir/Orx-A/Hcrt-1-ir neurons ($F_{(2, 42)} = 18.762; P \leq 0.001$, Figure 1a) as well as the percentage of total Orx-A/Hcrt-1-ir neurons stained with c-Fos ($F_{(2, 41)} = 23.18; P \leq 0.001$, Figure 1b). Further post hoc analysis using Fisher’s Protected LSD to determine group difference among treatments showed elevated expression of c-Fos within Orx-A/Hcrt-1-ir neurons in rats exposed to either AD or RD when compared to HCC (Figure 1a). Moreover, rodents exposed to RD, relative to AD rats, displayed decreased numbers of c-Fos-ir/Orx-A/Hcrt-1-ir neurons (Figure 1a). The percentage of Orx-A/Hcrt-1-ir neurons stained with c-Fos corroborated these findings with AD and RD rats, relative to HCC rats, displaying a higher percentage of c-Fos-ir/Orx-A/Hcrt-1-ir neurons, and there was a trend towards a greater percentage of c-Fos-ir/Orx-A/Hcrt-1-ir neurons in AD rats, relative to RD rats ($P = 0.052$, Figure 1b). Representative photomicrographs illustrating the
immunohistochemical staining of Orx-A/Hcrt-1 and c-Fos in the hypothalamus in HCC, AD, and RD subjects are depicted in Figure 2.

Figure 1. Graph illustrating the effects of acute defeat (AD), repeated defeat (RD), or home cage control conditions (HCC) on a) the total number of Orx-A/Hcrt-1-ir neurons (white bars) and the number of c-Fos-ir/Orx-A/Hcrt-1-ir neurons (shaded bars) as well as b) the percentage of Orx-A/Hcrt-1-ir neurons stained with c-Fos. Abbreviations: AD, acute social defeat; HCC, home cage control; LH, lateral hypothalamus; Pef, perifornical hypothalamus; Orx-A/Hcrt-1, orexin-A/hypocretin-1; RD, repeated social defeat. Data were analyzed by Fisher’s Protected LSD tests comparing HCC (n = 24-26), AD (n = 7), and RD (n = 10) rats and represent the means ± S.E.M. *P ≤ 0.05, ***P ≤ 0.001 compared with HCC rodents; !!P ≤ 0.01, ~ P ≤ 0.10 compared to AD rodents.
Figure 2. Photomicrographs illustrate c-Fos/Orx-A/Hcrt-1 immunostaining in the hypothalamus (−3.14 mm bregma) in representative rats exposed to home cage control (A and D), acute social defeat (B and E), and repeated social defeat (C and F). Black boxes in A, B, and C indicate regions displayed at higher magnification in D, E, and F. Black boxes in D, E, and F indicate regions shown at higher magnification in insets located in the lower right-hand corner of these respective panels. Black arrows indicate orexin-immunoreactive neurons (brown cytoplasmic staining); black arrowheads indicate c-Fos-immunoreactive/orexin-immunoreactive neurons (brown cytoplasmic staining with blue/black nuclear staining). Abbreviations: 3V; 3rd ventricle; f, fornix. Scale bar, A, B, C, 250 μm; D, E, F, 50 μm; insets, 25 μm.
3.3. Comparison of MCH-ir and c-Fos-ir/MCH-ir neurons in home cage control rodents

The comparison of HCC1 and HCC2 groups using independent Student’s *t*-tests revealed that there were no differences in the total number of MCH-ir neurons (e.g., c-Fos immunonegative/MCH-ir neurons and c-Fos-ir/MCH-ir neurons; \( t_{(26)} = -0.03, P = 0.976 \)) or the number of double-immunostained MCH-ir neurons (e.g., c-Fos-ir/MCH-ir neurons; \( t_{(24)} = 0.878, P = 0.388 \); data not shown). Both home cage control groups (i.e., HCC1 and HCC2) were combined for further statistical analysis because there were no statistically significant differences.

3.4. Effects of acute and repeated social defeat on MCH-ir and c-Fos-ir/MCH-ir neurons

The statistical test for outliers identified two outliers in the c-Fos-ir/MCH-ir (2/49 data points) data. The omnibus linear mixed model analysis reported no main effect of *treatment* (HCC, AD, and RD) on the total number of MCH-ir neurons (\( F_{(2, 49)} = 0.373; P = 0.691 \); open bars, Figure 3a), although there was significant main effect of *treatment* (\( F_{(2, 47)} = 5.107; P \leq 0.01 \); Figure 3a) on the number of double-immunostained c-Fos-ir/MCH-ir neurons in the hypothalamus. Further post hoc analysis using Fisher’s Protected LSD tests showed elevated expression of c-Fos within MCH-ir neurons in rats exposed to either AD or RD, relative to HCC (Figure 3a). Likewise, the percentage of MCH-ir neurons stained with c-Fos showed AD and RD rats, relative to HCC rats, display a higher percentage of c-Fos-ir/MCH-ir neurons (Figure 3b).
Figure 3. Graph illustrating the effects of acute defeat (AD), repeated defeat (RD), or home cage control conditions (HCC) on a) the total number of MCH-ir neurons (white bars) and the number of c-Fos-ir/MCH-ir neurons (shaded bars) as well as b) the percentage of MCH-ir neurons stained with c-Fos in the hypothalamus (−3.14mm from bregma). Abbreviations: AD, acute social defeat; HCC, home cage control; Hyp, hypothalamus; MCH, melanin-concentrating hormone; RD, repeated social defeat. Data were analyzed by Fisher’s Protected LSD tests comparing HCC (n = 26-28), AD (n = 11), and RD (n = 10) rats and represent the means ± S.E.M. *P ≤ 0.05 compared with HCC rodents.

3.5. Comparison of CRH-ir and c-Fos-ir/CRH-ir neurons in home cage control rodents

A linear mixed model analysis revealed no main effect of treatment (F(1, 27.931) = 0.311; P = 0.582) or interaction between treatment and region (F(5, 25.913) = 1.675; P = 0.176) on the total number of CRH neurons (e.g., CRH-ir/c-Fos immunonegative neurons and CRH-ir/c-Fos-ir neurons) in either the BnST or CeA between home cage control 1 and home cage control 2 groups (data not shown). Likewise, there was no main effect of treatment (F(1, 25.775) = 2.047; P = 0.165) or interaction between treatment and region (F(5, 24.333) = 1.449; P = 0.243) on the number of double-immunostained CRH neurons (e.g., CRH-ir/c-Fos-ir neurons) in the BnST or CeA.
between home cage control 1 and home cage control 2 groups (data not shown). Since there were no differences between home cage groups, the two groups were combined for further statistical analysis of treatment effects.

3.6. Effects of acute and repeated social defeat on CRH-ir and c-Fos-ir/CRH-ir neurons

Among all three rostrocaudal levels of the BnST, the statistical tests for outliers identified one outlier data point in the c-Fos-ir/CRH-ir data (1/147 data points); in regards to the levels of the CeA, the outlier tests identified one outlier data point in the total number of CRH-ir neuron data (1/147 data points) and two outliers in the c-Fos-ir/CRH-ir data (2/147 data points). After combining home cage groups, an analysis of the effects of home cage control, acute or repeated social defeat on the total numbers of CRH-ir neurons within the BnST or CeA discovered no effect of treatment ($F_{(2, 45.218)} = 2.305; P = 0.111$) or interaction between treatment and region ($F_{(10, 42.131)} = 1.281; P = 0.272$). In contrast, the analysis of c-Fos-ir/CRH-ir neurons showed a significant main effect of treatment ($F_{(2, 41.813)} = 3.423; P \leq 0.05$) and interaction between treatment and region ($F_{(10, 40.612)} = 2.101; P \leq 0.05$). The analysis of the percentage of CRH-ir neurons stained for c-Fos was in agreement, showing a main effect of treatment ($F_{(2, 45.543)} = 2.976; P = 0.061$) that approached significance and a significant interaction between treatment and region ($F_{(10, 41.353)} = 2.697; P \leq 0.05$). Further post hoc comparisons of HCC, AD, and RD groups, showed that rats exposed to RD, compared to both HCC and RD rats, had elevated c-Fos-ir/CRH-ir neurons (Figure 4c) and increased percentage of CRH-ir neurons stained for c-Fos (Figure 4f) in the CeA at –2.80 mm from bregma. There was a similar trend in the CeA at –2.30 mm, with an increased percentage of CRH-ir neurons stained for c-Fos in RD.
rats, compared to AD rats, although this trend only approached significance relative to HCC rats \((P = 0.066; \text{Figure 4d})\).

Figure 4. Repeated defeat activates topographically organized CRH neurons in the central nucleus of the amygdala. Graphs illustrate the effects of acute defeat (AD), repeated defeat (RD), or home cage control conditions (HCC) on a) the total number of CRH-ir neurons (white bars) and the number of c-Fos-ir/CRH-ir neurons (shaded bars; a-c) as well as b) the percentage of CRH-ir neurons stained with c-Fos (d-f) in specific rostrocaudal levels of the central nucleus of the amygdala. Abbreviations: AD, acute social defeat; CeA, central nucleus of the amygdala; CRH, corticotropin-releasing hormone; HCC, home cage control; RD, repeated social defeat. Data were analyzed by Fisher’s Protected LSD tests comparing HCC \((n = 23-25)\), AD \((n = 8-11)\), and RD \((n = 5-7)\) rats and represent the means ± S.E.M. *\(P \leq 0.05\), ~\(P \leq 0.10\) compared with HCC rodents; !!\(P \leq 0.01\) compared to AD rodents.

The analysis of the BnST showed a similar topographically-specific pattern with RD rodents, relative to both HCC and AD, displaying greater expression of c-Fos-ir/CRH-ir neurons (Figure 5b) and a robust increase in the percentage of CRH-ir neurons stained for c-Fos (Figure 5e) specifically at –0.28 mm from bregma. There was also a trend towards a reduced
percentage of CRH-ir neurons stained for c-Fos ($P = 0.091$; Figure 5e) in AD rats, compared with HCC rats.

Figure 5. Repeated defeat activates topographically organized CRH neurons in the bed nucleus of the stria terminalis. Graphs illustrate the effects of acute defeat (AD), repeated defeat (RD), or home cage control conditions (HCC) on a) the total number of CRH-ir neurons (white bars) and the number of c-Fos-ir/CRH-ir neurons (shaded bars; a-c) as well as b) the percentage of CRH-ir neurons stained with c-Fos (d-f) in specific rostrocaudal levels of in the bed nucleus of the stria terminalis. Abbreviations: AD, acute social defeat; BnST, bed nucleus of the stria terminalis; CRH, corticotropin-releasing hormone; HCC, home cage control; RD, repeated social defeat Data were analyzed by Fisher's Protected LSD tests comparing HCC ($n = 23-27$), AD ($n = 9-10$), and RD ($n = 6-8$) rats and represent the means ± S.E.M. *$P \leq 0.05$, **$P \leq 0.01$ compared with HCC rodents; !!$P \leq 0.01$ compared to AD rodents; ~$P \leq 0.10$, ***$P \leq 0.001$.

3.7. Correlations with c-Fos-ir/TPH-ir neurons in subregions of the DR
The Pearson Product Moment correlation test revealed a correlation between the number of c-Fos-ir/Orx-A/Hcrt-1-ir neurons in the hypothalamus and the number of c-Fos-ir/TPH-ir neurons in the DRD at −8.00 mm (r = 0.743, \( r^2 = 0.552, P \leq 0.001; \text{Figure 6a} \)), the DRVL/VLPAG at −8.18 mm bregma (r = 0.580, \( r^2 = 0.336, P \leq 0.001; \text{Figure 6b} \)), and DRV at −8.00 mm bregma (r = 0.542, \( r^2 = 0.294, P \leq 0.001; \) data not shown). Similarly, there were correlations between the number of hypothalamic MCH-ir/c-Fos-ir neurons and the number of c-Fos-ir/TPH-ir neurons in the DRD at −8.00 mm (r = 0.420, \( r^2 = 0.176, P \leq 0.01; \text{Figure 6c} \)) and the DRVL/VLPAG at −8.18 mm (r = 0.505, \( r^2 = 0.255, P \leq 0.001; \text{Figure 6d} \)). There were no correlations between the number of c-Fos-ir/CRH-ir neurons located in either the BnST or CeA and the number of c-Fos-ir/TPH-ir neurons in any DR subregion.
Figure 6. Graphs illustrating the correlation between (a,b) the number of c-Fos-immunoreactive/orexin-A/hypocretin-1-immunoreactive neurons or (c, d) c-Fos-immunoreactive/MCH-immunoreactive neurons within the hypothalamus (–3.14 mm bregma) and the number of c-Fos-immunoreactive/trypophan hydroxylase (TPH)-immunoreactive serotonergic neurons within (a, c) the dorsal raphe nucleus, dorsal part (DRD; –8.00 mm bregma) or (b, d) the dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG; –8.18 mm bregma). Abbreviations: AD, acute social defeat; DRD, dorsal raphe nucleus, dorsal part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; HCC, home cage control; MCH, melanin concentrating hormone; Orx-A/Hcrt-1, orexin-A/hypocretin-1; RD, repeated social defeat; TPH, tryptophan hydroxylase.
3.8. Correlations with behavior during social defeat

Although no particular behavioral coping strategy (e.g., proactive or reactive) was correlated with the numbers of c-Fos-ir/Orx-A/Hcrt-1-ir neurons, there were correlations with individual behaviors associated with proactive coping and arousal, including the frequency \( (r = 0.577, r^2 = 0.332, P \leq 0.05; \text{data not shown}) \) and duration \( (r = 0.587, r^2 = 0.345, P \leq 0.05; \text{Figure 7a}) \) of rearing and the duration of locomotion \( (r = 0.616, r^2 = 0.379, P \leq 0.05; \text{Figure 7b}) \). There were no correlations between any individual behaviors during the predefeat phase of social defeat and c-Fos-ir/Orx-A/Hcrt-1-ir neurons. Similarly, MCH-ir neurons stained with c-Fos were not correlated with any particular behavior during the predefeat phase of social defeat or any behavioral coping strategy during the defeat phase, but they were negatively correlated with self-grooming behavior \( (r = -0.514, r^2 = 0.264, P \leq 0.05; \text{Figure 7c}) \) during the defeat phase of social defeat.

![Figure 7](image)

**Figure 7.** Graphs illustrating the correlation between the number of c-Fos-ir/orexin-A/hypocretin-1-ir neurons within the hypothalamus \((-3.14 \text{ mm bregma})\) and behaviors related to proactive coping and arousal, including the duration of rearing (a) and the duration of locomotion (b) during social defeat. The number of c-Fos-ir/MCH-ir neurons was correlated with the frequency of self-grooming (c) during social defeat. Abbreviations: AD, acute social defeat; MCH, melanin concentrating hormone; Orx-A/Hcrt-1, orexin-A/hypocretin-1; RD, repeated social defeat.
The correlation analysis revealed that c-Fos-ir/CRH-ir neurons located in the CeA (−2.80 mm from bregma) were correlated with predefeat behaviors associated with reactive coping and reduced arousal, including the frequency ($r = 0.608$, $r^2 = 0.370$, $P \leq 0.01$; data not shown) and duration ($r = 0.574$, $r^2 = 0.329$, $P \leq 0.05$; data not shown) of freezing and the frequency ($r = 0.572$, $r^2 = 0.327$, $P \leq 0.05$; data not shown) and duration ($r = 0.713$, $r^2 = 0.508$, $P \leq 0.01$; data not shown) of inactivity. Consistent with this, the amount of double-labeled c-Fos-ir/CRH-ir neurons was correlated with the duration of reactive coping during the defeat phase of social defeat ($r = 0.568$, $r^2 = 0.322$, $P \leq 0.05$; Figure 8a), as well as the duration of freezing ($r = 0.572$, $r^2 = 0.327$, $P \leq 0.05$; Figure 8b), the predominant behavior of reactive coping. The CeA CRH-ir neurons stained with c-Fos were also correlated with neutral arousal-related behaviors, including the frequency ($r = 0.569$, $r^2 = 0.324$, $P \leq 0.05$; Figure 8c) of inactivity and negatively correlated with the frequency ($r = −0.638$, $r^2 = 0.407$, $P \leq 0.01$; Figure 8d) and duration ($r = −0.582$, $r^2 = 0.339$, $P \leq 0.05$; data not shown) of locomotion. In contrast to double-labeled c-Fos-ir/CRH-ir neurons in the CeA, there were no correlations between double-labeled c-Fos-ir/CRH-ir neurons in the BnST (−0.28 mm from bregma) and any particular behavioral coping strategy or individual behaviors during the predefeat and defeat phases of social defeat.
4. Discussion

Acute and repeated social defeat differentially altered c-Fos expression in forebrain neuropeptidergic neurons involved in eliciting behavioral and physiological responses to stressful stimuli. Consistent with previous studies showing that chronic social defeat perturbs...
CRH systems in the BnST (Choi et al., 2006) and CeA (Albeck et al., 1997), repeated social defeat, but not acute social defeat, increased the expression of the neuronal activation marker, c-Fos, in CRH neurons located in subregions of the BnST and CeA. Activation of CeA CRH neurons was correlated with a reactive coping style and freezing behavior, an association that is consistent with evidence suggesting that a reactive (passive-submissive) coping strategy during chronic social defeat is mediated by increased activity of CRH systems (Wood et al., 2010; Wood et al., 2012). In contrast to c-Fos expression in CRH neurons that was specific to repeated defeat, exposure to either acute or repeated social defeat increased c-Fos expression in Orx-A/Hcrt-1 neurons located in the hypothalamus, although this effect was more pronounced in rats exposed to AD. This is not surprising considering the role of orexinergic-/hypocretinergic-circuits in stress-induced behavioral arousal (Kuwaki, 2011; Sakurai, 2007; Sakurai and Mieda, 2011; Tsujino and Sakurai, 2013; Zhang et al., 2006) and previously reported findings from the same rats used here showing that AD rats, relative to RD rats, respond to social defeat with more proactive coping strategies and greater behavioral arousal (Paul et al., 2011). Consistent with a role for MCH neurons in stress- and anxiety-related responses (Hervieu et al., 2000; Hervieu, 2006), exposure to either acute or repeated social defeat increased c-Fos expression in a small portion of hypothalamic MCH neurons. Together, these findings suggest forebrain neuropeptides respond differently to acute or repeated social defeat and these differences in responsiveness may be relevant for the expression of different patterns of behavior during social defeat.

Repeated social defeat, relative to both acute social defeat and home cage control, increased the activity of CRH neurons in the CeA, and these alterations were associated with behavioral changes that were previously observed in the same rodents (Paul et al., 2011), including increased reactive coping and freezing behavior as well as reduced behavioral arousal.
(i.e., increased inactivity and reduced locomotion) during social defeat. Many studies have reported stress-induced activation of CRH neurons in the CeA using a variety of diverse stressors, including foot shock, restraint, acute and chronic social defeat (Albeck et al., 1997; Funk et al., 2006; Hsu et al., 1998; Hwang et al., 2005; Makino et al., 1999), as well as administration of anxiogenic drugs, including yohimbine, an α₂-receptor antagonist with actions on other monoaminergic receptors (Millan et al., 2000), and FG-7142 (Funk et al., 2006), a partial inverse agonist at the benzodiazepine allosteric site of the GABAₐ receptor. Consistent with stress-induced activation of CeA CRH, acute or chronic glucocorticoid exposure, in the absence of stress, elevates CRH mRNA expression in the CeA (Makino et al., 1994a; Makino et al., 1994b; Shepard et al., 2000; Thompson et al., 2004), and also increases anxiety-like behavior (Shepard et al., 2000) and conditioned contextual fear responses (Thompson et al., 2004).

Highlighting the importance of CeA CRH in stress-related processes, a recent study reported that selective silencing of CeA CRH using RNA interference attenuates the stress-induced activation of the HPA axis (Callahan et al., 2013). The lack of any effect of acute defeat on c-Fos expression in CRH neurons is at odds with another study showing that acute defeat increases CRH mRNA (Funk et al., 2006), although it is more likely due to different methodology (i.e., longer duration social defeat) and assessment (immunohistochemistry vs in situ hybridization histochemistry) of serotonergic function. Nevertheless, repeated defeat did activate CRH neurons, consistent with the idea that CRH neurons in the CeA comprise a corticosterone- and stress-sensitive subsystem involved in facilitating neuroendocrine activation and anxiety- and fear-like behavioral responses.

The repeated defeat-induced changes in CRH activity, relative to HCC rats, were only apparent at one rostrocaudal level (e.g., −2.80) of the CeA, suggesting topographically specific effects of RD on c-Fos expression in CRH neurons. This rostrocaudal level contains a high
density of CRH neurons that target the DRVL/VLPAG region (Gray and Magnuson, 1992; Oka et al., 2008; Petrovich and Swanson, 1997), a brain region containing a subpopulation of serotonergic neurons implicated in inhibiting fight-or-flight behaviors and promoting reactive coping behaviors (Hale and Lowry, 2011; Johnson et al., 2004; Keay and Bandler, 2001; Paul and Lowry, 2013). CRH-ir terminals in the dorsal raphe nucleus, some of which appear to originate in the CeA, display a rostrocaudal topographical organization with a preference towards innervating the dorsolateral region (e.g., DRVL/VLPAG) in the caudal DR and the ventromedial area (e.g., DRD and DRV subregions) in the more rostral portions of the DR (Kirby et al., 2000; Lowry et al., 2000; Valentino et al., 2001). Ultrastructural visualization of CRH-ir terminals reveal direct contacts with serotonergic neurons and the type of synaptic specialization depends on the topographical organization; for example, CRH-ir terminals in the DRVL/VLPAG subregion form asymmetric (type 1) excitatory synapses that predominantly innervate dendrites, whereas CRH-ir terminals in the DRD and DRV form symmetric (type II) inhibitory synapses that favor axon terminals (Valentino et al., 2001). These topographically organized CRH projections suggest that activation of CeA CRH neurons would have opposing effects depending on the specific subpopulation of DR serotonergic neurons being targeted.

Considering this arrangement, the increased CeA CRH activation in repeated defeat rats may be responsible for the reduced activation of DRD and DRV serotonergic neurons observed in the same rats (Paul et al., 2011) as CRH-terminals in these regions primarily form inhibitory synapses. Likewise the increased activation of DRVL/VLPAG serotonergic neurons observed in both acute and repeated defeat rats (Paul et al., 2011) could be due to increased CRH excitatory afferent input as CRH terminals in this region preferentially form excitatory synapses. This is consistent with the notion that DRVL/VLPAG serotonergic neurons are stress-sensitive due to their increased intrinsic excitability (Crawford et al., 2010). Altered CRH afferent input to
specific subregions of the DR could be the underlying neurobiological mechanism for altered patterns of serotonergic activity and shift to reactive coping observed in response to repeated social defeat. Indeed, CRH activation (i.e., increased c-Fos expression) was correlated with reactive coping and freezing, as well as other neutral behaviors consistent with the hypoactivity observed in reactive coping, including a correlation with inactivity and a negative correlation with locomotion. Overall, these data provide further support for the well-established role of the CeA mediating anxiety- and fear-related responses such as freezing behavior through projections to the VLPAG (LeDoux et al., 1988); moreover, our data suggest this behavioral response may be controlled by a subpopulation of CRH neurons that project to serotonergic neurons in the DRVL/VLPAG and DRD that are known to coordinate coping responses to stress.

Evidence pointing to a role of altered CRH input to subregions of the DR in the behavioral coping responses to stress is also supported by two converging lines of evidence using different resident-intruder paradigms. Using a model of chronic social defeat (i.e., 7 days of daily defeat), Woods and colleagues report that rats responding with short latencies to defeat exhibit more reactive coping behaviors during a subsequent forced swim test, including increased immobility, and this increased behavioral reactivity is thought to be due to altered CRH neurotransmission in subdivisions of the DR (Wood et al., 2010; Wood et al., 2012; Wood et al., 2013). Likewise, following social defeat, Syrian hamsters display decreased territorial aggression and respond to conspecific aggression with more submissive/defensive behaviors, behavioral phenomena called conditioned defeat (Huhman et al., 2003). The behavioral alterations in conditioned defeat strikingly resemble the increased reactive coping observed in rodents exposed to repeated defeat (Paul et al., 2011) and chronic social defeat (Wood et al., 2010; Wood et al., 2012; Wood et al., 2013). Conditioned defeat appears to involve CRH projections to the DR as administration of anti-sauvagine-30, a CRHR2 antagonist, either
intracerebroventricularly or directly into the DR, attenuates the expression of submissive/defensive behaviors (Cooper and Huhman, 2007; Cooper and Huhman, 2010). Underlining the importance of serotonergic circuits, conditioned defeat alters serotonergic activity in specific DR subregions (e.g., rostral DRV) and reduces 5-HT$_{1A}$ receptor mRNA expression, encoding an inhibitory autoreceptor, throughout the entire DR (Cooper et al., 2009a). Likewise, the behavioral consequences of conditioned defeat are blocked by prior administration of 5-HT$_{1A}$ receptor agonists (Cooper et al., 2008a). Altogether, three different models of conspecific aggression using rodent/intruder models, namely repeated social defeat, chronic social defeat, and conditioned defeat, implicate CRH systems in the stress-induced activation of DR serotonergic systems and consequent alterations in behavioral and emotional reactivity towards stress. Our previous results suggesting altered patterns of serotonergic activity in repeated social defeat animals (Paul et al., 2011) taken together with the current observation that CeA CRH neurons show greater activation in RD rats, strongly suggest this overactive CRH system may originate in the CeA in models of conspecific aggression.

In addition to altering CeA CRH activity, exposure to RD, but not AD, elevated c-Fos expression in CRH neurons in topographically specific rostrocaudal levels of the BnST (i.e., – 0.28 mm from bregma), the same rostrocaudal level where lentiviral overexpression of CRH alters conditioned emotional memory and reduces CRHR1 receptor expression in the BnST and CRHR2 in the DRD (Sink et al., 2013), strengthening the notion that BnST CRH neurons and DRD serotonergic neurons are part of a conflict-anxiety facilitation circuit (Hale and Lowry, 2011; Lowry et al., 2008c; Paul and Lowry, 2013). Similarly, priming of the BnST by repeated injections of the stress- and CRH-related neuropeptide, urocortin I (Ucn I) results in a chronic social anxiety-like state that is reversed by previous microinjection of the CRHR1 antagonist, astressin, directly into the BnST (Lee et al., 2008). Consistent with BnST CRH receptors in
anxiety-like responses, prior antagonism of BnST CRHR2 receptors blocks the submissive/defensive behaviors observed in conditioned defeat hamsters (Cooper and Huhman, 2005; Jasnow et al., 2004b). The finding that RD increased c-Fos expression in BnST CRH neurons is consistent with a number of other studies confirming BNST CRH neurons are sensitive to complex social and environmental stressors, including subordination stress in the visible burrow system, chronic mild stress, foot shock, and acute social defeat (Choi et al., 2006; Funk et al., 2006; Jasnow et al., 2004b; Kim et al., 2006). Although acute defeat failed to activate CRH neurons in the currently study, this may be due to methodology and is offset by the finding that repeated defeat activates CRH neurons. Nevertheless, these data are still consistent with the hypothesis that CRH neurons in the BnST respond to diverse stressful stimuli and mediate behavioral responses generally characterized by increased anxiety-related behavior. Overall this is consistent with the notion that the BnST is critical for the expression of anxiety-related behaviors (i.e., sustained anxiety), whereas the CeA is critical for phasic fear responses (Davis et al., 2010; Davis and Shi, 1999).

Similar to CRH neurons in the CeA, the CRH neurons of the BnST send a substantial projection to the lateral wings of the DR (i.e., DRVL/VLPAG), a subregion involved in inhibiting fight-or-flight and panic-like responses (Hale and Lowry, 2011; Johnson et al., 2004; Keay and Bandler, 2001; Paul and Lowry, 2013); BnST neurons also project to the DRD, a region involved in conflict-anxiety (Hale and Lowry, 2011; Lowry et al., 2008c; Paul and Lowry, 2013), and this innervation may originate from CRH neurons because chronic overexpression of BnST CRH results in a compensatory decrease in CRHR2 expression selectively in the DRD subregion (Sink et al., 2013). Consistent with these findings, Ucn I priming of the BnST increases tph2 and slc6a4 mRNA expression selectively in the mid-rostralcaudal DRD (Mani, 2013; Mani et al. unpublished). Neurons in the DRD project back to the BnST, including BnST subregions that
contain CRH neurons (Peyron et al., 1998a), and some of these BnST-projecting neurons in the DRD may express CRH themselves (Commons et al., 2003). Taken together with evidence that 5-HT modulates BnST activity through complex actions on multiple BnST neuron types expressing many different receptor subtypes (e.g., 5-HT1A, 5-HT2A, 5-HT2C, and 5-HT7; Guo et al., 2009; Hammack et al., 2009), a BnST CRH-DR 5-HT circuit appears to play an important role in controlling anxiety-related behaviors in response to stress and anxiety-provoking stimuli. Considering these anatomical interconnections between the BnST and subregions of the DR and the heterogeneity of CRH-terminals in subregions of the DR, the increased BnST CRH activation observed in rats exposed to repeated social defeat may be relevant for the changes in activity of specific subpopulations of serotonergic neurons and the adoption of a reactive coping style that was previously observed in the same animals (Paul et al., 2011). Based on evidence that CRH terminals display heterogeneous synapses in subregions of the DRD, increased activation of BnST CRH projections as seen in RD rats, but not AD rats, would be predicted to inhibit DRD 5-HT neurons or activate DRVL/VLPAG neurons, which could then inhibit DRD neurons through intra-raphe projections. The observations that RD rats, relative to AD rats, displayed reduced serotonergic activity in the DRD support this notion (Paul et al., 2011).

Both social defeat procedures (AD and RD) strongly increased the cellular expression of the neuronal activation marker, c-Fos, in Orx-A/Hcrt-1 neurons, confirming reports that orexinergic/hypocretinergic systems are stress-sensitive and capable of modulating diverse aspects of the stress response (Sakurai and Mieda, 2011; Winsky-Sommerer et al., 2004; Winsky-Sommerer et al., 2005), especially the behavioral and sympathetic arousal components. This is supported by correlations between c-Fos-ir/Orx-A/Hcrt-1-ir neurons and arousal-related behaviors such as locomotion and rearing that were previously observed in the same rodents (Paul et al., 2011). The magnitude of this defeat-induced activation of Orx-A/Hcrt-
1 neurons tended to be greater in AD rodents, relative to RD rodents, an effect that may be relevant for the increased behavioral arousal (e.g., increased locomotion) and proactive coping observed in AD rats (Paul et al., 2011). Our finding that social defeat activates Orx-A/Hcrt-1-ir neurons is consistent with other reports that Orx-A/Hcrt-1-ir neurons are responsive to diverse stressors, including cold exposure (Ida et al., 2000; Sakamoto et al., 2004), peripheral inflammation (Watanabe et al., 2005), immobilization (Ida et al., 2000; Sakamoto et al., 2004), restraint (Reyes et al., 2003; Winsky-Sommerer et al., 2004), foot shock (Watanabe et al., 2005; Zhu et al., 2002), novelty stress (i.e., brightly lit novel environment; Berridge et al., 2010) and high-arousal waking (i.e., diurnal novelty-stress; Espana et al., 2003).

Orexinergic/hypocretinergic neurons are anatomically positioned to control behavioral and sympathetic responses to emotionally-salient events with diffuse projections to limbic structures (e.g., CeA and BnST) and brainstem (e.g., raphe nuclei) and spinal cord structures implicated in arousal and stress responses. The expression of c-Fos in Orx-A/Hcrt-1 neurons in the current study was correlated with the expression of c-Fos in TPH neurons observed in the same animals (Paul et al., 2011) in specific subregions of the DR, including the DRD, a component of a conflict-anxiety facilitation circuit, the DRVL/VLPAG, a key node in a fight-or-flight (panic) inhibition circuit, and to a lesser extent the DRV, a subregion with extensive interconnections with cortical regions and thought to be involved in motor function and cognitive tasks (Hale and Lowry, 2011; Johnson et al., 2004; Lowry et al., 2008c; Lowry et al., 2008a; Paul and Lowry, 2013). This is consistent with anatomical tracing studies revealing dense orexin/hypocretin (Lee et al., 2005; Nambu et al., 1999; Peyron et al., 1998b) projections to these subregions of the DR and some of these projections directly innervate serotonergic neurons (Wang et al., 2005). The DR contains an abundance of both Gq-protein linked Orx/Hcrt receptors (Cluderay et al., 2002; Greco and Shiromani, 2001; Hervieu et al., 2001; Kilduff and de
Lecea, 2001; Marcus et al., 2001; Trivedi et al., 1998), Orx/Hcrt-1 and Orx/Hcrt-2, which have predominantly excitatory effects on serotonergic neurons (Brown et al., 2001; Brown et al., 2002; Kohlmeier et al., 2008; Liu et al., 2002; Takahashi et al., 2005; Wang et al., 2005), although in some circumstances serotonergic inhibition is observed presumably through activation of local GABAergic interneurons (Liu et al., 2002). In agreement with Orx/Hcrt exciting serotonergic neurons, reverse microdialysis infusion of either Orx-A/Hcrt-1 or Orx-B/Hcrt-2 directly into the DR increases extracellular 5-HT concentrations within the DR, and these increases in 5-HT are particularly robust following Orx-A/Hcrt-1 infusion (Tao et al., 2006). The serotonergic system in turn innervates Orx/Hcrt neurons, although the majority of this serotonergic input originates from the median raphe nucleus and caudal raphe nuclei (e.g., raphe pallidus and raphe magnus nuclei), rather than the DR (Sakurai et al., 2005). The function of an Orx-A/Hcrt-1-serotonergic circuit is unclear, but evidence suggests serotonergic systems are necessary for orexin-mediated behaviors. For example, centrally administered Orx-A/Hcrt-1 induces stereotypic behaviors such as increased grooming, face washing, and wet dog shaking in rats, and these Orx-A/Hcrt-1-elicited behaviors are attenuated by serotonin receptor antagonists, especially 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor antagonists (Duxon et al., 2001; Matsuzaki et al., 2002). Taken together, these data suggest an Orx-A/Hcrt-1-serotonergic circuit is important for coordinating behavior and potentially stress-related arousal behaviors.

Similar to the effects of defeat on Orx-A/Hcrt-1 neurons, both AD and RD increased c-Fos expression in a small portion of the total number of hypothalamic MCH neurons, effects that are consistent with the view that MCH modulates stress and anxiety-related behavior (Hervieu, 2006; Hervieu et al., 2000; Kerman et al., 2007; Kerman, 2008; Smith et al., 2006). Studies investigating the actions of MCH on anxiety-like behavior are conflicting with some studies reporting anxiogenic (Gonzalez et al., 1996; Smith et al., 2006) or anxiolytic (Kela et al.,
2003; McBride et al., 1994; Monzon et al., 2001; Monzon and De, Sr., 1999) effects of centrally administered MCH; antagonism of the MCH1 receptor has predominantly anxiolytic-/antidepressant-like effects (Borowsky et al., 2002; Chaki et al., 2005a; Chaki et al., 2005b; David et al., 2007; Lee et al., 2011; Roy et al., 2007; Shimazaki et al., 2006), although not all studies replicate these findings (Basso et al., 2006). These conflicting results are likely due to a number of factors, including the behavioral test used to assess anxiety, the type of antagonist, and the route and time period of administration (e.g., acute or chronic). Nevertheless, in the current study, MCH neurons stained with c-Fos were negatively correlated with self-grooming behavior (Paul et al., 2011), consistent with evidence that MCH antagonizes pharmacologically-elicited grooming behavior (Sanchez et al., 1997). Considering that self-grooming is elicited by HPA-axis activation (Gispen et al., 1975; Gispen and Isaacson, 1981), a number of diverse stressors (Spruijt et al., 1992; van Erp et al., 1994), including social defeat, and has utility as a behavioral index of stress or anxiety levels (Kalueff and Tuohimaa, 2005a; Kalueff and Tuohimaa, 2005b; Steimer and Driscoll, 2003), the link between MCH and grooming behaviors is worth exploring. Overall, our data are consistent with the hypothesis that MCH neurons respond to stressful stimuli and may be relevant for the expression (or suppression) of different anxiety-related behaviors such as self-grooming behavior. Clarifying the role of MCH in behavioral and physiological responses to stress should be an important objective for future research.

Although the Orx-A/Hcrt-1 system provides relatively denser projections to the DR, MCH neurons moderately innervate both the DRD and DRVL/VLPAG subregions of the DR (Elias and Bittencourt, 1997; Yoon and Lee, 2013), an observation that is consistent with the expression of MCH-1 receptor (MCHR1) mRNA and protein in the DR (Hervieu et al., 2000). The correlations between the expression of c-Fos in MCH-ir neurons in the current study and the expression of c-Fos in TPH neurons in DRD and DRVL subregions in the same animals
reported previously (Paul et al., 2011) also support a connection between MCH and serotonergic subpopulations in the DR. The functional role of an MCH-DR projection is unclear, however, there is evidence that MCH actions in the DR facilitate sleep states like REM (Lagos et al., 2009; Lagos et al., 2011a) and also behavioral reactivity towards stress (Lagos et al., 2011b). For example, microinjection of MCH directly into the DR increases reactive coping behavior (e.g., immobility) and decreases proactive coping behavior (e.g., climbing) in the forced swim test, and this MCH-induced increase in reactive coping is blocked by pretreatment with the selective serotonin reuptake inhibitor (SSRI), fluoxetine (Lagos et al., 2011b), suggesting involvement of serotonergic systems in the DR. Our data are consistent with a role for MCH in modulating stress and anxiety-related behavior (Hervieu, 2006; Hervieu et al., 2000; Kerman et al., 2007; Kerman, 2008; Smith et al., 2006) and further suggest MCH may interact with serotonergic systems in the DR to alter behavioral responses to stress.

5. Conclusions

Altogether, these data suggest that stress- and anxiety-related neuropeptidergic systems respond differently to acute and repeated social defeat and these differential responses to social stress may be involved in the shift toward a more reactive coping strategy and away from a proactive coping strategy in rats exposed to repeated defeat. Rather than pointing toward a unitary role of a single neuropeptidergic system in behavioral adaptations to repeated defeat, these data support unique but important contributions of each of the neuropeptidergic systems in these behavioral adaptations, potentially resulting in a fine tuning of defensive behavior following repeated defeat. Extrahypothalamic CRH systems in the BnST and CeA were activated by repeated social defeat, relative to home cage control conditions or acute social
defeat, and were associated with increased reactive coping during social defeat. Both acute and repeated defeat activated MCH neurons and Orx-A/Hcrt-1 neurons. Activation of Orx-A/Hcrt-1 neurons was correlated with increased arousal-related behaviors, whereas activation of MCH neurons was correlated with the anxiety-related and stress-related behavior, self-grooming. These stress-sensitive neuropeptidergic systems may modulate behavior in part through interactions with serotonergic circuits involved in coordinating the appropriate behavioral and physiologic response to stress.
1. Adaptive value of proactive and reactive coping strategies

Rats exposed to neonatal maternal separation responded to social defeat in adulthood with a shift towards a more reactive coping style (e.g., increased freezing) and away from proactive coping (e.g., decreased rearing), consistent with prior studies documenting an anxiety- or depressive-like phenotype in maternally separated rodents (Huot et al., 2001; Kalinichev et al., 2002a; Kalinichev et al., 2002b; Ladd et al., 2000; Wigger and Neumann, 1999). In addition, repeated exposure to social defeat (i.e., two social defeat episodes separated by 24 hrs) produced a very similar shift towards a reactive coping strategy. This is consistent with studies showing that even after a single social defeat encounter animals react to subsequent conspecific aggression with reduced aggression and increased defensive/submissive behavior (Markham et al., 2009b; Markham et al., 2010b; Razzoli et al., 2006b). Together, these data suggest that previous stress, whether early in life or in adulthood, is sufficient to alter behavioral coping strategies during subsequent social defeat. This behavioral shift, although adaptive, is thought to increase vulnerability to the deleterious consequences of stress. As mentioned in Chapter I, the two behavioral coping strategies are associated with two distinct physiological profiles (De Boer and Koolhaas, 2003; Koolhaas et al., 1999), with reactive coping showing increased activity of the HPA axis and increased parasympathetic activity and proactive coping associated with reduced HPA axis activity and increased sympathetic activity.
Rodents with proactive and reactive coping strategies also show other behavioral differences related to routine formation and behavioral flexibility. Rodents with reactive coping strategies have high conditioned immobility and highly flexible behavioral patterns; in contrast, rodents with proactive coping strategies show the opposite behavioral profile. This suggests that rodents with proactive coping styles are more aggressive and rigid in their behavioral repertoire, whereas rodents with reactive coping styles are more docile and flexible in patterns of behavior (Coppens et al., 2010). Koolhaas and colleagues (1999;2007) have argued that proactive coping would be more adaptive in environments that have stable social structures and adequate food and water availability where routine behavior formation can thrive, whereas reactive coping has adaptive value in unstable environments where consistent environmental demands warrant highly flexible, reactive behaviors. Although both coping strategies may have adaptive value in the right circumstances, when a particular coping mechanism fails to properly master the environmental demand, both coping strategies can increase vulnerability to different stress-related disease processes. For example, reactive coping may increase susceptibility to stress-related disorders that show hypersensitive HPA axis responses such as depression and certain anxiety disorders (Frank et al., 2006;Graeff, 2007;Koolhaas et al., 1999;Koolhaas et al., 2007;Pariante and Lightman, 2008); on the other end of the spectrum, proactive coping may increase vulnerability to cardiovascular diseases associated with high sympathetic arousal and hypertension (De Boer and Koolhaas, 2003;Koolhaas et al., 1999).

2. Conclusions

Here we identify two rodent models that result in a shift to a more reactive coping strategy, characterized by increased anxiety- and fear-like behavior, and away from a proactive
coping strategy, characterized by increased confrontation, exploration, and escape. Considering that each coping strategy is associated with vulnerability to particular stressors and disease processes, these two models appear to have utility investigating susceptibility to psychiatric diseases and other medical conditions. In addition, the switch to a reactive coping style was associated with altered patterns of *tph2* mRNA expression in specific subpopulations of serotonergic neurons, including the DRVL/VLPAG, implicated in reactive coping and inhibiting fight-or-flight, and the DRD, part of a neural circuit involved in facilitating conflict-anxiety. Adverse early life experience altered basal *tph2* mRNA in caudal DRVL/VLPAG, suggesting this subregion is particularly sensitive to early life experiences, an effect that may constitute a vulnerability to stress and stress-related disorders (e.g., panic disorder). These altered gene expression patterns may result in the adoption of a reactive or proactive coping style. These data partially agree with the functionally distinct serotonergic pathways proposed in the Deakin and Graeff hypothesis. Altered patterns of serotonergic activity in the DRVL/VLPAG were associated with reactive coping behavior, which is consistent with this subregion's function in promoting reactive coping (e.g., freezing) and inhibiting flight-or-flight behaviors. On the other hand, the MnR was relatively unaltered by maternal separation followed by social defeat in adulthood, in contradiction to what we would predict based on the Deakin and Graeff hypothesis. The MnR, however, may show dysregulation in other aspects of serotonergic neurotransmission.

Exposure to acute or repeated social defeat differentially altered forebrain neuropeptidergic neurons, including Orx-A/Hcrt-1 and MCH systems in the hypothalamus and extrahypothalamic CRH systems in the BnST and CeA. These neuropeptides densely innervate subpopulations of serotonergic neurons in a topographical manner, suggesting they may be involved in stress-induced changes in serotonergic activity. Altogether, these data support the
original hypothesis of this dissertation, that specific subpopulations of serotonergic neurons are sensitive to the effects of stress and are important for stress-induced changes in behavior. Future studies should aim to manipulate the activity of specific serotonergic and neuropeptidergic circuits in order to determine whether altered forebrain neuropeptide afferent input is responsible for the stress-induced changes in serotonergic circuit.
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