EFFECTS OF ADOLESCENT STRESS ON DEPRESSIVE- AND ANXIETY-LIKE BEHAVIORS AND HIPPOCAMPAL MOSSY FIBRE-CA3 REMODELING IN THE NOVELTY-SEEKING PHENOTYPE: IMPLICATIONS FOR EPIGENETIC REGULATION OF THE BDNF GENE

by

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ABSTRACT

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Title: Effects of Adolescent Stress on Depressive- and Anxiety-like Behaviors and Hippocampal Mossy Fibre-CA3 Remodeling in the Novelty-seeking Phenotype: Implications for Epigenetic Regulation of the BDNF Gene

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Experimentally naive rats show variance in their locomotor reactivity to novelty, some displaying higher (HR) while others displaying lower (LR) reactivity, associated with vulnerability to stress. LRHR phenotype is proposed as an antecedent to the development of stress hyper responsiveness. Results presented here show emergence of antidepressive-like behavior following peripubertal-juvenile exposure to chronic variable physical (CVP) and chronic variable social stress (CVS) in HR rats, and depressive-like behavior following CVP in the LRs. The antidepressive-like behavior in HR rats was accompanied by increased levels of acetylated Histone3 (acH3) and acetylated Histone4 (acH4) at the hippocampal brain-derived neurotrophic factor (BDNF) P2 and P4 promoters respectively. This effect may mediate increased mossy fibre (MF) terminal field size, particularly the suprapyramidal mossy fibre projection volume (SP-MF), in the HR animals following both stress regimens. Concurrent with depressive-like behavior in

LRs, decreased SP-MF volume was observed following CVP exposure that is accompanied by decreased acH4 at the BDNF promoter P4. Moreover, CVP resulted in increased social anxiety-like behavior in both LRs and HRs and this effect was accompanied by upregulation of BDNF mRNA in the basolateral amygdala (BLA). In parallel with changes on stress-induced epigenetic regulation of BDNF expression and mossy fibre remodeling, dendritic processes of hippocampal CA3 neurons were affected by CVP and CVS in LRHR rats. Dendritic length, complexity and spine density were decreased in dendritic arbors of CA3 neurons with both CVP and CVS in juvenile LRs whereas in HRs, intensity of CA3 dendritic remodeling was much smaller and restricted to CVS. Moreover, stress-induced depressive-like behavior in LRs was reversed with the pan-HDAC inhibitor, TSA, by increasing the acetylation levels of H4 at the BDNF promoter P4. In HRs, TSA treatment resulted in effects similar to those observed with chronic variable stress exposure on acetylation levels of histones at BDNF promoters and a corresponding antidepressive behavior. These findings show that chronic variable stress during adolescence induces individual differences in molecular, neuromorphological and behavioral parameters between LRs and HRs, which provides further evidence that individual differences in stress responsiveness is an important factor in resistance or vulnerability to stress-induced depression and/or anxiety.

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ABBREVIATIONS

5HT: 5-hydroxytryptamine (Serotonin)

acH3: acetylated Histone3

acH4: acetylated Histone4

ACTH: adrenocorticotropic hormone

AVP: arginine vasopressin

BDNF: brain derived neurotrophic factor

BLA: basolateral nucleus of the amygdala

CeA: central nucleus of the amygdala

CORT: corticosterone

CRF: corticotropin releasing factor

CVP: chronic variable physical stress

CVS: chronic variable social stress

DG: Dentate gyrus

GR: Glucocorticoid receptor

HPA: Hypothalamic pituitary-adrenal axis

HR: High responder

IIP – MF: Intra-infra pyramidal mossy fibre

IR: Intermediary responders

LR: Low responder

MF: Mossy fiber

mPFC: medial prefrontal cortex

NAc: Nucleus accumbens

NE: Norepinephrine

PD: Postnatal day

PVN: Paraventricular nucleus of the hypothalamus

SP-MF: Suprapyramidal mossy fiber

GENERAL INTRODUCTION

A. *The novelty-seeking (LRHR) phenotype is a model of stress responsiveness.*

Neurobiological differences between individuals contribute to significant variance in reactivity to stress (Zuckerman, 1990; Anisman and Zacharko, 1992; Holsboer et al., 1995). Individual differences exist in the degree to which humans voluntarily participate in activities that are associated with personal risk, which is termed sensation-seeking/risk taking personality trait (Zuckerman, 1984). Sensation-seeking/risk-taking behavior in humans initiates stress and anxiety but gives the participant a "thrill", and is associated with vulnerability to drug abuse and emergence of affective disorders including depression and anxiety later in life (Zuckerman and Neeb, 1979). To study such individual differences in stress responsiveness, an outbred rat model of novelty-seeking phenotype (animal model of human sensation-seeking trait) was introduced (Piazza et al., 1989), where experimentally naïve rats were screened based on degrees of locomotor reactivity in a novel environment. In such settings, some rats display high rates of locomotor reactivity to novelty (high responders; HR), and others display low rates (low responders; LR, Figure 1). HR rats repeatedly choose novel environments over familiar ones (Piazza et al., 1989), appear less anxious in the light–dark box and the elevated plus maze (Kabbaj et al., 2000), and demonstrate prolonged stress-induced secretion of corticosterone (CORT) when compared to LR rats (Dellu et al., 1996), suggesting a hyperactive hypothalamic pituitary adrenal axis (HPA). HR and LR rats also have

distinct patterns of basal gene expression for stress-related molecules in regions of the brain critical for the control of stress responsiveness (Kabbaj et al., 2000). HR rats express lower levels of basal corticotrophin releasing hormone (CRH) mRNA in the central nucleus of the amygdala (CeA) and show higher levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) compared to LR counterparts (Kabbaj et al., 2000). In the hippocampus, HR rats show lower glucocorticoid receptor (GR) expression compared to LR rats, and hippocampal microinjection of a GR antagonist results in a phenotypic switch in LR rats towards HR-like anxiety and novelty-seeking behaviors, suggesting that deficits in hippocampal GR activation may promote the novelty-seeking behavior (Kabbaj et al., 2000). Physiologically, novelty exploration is accompanied by increased stress-induced plasma CORT levels in HR rats, thus novelty exploration in HRs does not appear to be less "stressful". In fact, HR rats self-administer CORT more readily than LR rats (Piazza et al., 1993), suggesting that HR rats might perceive stress-induced increases in plasma CORT rewarding. In sum, HRs prefer to engage in novelty-seeking behaviors either despite or because of their ability to activate the stress axis.

HR and LR rats also differ in basal expression levels of serotonin receptors; 5-HT2a mRNA in the parietal cortex and 5-HT1a mRNA in the dorsal raphe (Calvo et al., 2011). When compared to HR, LR rats show higher 5-HT2a mRNA expression in the parietal cortex and lower 5-HT1a mRNA expression in the dorsal raphe (Calvo et al., 2011). Decrease in 5-HT1a receptor function in dorsal raphe is linked to anxiogenic behavior in rats (Pobbe and Zangrossi, 2005) and 5-HT1a knockout mice show significant increase in anxiety-like behavior (Ramboz et al., 1998). These findings are

particularly relevant since these receptors are dysregulated in patients with depression and/or anxiety (Arango et al., 1990; Schechter et al., 1990). Thus, the higher anxiety level in LR rats is associated with dysregulated serotonin neurotransmission in parietal cortex and dorsal raphe, as well as high levels of hippocampal GR expression that implicate alterations in mood as tested on experimental paradigms for anxiety- and depressive-like behaviors. Additionally, HR and LR rats show differences in neuronal activation as shown by mRNA expression levels of the immediate early gene, c-fos, in response to acute exposure to light-dark box anxiety test (Kabbaj and Akil, 2001). HR rats show low expression of c-fos mRNA in the CA1 area of the hippocampus, but high c-fos mRNA levels in olfactory areas, the orbital cortex, the cingulate cortex, the dorsal striatum and the PVN compared to LR rats (Kabbaj and Akil, 2001). Since c-fos reflects neuronal activity, differential regulation of c-fos mRNA expression following exposure to anxiogenic stressors in the LRHR rats suggests that the same stress exposure leads to activation of anatomically distinct brain regions in the LR versus HR phenotype. This is proposed as a basis for observed individual differences in stress responsiveness in the novelty-seeking phenotype (Kabbaj and Akil, 2001).

HR and LR rats also exhibit different molecular and behavioral alterations relevant to depression and anxiety in response to chronic stress. It has been shown that basally HR rats consume more sucrose solution than LR rats, but exposure to four daily consecutive bouts of social defeat stress results in decreased sucrose consumption in HR rats, indicative of depressive-like behavior. However, LR rats' sucrose intake is not affected by social defeat (Hollis et al., 2011). Moreover, levels of Histone 3(H3) and Histone 2B (H2B) acetylation are higher in the hippocampus of HR rats compared to LR

rats in non-stress conditions. Following social defeat, levels of H3 and H2B acetylation are decreased in HR rats, while acetylation of H3 is increased in LR rats, suggesting that histone modifications are potential mechanisms behind observed behavioral differences in depressive-like states in LRHR rats (Hollis et al., 2011). Additionally, another study analyzed the effects of social defeat stress on depressive- and anxiety-like behaviors in HR and LR rats (Duclot et al., 2011). Two weeks after four daily social defeat exposures, HR animals exhibited higher anxiety levels and social avoidance, together with reduced body weight gain and sucrose preference. However, LR animals remained unaffected, suggesting that HR rats but not LR rats demonstrate stress-induced anhedonia, a core symptom of depression, in response to social defeat stress (Duclot et al., 2011). These findings provide compelling evidence that the novelty-seeking phenotype is a valid model to study individual differences in vulnerability or resistance to stress-induced depression- and/or anxiety-like behaviors.

Moreover, the LRHR model has a predictive value for psychostimulant taking behavior (Zuckerman & Neeb, 1979). It has been shown that HR animals acquire self-administration of psychostimulants such as amphetamine and cocaine faster and at lower drug doses when compared to LR rats (Piazza et al., 1989; Hooks et al., 1991; Piazza et al., 2000). Furthermore, LRHR phenotypes are reported to be useful for predicting propensity to self-administer nicotine (Suto et al., 2001). Our laboratory previously showed that HR animals are more vulnerable to the behaviorally sensitizing effects of nicotine, and hippocampal anatomical plasticity is implicated in this behavior (Bhatti et al., 2007, 2009), all of which suggest that HR animals are more vulnerable to psychostimulant-taking behavior. Together, these findings provide compelling evidence

for the validity of the rodent LRHR phenotype model in studying molecular and neural mechanisms that underline the development of stress responsiveness under the general umbrella of vulnerability to emotional reactivity.

Accumulation of stressful life events renders individuals to develop psychiatric disorders such as depression and anxiety (Anisman and Zacharko, 1992) and personality traits play an important role in an individual's vulnerability to mood disorders. Moreover, clinical evidence suggests that vulnerability to mood disorders can be predicted by personality assessment (Cloninger et al., 2006; Kotov et al., 2010). It is therefore crucial to study individual differences in stress responsiveness in an animal model mimicking intra-species stress vulnerabilities, in order to better understand the mechanisms behind the neurobiology of depression and anxiety. Sensation-seeking/risk-taking trait in humans emerges during adolescence (Zuckerman, 2004) and accumulation of stressful life events early on is known to be a predisposing factor in an individual's vulnerability to psychopathologies later in life (Hoffmann & Su, 1998; Cerbone & Larison, 2000; Hoffman et al., 2000). Therefore, the sensation-seeking trait can account for some of the individual differences in emotional reactivity to stress in the human population. Animal model of human sensation-seeking/risk taking behavior (LRHR) is therefore used to understand the role of individual differences in the neurobiology of stress-induced depression and anxiety. Thus, identifying individual mechanistic differences in stress responsiveness will be very valuable in developing potential treatments for depression and/or anxiety to overcome personality trait-induced resistances to current treatments.

Although the aforementioned animal data was obtained from adults, the effects of chronic stress exposure in juvenile LRHR rats have not been investigated. Since this

period is marked by heightened responsiveness to environmental perturbations, it is important to understand how adolescent brain responds to stress. In this dissertation, I will use the LRHR rat model to study individual differences in vulnerability to emotional reactivity defined as depressive- and anxiety-like behaviors in response to chronic stress exposure during a critical period in adolescence (i.e., the peripubertal-juvenileperiod).

A. **B.**

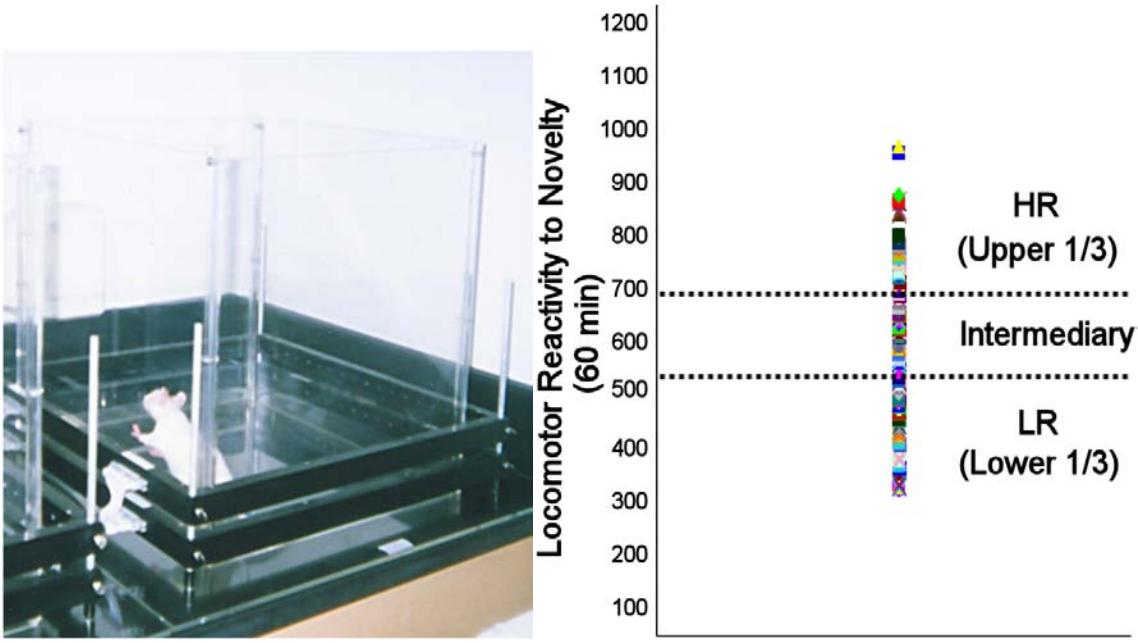
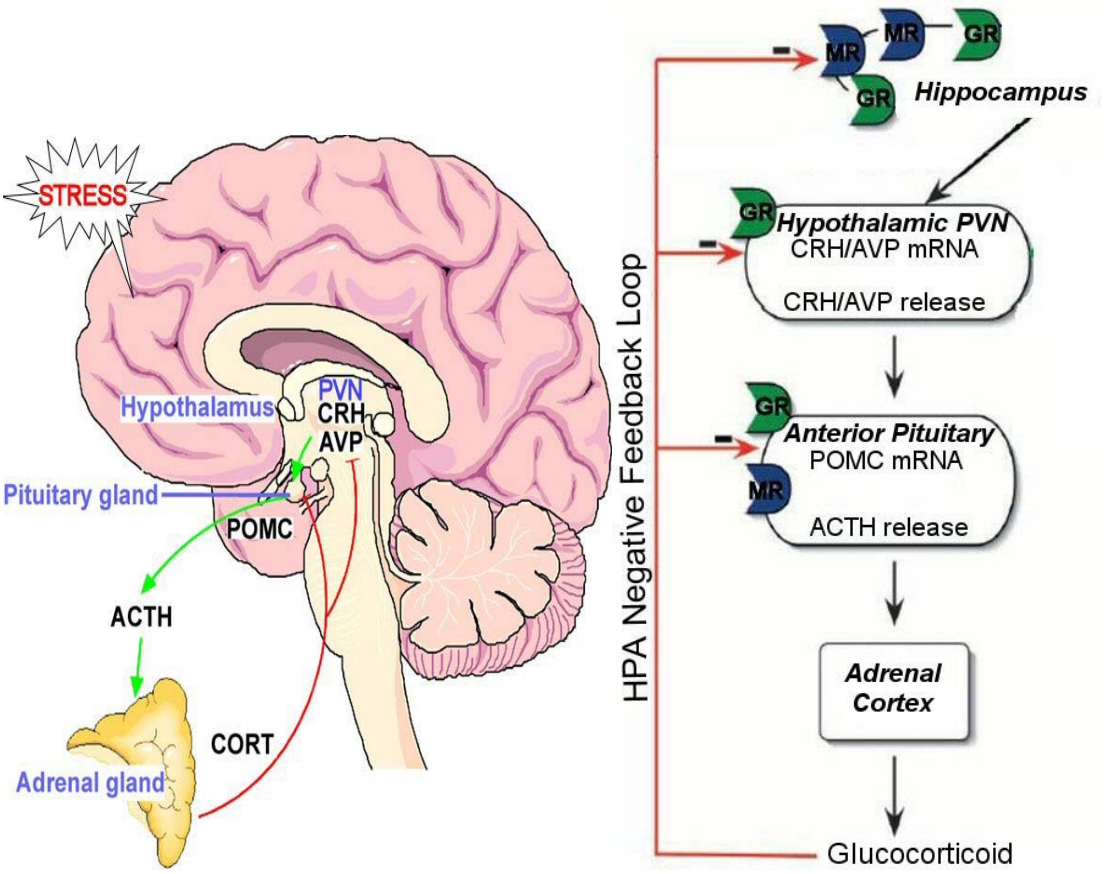


Figure 1: LRHR phenotype screening. A) Locomotor reactivity to novelty is tested in 43 X 43 X 24.5 cm clear Plexiglas cages with stainless steel grid flooring. Activity is monitored by means of two banks of photocells (total of photocells X=16; Y=16) connected to a microprocessor. Rearing is monitored by activity on the upper bank of photocells. B) At the end of a 60 min screening session, total locomotor activity (i.e., X, Y and Z locomotion) are pooled and the distribution of the rat population according to their locomotor reactivity to novelty is determined. Rats are ranked as HRs (i.e., rats that exhibit locomotor scores in the highest third of the sample) or LRs (i.e., rats that exhibit locomotor scores in the lowest third of the sample).

B. ***Physiological, molecular and structural effects of chronic stress.***

The HPA axis is the primary neuroendocrine axis that mediates the stress response (Herman et al., 2003; Smith et al., 2006). In response to stress, stress-related hormones [such as CRH, adrenocorticotropic hormone (ACTH), arginine vasopressin (AVP), glucocorticoids] are secreted by the HPA axis and these help animals to cope with adverse effects of stressful events (Sapolsky et al., 2000b; McEwen, 2007). The release of stress hormones by the HPA axis is driven by the release of AVP and CRH from the PVN. These two peptides are released into the portal system and stimulate the secretion of ACTH from anterior pituitary, which in turn induces the secretion of glucocorticoids (CORT in rodents and cortisol in humans) from the adrenal cortex (Figure 2A). To maintain HPA axis activity at homeostatic levels, glucocorticoids exert negative feedback on the PVN, hippocampus and pituitary gland to inhibit the further synthesis and secretion of CRH, AVP and ACTH through a GR-mediated shutdown mechanism (Figure 2B; Herman et al., 1997; Sapolsky et al., 2000b), the net effect of which is a return to baseline levels. However, repeated exposure to stress results in elevated concentrations of glucocorticoids over an extended periods of time due to inability to shut down the HPA axis, and causes impaired negative feedback by way of deficits in GR signaling, thereby resulting in a hyperactive HPA axis response (Figure 2B; Sapolsky et al., 1984; Hermanet al., 1995; Mizoguchi et al., 2003).

A. **B.**



Adapted from Murgatroyd and Spengler, 2011

Figure 2: A) A schematic of HPA axis activity in response to stress. The release of CRH and AVP from the PVN into the portal blood vessels leads to secretion of ACTH from the anterior pituitary, which then acts on the adrenal cortex to synthesize and release glucocorticoids. B) A negative feedback loop is critical for shutting down the HPA axis. The activational effects of the HPA axis are counteracted by the inhibitory effects of glucocorticoid receptors expressed in the hippocampus, hypothalamus, and anterior pituitary (Murgatroyd and Spengler, 2011).

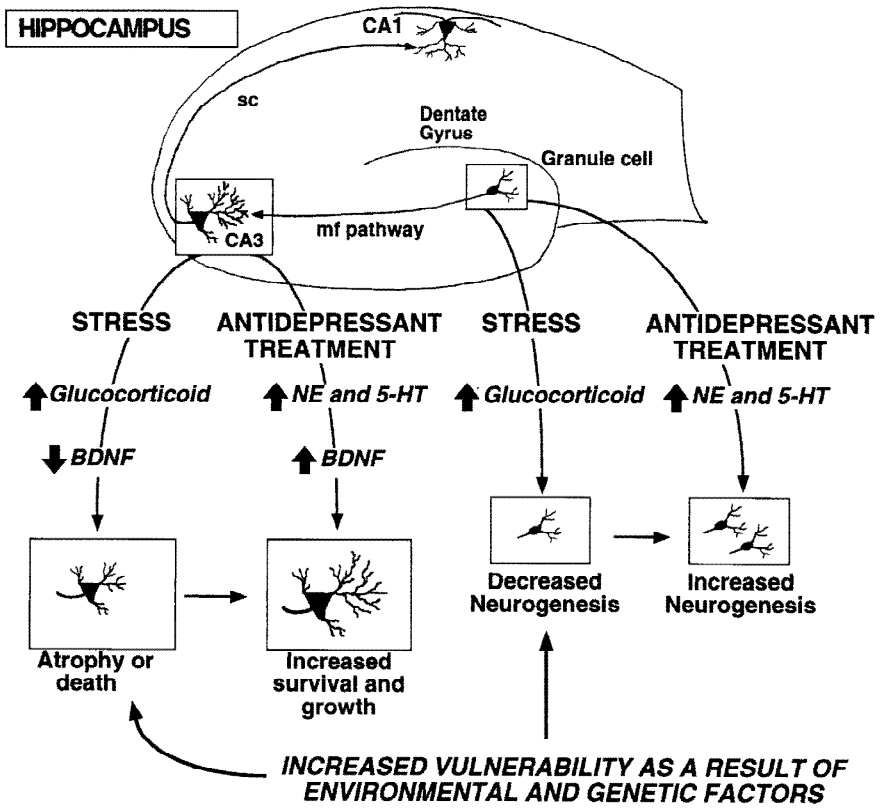
HPA axis hyperactivity has been implicated in patients with major depressive (Nemeroff et al., 1992; Holsboer, 2000; Cowen, 2002; Pariante and Lightman, 2008; Pariante, 2009) and anxiety disorders (Condren et al., 2002; Risbrough and Stein, 2006; Abelson et al., 2007; Martin et al., 2009). Impaired GR-mediated negative feedback inhibition of the HPA axis in depressed patients has been demonstrated by administration of the synthetic glucocorticoid, dexamethasone, which fails to suppress elevated plasma levels of cortisol due to desensitization of GRs (reviewed in Arana et al. 1985; Baldessarini and Arana 1985; Ribeiro et al. 1993). In parallel with clinical research, detrimental effects of long-term CORT elevation have been shown in animal studies using chronic CORT treatments that induce depressive- and anxiety-like behaviors (Ardayfio and Kim, 2006; Murray et al., 2008; David et al., 2009). Moreover, hypersecretion of CRH has been implicated in patients with depression, and anxiety (de Kloet et al., 2005; Arborelius et al., 1999; Holsboer, 1999; Holsboer and Ising, 2010). Consistent with human data, central administration of CRH in rodents produces phenotypic alterations reminiscent of symptoms of depression and anxiety (for review see; Holsboer, 1999). Taken together, these findings show that chronic stress is a model to study physiological and molecular mechanisms underlying depressive- and anxiety-like behaviors in animal studies, since HPA axis dysfunction is evident in both clinical and laboratory settings following chronic exposure to stress.

Long-term elevation of CORT with chronic stress causes atrophy and, in severe cases, death of hippocampal CA3 neurons (Sapolsky et al., 1990; McEwen, 2000; Sapolsky, 2000a). In addition, sustained elevation of CORT results in decreased numbers of newly generated granule neurons in the adult hippocampal dentate gyrus (DG, for

review see; Fuchs and Gould, 2000; see McEwen 1999; Malberg and Duman 2003; Vollmayr et al 2003). These stress-induced changes in hippocampal morphology observed in laboratory models could be a factor in the reduced hippocampal volume observed in some depressed patients, based on post mortem brain analyses (Sheline et al., 1999; Bremner et al., 2000). Moreover, animal studies have shown that antidepressant treatments oppose these stress-induced morphological changes by preventing the atrophy of CA3 pyramidal cells (Watanabe et al 1992; Norrholm and Ouimet, 2001) and up-regulating adult neurogenesis of dentate gyrus granule neurons (Duman and Malberg, 1998; for review see Duman et al. 2000; Malberg et al. 2000). These findings indicate a common neuronal pathology linking depression and chronic stress in animal models, confirming the validity of these animal models in studying physiological, molecular and structural outcomes of depression.

Investigation of the mechanisms underlying chronic stress-induced hippocampal remodeling implicates a critical role for brain-derived neurotrophic factor (BDNF). BDNF is a peptide neurotrophic factor in the brain that is important for neuronal morphological differentiation and synapse formation during development, but is also required for the survival and plasticity of existing neurons in the adult brain (Thoenen, 1995; McAllister et al., 1999; Lu et al., 2008; Rauskolb et al., 2010). Chronic stress such as occurs with social defeat (Tsankova et al., 2006) or immobilization (Smith et al., 1995; Vollmayr et al., 2001; Roceri et al., 2002; Nair et al., 2007) in adult animals is reported to decrease BDNF expression in the hippocampus, which is associated with atrophy of CA3 pyramidal neurons (for review see Duman et al., 1999). This reduction in BDNF is partly regulated by stress-induced glucocorticoids and partly by other mechanisms such as

stress-induced deficits in cyclic adenosine monophosphate (cAMP)-cAMP response element-binding protein (CREB) signaling. CREB phosphorylation levels affect BDNF expression through CREB transcriptional regulation. Lower levels of brain BDNF gene expression in response to chronic stress is associated with depression, and can be reversed by antidepressant treatments (for review see; Duman et al., 1999; 2000 and Nestler et al., 2002). Indeed, the 'neurotrophic hypothesis of depression' postulates that antidepressant treatments can reverse the inhibition of BDNF gene expression caused by chronic stress via the cAMP-CREB cascade (Duman et al., 1997; Altar, 1999), which in turn either blocks or reverses atrophy of CA3 cells and/or CA3 cell death (Figure 3). Moreover, BDNF plays an important role in another stress-reactive limbic region, the amygdala, in terms of synaptic plasticity and emotional behavior. In particular, BDNF in the amygdala is critical for molecular and structural changes associated with chronic stress-induced anxiety. For instance, BDNF expression levels are increased in the amygdala in a sustained manner following chronic social defeat stress (Fanous et al., 2010). Chronic stress induced anxiety-like behavior has been shown following adolescent social isolation (Lukkes et al., 2009), early life maternal separation, and chronic restraint (Eiland and McEwen, 2010). These findings are linked to increased spine density in the basolateral amygdala (BLA, Vyas et al., 2006; Mitra et al., 2005), together with a long-term increase in amygdalar BDNF (Fanous et al., 2010) that is proposed to mediate this morphological plasticity. These reports provide compelling evidence that BDNF is a critical neurotrophin that mediates the cellular effects of chronic stress in depressive- and anxiety-like behaviors in the hippocampus and amygdala, respectively.

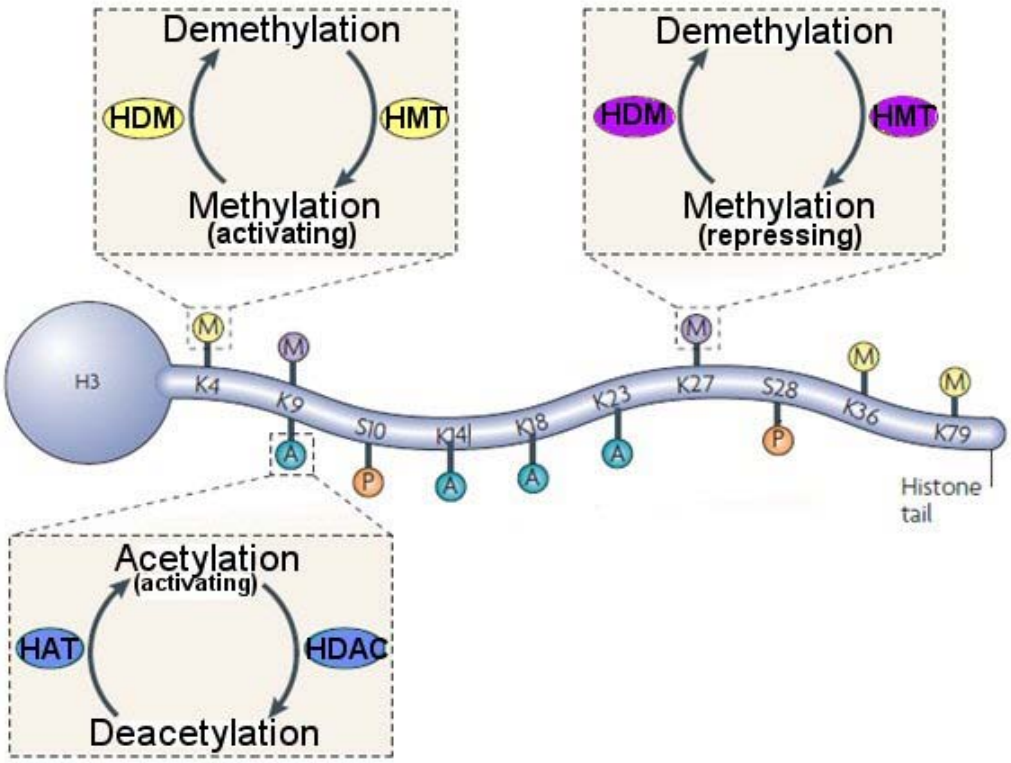


Adapted from Duman et al., 1999

Figure 3: Diagrammatic representation of the actions of stress and antidepressant treatment on hippocampal neurons. Chronic stress and subsequent long-term elevation of glucocorticoids cause atrophy and, in severe cases, death of CA3 pyramidal neurons and decrease neurogenesis of dentate gyrus granule cells in the hippocampus. Antidepressant treatments are proposed to oppose these actions of stress and either block or reverse hippocampal atrophy and/or cell death and decreased neurogenesis. The mechanisms underlying such actions of antidepressants involve upregulation of BDNF via increased Norepinephrine (NE) and 5-hydroxytryptamine (5-HT) signal transduction. CA1: CA1 pyramidal cells, mf pathway: Mossy fibre pathway, sc: Schaffer collaterals (Duman et al., 1999)

In addition to direct regulation of the BDNF gene by transcriptional factors such as CREB, there are also epigenetic regulators that can be important for the modulation of BDNF gene transcription. Epigenetic regulation is defined as histone and DNA modifications that influence the variety of gene expression (Jaenisch and Bird, 2003; Margueron et al., 2005). Acetylation/deacetylation of N-terminal domains of core histone proteins regulates chromatin structure and gene expression (Strahl and Allis, 2000). Histone acetylation is catalyzed by histone acetyltransferases (HATs) which add acetyl groups to N-terminal domains of histones, thereby reducing the affinity between histones and DNA, resulting in increased gene expression or activation (Sterner and Berger, 2000). Histone deacetylation is catalyzed by a family of histone deacetylase (HDAC) proteins (see Figure 4 for an example of modifications on the Histone 3). There are three major classes of HDACs; class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), and the Sir2 family of NAD^+ - dependent HDACs (Marks et al., 2003). HDACs remove acetyl groups from N-terminal domains of histones, lead to tighter DNA coiling and thereby result in gene repression or silencing (Hong et al., 1993; Strahl and Allis, 2000). Methylation is mediated by histone methyltransferases (HMTs) at lysine or arginine residues of histones and is reversed by histone demethylases (HDMs). Methylation of histones at lysine residues can either activate transcription (H3-K4 and H3-K36) or repress (H3-K9 and H3-K27) transcription of the genomic region (Shi et al., 2004). Thus, interplay between HATs and HDACs, or HMTs and HDMs appear to be important for chromatin remodeling of a variety of genes, such as the BDNF gene. Chronic social defeat stress (an animal model of depressive-like state), has been shown to induce social avoidance behavior and long-lasting increases in H3-K27 demethylation, a

repressive modification, at the P3 and P4 promotors in the BDNF gene, as well as long-lasting downregulation of total BDNF mRNA levels in the mouse hippocampus (Tsankova et al., 2006). However, antidepressant treatment reverses the repression of BDNF gene expression via Histone 3 (H3) acetylation, as well as H3-K4 methylation, an activating modification, at the same BDNF promoters (Tsankova et al., 2006). These effects of antidepressant treatment appear to be mediated at least in part by downregulation of HDAC5 in the hippocampus (Tsankova et al., 2006). Moreover, chronic electroconvulsive seizures (ECS; an effective treatment for depressed patients) have been shown in rats to induce increases in H3 acetylation at the BDNF P3 and P4 promoters, in correlation with increased BDNF mRNA levels in rat hippocampus (Tsankova et al., 2004). Therefore, these studies provide compelling evidence for epigenetic regulation of hippocampal BDNF expression in development of the depressive state. The present dissertation will investigate the effects of chronic variable stress, along with systemic administration of a HDAC inhibitor, on epigenetic regulation of hippocampal BDNF gene expression and associated changes in depressive- and anxiety-like behaviors in LRHR rats.



Adapted from Tsankova et al., 2007

Figure 4: Representation of chromatin remodeling via common covalent modifications on Histone 3 (H3), which include acetylation and methylation at several amino acid residues. Acetylation is catalyzed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs); lysine methylation (which can be either activating or repressing) is catalyzed by histone methyltransferases (HMTs) and reversed by histone demethylases (HDMs; Tsankova et al., 2007).

In this dissertation, a chronic variable stress paradigm will be used to investigate individual differences in the effects of chronic stress using the LRHR stress vulnerability rat model. This paradigm was originally described in a protocol by Katz and colleagues (1981) to counter the habituating effects of repeated stress in animal studies. The original regimen incorporated physical stressors (e.g., restraint, cold exposure, and loud noise) assigned in a random exposure sequence at varying times in one day and at varying durations. In addition to this, Isgor and colleagues (2004a) developed a parallel regimen using social stressors (e.g., isolation, novel environment, crowding) that are commonly utilized in the literature and applied with the same parameters. Hence, a chronic variable social stress regimen was designed as a model for psychosocial stressors that may have relevance for human stress conditions. Even though chronic variable physical (CVP) and chronic variable social (CVS) stressors are physiologically comparable when administered acutely (Isgor et al., 2004a), when they are applied chronically during the peripubertal-juvenile period (PD28-56), they appear to result in long term differential alterations in the physiological response to stress, in hippocampal GR expression and in the morphology of the hippocampus (Isgor et al., 2004a). Particularly, CVP exposure leads to reduced hippocampal volume in the CA1, DG and the CA3 cell layers during transition into young adulthood (following 3 weeks of recovery from stress), effects that are associated with impairments in Morris water-maze navigation, downregulation of hippocampal GR expression, and deficits in feedback shutdown of acute stress-induced CORT secretion. Even though similar downregulation of hippocampal GRs and elevated CORT responses to acute stress are observed with CVS, these effects are reversed with 3 weeks of recovery into young adulthood (PD 77). Moreover, these two stress regimens

have been characterized under exposure to a pharmacological stressor, namely repeated amphetamine treatment; showing that although CVP leads to robust expression of locomotor sensitization to amphetamine, CVS leads to inhibited locomotor activity to amphetamine challenge (Kabbaj et al., 2002). This suggests differential neural adaptations in the brain reward circuitry (Kabbaj and Isgor, 2007). These findings suggest that CVP and CVS exposure during adolescence differentially modulate HPA axis function, hippocampal morphology and brain reward circuitry. In this dissertation, I will study CVP vs. CVS-induced neuronal mechanisms that play important roles in the emergence of individual differences in behavioral and structural responses to chronic stress, using the LRHR animal model.

C. ***Adolescence is a critical developmental period for stress vulnerability.***

Adolescence is characterized by major changes in neural, hormonal, and behavioral systems. Despite its gradual onset and offset there are differences in these systems between adolescence and adulthood. Adolescence in laboratory rodents consists of three stages; a prepubescence/early adolescence period from PD 21 to PD 34, a mid-adolescence period from PD 34 to PD 46, and a late adolescence period from PD 46 to PD 59 (Spear, 2000; Tirelli et al., 2003; McCormick et al., 2010). Human and animal studies support the notion that adolescence is a developmental period of stress vulnerability, and accumulation of stressful experiences during pubertal maturation increases the frequency of psychological disorders later in life, such as depression, anxiety and drug abuse (Kessler & Magee, 1993; Andersen et al., 2003; Costello et al., 2003; Penza et al., 2003; Dahl R.E., 2004; Heim, Plotsky, & Nemeroff, 2004; Patton et al., 2007). During adolescence, both progressive (i.e., neurite fiber sprouting,

myelination) as well as regressive (i.e., synapse pruning) processes take place in the brain, suggesting that this period marks the maturation of neuronal connectivity (Giedd et al., 1999; Cunningham et al., 2002). Brain areas involved in emotional behavior such as the hippocampus continue to grow across the peripubertal-juvenile period into young adulthood (Bayer et al., 1982; Sousa et al., 1998; Isgor et al., 2004a). Stress-induced structural and functional changes in brain regions implicated in stress reactivity and emotionality are hypothesized to render the adolescent individual more vulnerable to develop psychopathologies later on. In this dissertation, I will use the LRHR phenotype to assess individual differences in the neurobiology of stress responsiveness during a developmental period that falls inside the peripubertal- juvenile period (PD 28 and PD 42, Toledo-Rodriguez and Sandi, 2011), which is characterized by heightened responsiveness to environmental perturbations.

In fact, chronic stress applied during adolescence leads to robust behavioral effects in adulthood in the laboratory setting. For instance, three days of unpredictable physical stress exposure during the juvenile period leads to heightened anxiety-like behaviors in rats in over a third of the stressed population, demonstrated by decreased time spent in the open arms of the elevated plus maze. An additional third of the population displays depressive-like behavior as evidenced by decreased avoidance shuttles and increased escape failures during a two-way passive avoidance task in adulthood (Tsoory et al., 2007). These results suggest that even short periods of variable stress during the juvenile period can induce long term alterations in emotional behaviors. Moreover, another study examined the effects of early chronic social stress (1 h isolation, daily and change of cage partner between PD 30 and 45) on anxiety-like behavior in

adulthood and revealed that adult rats exhibited increased anxiety behavior as indicated by decreased time spent in the open arms of an elevated plus maze (McCormick et al., 2008). In a different study Wilkin et al (2012) showed that severe chronic variable stress exposure (water immersion, elevated platform, foot shock) from PD 22 to 33, results in increased anxiety (tested on elevated plus maze) and depressive behaviors (tested on forced swim test) in adult male rats. Thus, these reported effects support the notion that juvenile stress induces lasting impairments in stress-coping responses, and vulnerability to depression and anxiety may be shaped by experiences in adolescence.

In contrast to the reported deleterious effects of chronic stress during adolescence on depressive- and anxiety-like behaviors, there are also reports that show protection from later stress-induced effects when stressors are applied during adolescence. For instance, chronic unpredictable stress exposures (daily 1 h isolation and new cage partner on PD 30–45) produced no difference between stressed and control adolescent rats in terms of depressive behavior assessed during the forced swim test, in spite of increased corticosterone release in chronically stressed male rats (Mathews et al., 2008). Similarly, in response to chronic variable stress (CVS, repeated exposure of restraint, hypoxia, open field, cold and shaker stress between PD 35-48) adolescent animals did not show a latter increase in immobility on the forced swim test, but demonstrated resistance to the effects of CVS on depressive behavior (Jankord et al., 2011). Interestingly, psychogenic stress (novelty, odor exposure and placement on an elevated platform) during PD 28-30 and PD 40-42 decreased anxiety-like behaviors measured on the elevated plus maze, and increased risk-taking and novelty-seeking behaviors in adolescent rats, with no effect on depressive behaviors and the HPA response (Toledo-Rodrigez and Sandi, 2011).

Corroborating findings in additional recent studies also showed increased risk-taking and decreased anxiety-like behaviors following other forms of adolescent stress (Burke et al., 2010; Ito et al., 2010; Jacobson-Pick and Richter-Levin, 2010; Jacobson-Pick et al., 2011). Thus, some of the literature suggests resilience in response to the adverse effects of stress during adolescence. The discrepancies in these reported effects may very well be due to individual differences in stress responsiveness, and the impact of different types of specific stressors, which will be investigated in this dissertation.

In this dissertation, the LRHR rat model will be used to investigate the behavioral, neurobiological and structural effects of chronic variable stress during the peripubertal-juvenile period. In **Chapter 1**, the effects of chronic variable stress on depressive- and anxiety-like behaviors and related changes in hippocampal and amygdalar BDNF levels will be assessed. Particularly, stress-induced changes in epigenetic regulation of hippocampal BDNF gene expression will be studied. These changes then will be linked with changes in MFs, the primary innervation of the stress-responsive CA3 neurons in hippocampus. In **Chapter 2**, I will further assess remodeling of projections induced by chronic variable stress during adolescence, as measured on receptive CA3 dendritic surfaces in LRHR rats, to determine if structural changes in mossy fibre terminals are accompanied by changes in CA3 dendritic arbors. In **Chapter 3,** following administration of a pan-HDAC inhibitor during chronic variable stress exposure, I will investigate interactions between depressive-like behavior and epigenetic regulation of hippocampal BDNF gene expression to assess molecular mechanisms that may underlie behavioral changes induced by chronic variable stress during the peripubertal-juvenile period.

CHAPTER 1: CHRONIC VARIABLE STRESS DURING PERIPUBERTAL-JUVENILE PERIOD CAUSES DIFFERENTIAL DEPRESSIVE AND ANXIETY EFFECTS IN THE NOVELTY-SEEKING PHENOTYPE: EFFECTS IN HIPPOCAMPAL AND AMYGDALAR BDNF AND THE MOSSY FIBRE TERMINAL FIELDS

Introduction

The hippocampus, and specifically the MF system, is critically involved in detection of novelty (Vinogradova, 2001). Moreover, morpho-behavioral correlations in genetically-altered mice have implicated hippocampal MF in exploratory behavior (Roullet and Lasalle, 1993; Ivanco and Greenough, 2002; Mineur and Crusio, 2002). The MF axons of dentate granule cells relay multimodal sensory information from the entorhinal cortex to the hippocampus proper (CA3 dendrites), which upon receipt is compared with previously stored information (Amaral and Witter, 1989; Witter et al., 1989; Vinogradova, 2001). It is plausible that such a mechanism to detect environmental novelty may be a necessary component of the novelty-seeking phenotype (i.e., LRHR phenotype). Hippocampal MF remodeling continues throughout the peripubertal-juvenile period, and experiential factors affect the sprouting and/or pruning of these axon terminals during this time (Gomez-Di Cesare et al., 1997). The mossy fibres comprise the axons of the dentate gyrus (DG) granule neurons that innervate the CA3 neurons (for review see; Gaarskjaer, 1986) and stress-induced dendritic atrophy occurs in these neurons. The granule cell axons contains high levels of BDNF (Conner et al., 1997; Yan et al., 1997; Dieni et al., 2012) and hippocampal BDNF is implicated in chronic

stress-induced morphological changes in hippocampus (for review see; Duman et al., 1999). Therefore, alterations in hippocampal BDNF levels in response to chronic stress exposure during the peripubertal-juvenile period in LRHR rats could mediate structural changes in the MF-CA3 synapse, during the time when this fibre pathway normally undergoes extensive remodeling.

It has been previously shown that decreased levels of hippocampal BDNF, and subsequent neuronal atrophy is correlated with depression, whereas anxiety is associated with increased levels of amygdalar BDNF and increased dendritic spine density in the BLA in adult animals (for review see Vaidya and Duman, 2001; Vyas et al., 2002; 2006; Mitra et al., 2005; Fanous et al., 2010). Thus, BDNF is thought to play an important role in the regulation of stress-induced cellular changes through its binding of full length TrkB receptors, mediating region-specific responses to stress in the brain. In this chapter of the dissertation, I will use a previously described, chronic variable stress regimen (CVP and CVS; Isgor et al., 2004a) to study individual differences in the neurobiology of stress reactivity using the outbred rat model of the novelty-seeking phenotype (i.e., LRHR). This experiment will investigate the effects of chronic variable stress during the peripubertal-juvenile period on depressive- and social anxiety-like behaviors and the associated changes in hippocampal and amygdalar BDNF and MF plasticity in the novelty-seeking phenotype. I will test the hypotheses that the LRHR model can reliably predict stress-specific alterations in hippocampal MF plasticity, and that BDNF modulation in the hippocampus and amygdala will be associated with stress-induced depressive- and anxiety-like behaviors.

Methods

LRHR phenotype screening

Animals were treated in accordance with the National Institutes of Health guidelines on laboratory animal use and care. Male Sprague-Dawley rats (N=108, Charles River, Wilmington, MA) arrived at weaning (postnatal day, PN 22), were housed in 43 X 21.5 X 25 cm clear acrylic cages, and were allowed ad libitum access to food and water. Animals were kept on a 12 hr light/dark cycle (lights on at 7:00 A.M.). Animals were allowed to habituate to the housing conditions and were handled daily for 2 days. On PN 25, animals underwent locomotor screening for 60 min in commercially-available locomotion chambers (San Diego Instruments, San Diego, CA). Locomotor reactivity to novelty was tested in 43 X 43 X 24.5 cm clear Plexiglas cages with stainless steel grid flooring. Activity was monitored by means of two banks of photocells (total of photocells X=16; Y=16) connected to a microprocessor. Two frames of photocells were used; the lower frame was located 2.5 cm above the grid floor and the upper frame was located 12.5 cm above the floor of the box. Horizontal locomotion was monitored by the lower bank of photocells. Each horizontal activity count recorded a minimum 14.3 cm traversing of the cage. Rearing was monitored by activity on the upper bank of photocells. At the end of a 60 min screening session, total locomotor activity (i.e., X, Y and Z locomotion) were pooled and the rats were ranked as HRs (i.e., rats that exhibited locomotor scores in the highest third of the sample) or LRs (i.e., rats that exhibited locomotor scores in the lowest third of the sample).

Chronic variable stress exposure

For the chronic stress regimen, three stressors were selected from the published literature, and utilized in our previously published work (Isgor et al., 2004a; Kabbaj and Isgor, 2007). Stressors were applied in a systematic random order and at varying times of day (one or two stressors per day) for a total of 15 stress exposures to avoid habituation. Control animals were handled daily. Chronic stress or control handling occurred between PN 28-42 (Table 1).

Physical stressors: 1) Ether exposure: Animals were transferred to an ether chamber located outside of the home colony in groups of four, for a maximum of 30 s. After complete recovery from ether, animals were returned to the home colony. 2) Restraint: Animals were wrapped individually in flexible Teflon, which was secured with Velcro closures to limit movement for 2-hr. 3) Cold exposure: Animals were transferred to a cold box (4^0C) for 2-hr.

Social stressors: 1) Isolation: Animals were transferred into cages similar to home cages and placed in a room other than the home colony for 2-hr with free access to food and water. 2) Novel environment: Animals were individually placed in novel environments that consist of boxes with different geometric shapes (circular, rectangular, triangular), color and luminosity for 2-hr. 3) Crowding: A group of animals (n=16) were placed in one cage similar to the home cage for 2-hr in the home colony. As animals matured (PD 35-42), total number of animals placed in a cage during crowding session was decreased to n=8. Twenty-four hours after the last stress exposure or control handling (PN 43), all animals were tested on the social interaction and forced swim tests.

Table 1: Behavioral procedure for phenotype screening and chronic variable stress exposure.

Animals arrive	Rest	Phenotype screening (LRHR)	Rest	CVP,CVS or control handling	Behavioral testing and sacrifice
PD 22	PD 23-24	PD 25	PD 26-27	PD 28-42	PD 43

CVP: Chronic Variable Physical stress, CVS: Chronic Variable Social stress, PD: Postnatal day

Social interaction test

The social interaction (SI) test was developed by File and Hyde (1978) to measure anxiety-like behavior in rodents. The SI test is sensitive to both anxiolytic and anxiogenic effects and does not require to use of aversive (e.g. electric shock) or appetitive (e.g. food or water deprivation) conditions (for review see File and Seth, 2003). The SI test in rodents is commonly used in research on anxiety-like behavior and this test reveals social behaviors that loosely reflect altered social communication and interactions (for review see File and Seth, 2003). Anxiety disorder in humans indeed affects an individual's interpersonal relationships and ability to interact socially with people around them (Gelder et al., 2005). Therefore, the SI test was used to assess anxiety-like behavior in a social context following chronic variable stress regimen in LRHR phenotype to model the human social fobia state. An experimentally naive resident rat was placed in a 23 X 15 X 13 cm rubber rectangular box and allowed to habituate to its surroundings for 8 min. After the initial 8 min, an age- and weight-matched experimental rat was placed in the box and both rats were allowed to interact for 5 min. Resident rats were used only once and, between each trial, the box was wiped thoroughly with ethyl alcohol. Social behaviors elicited by the experimental rat towards the resident rat were measured (i.e., sniffing, following, grooming and crawling over or under) as described by File (1980) and published by our laboratory (Aydin et al., 2010). No aggressive behaviors were observed. Total amount of social interaction per animal was measured by adding the duration of all of the social interaction behaviors demonstrated by the experimental animal, and percent time spent in social interaction was calculated.

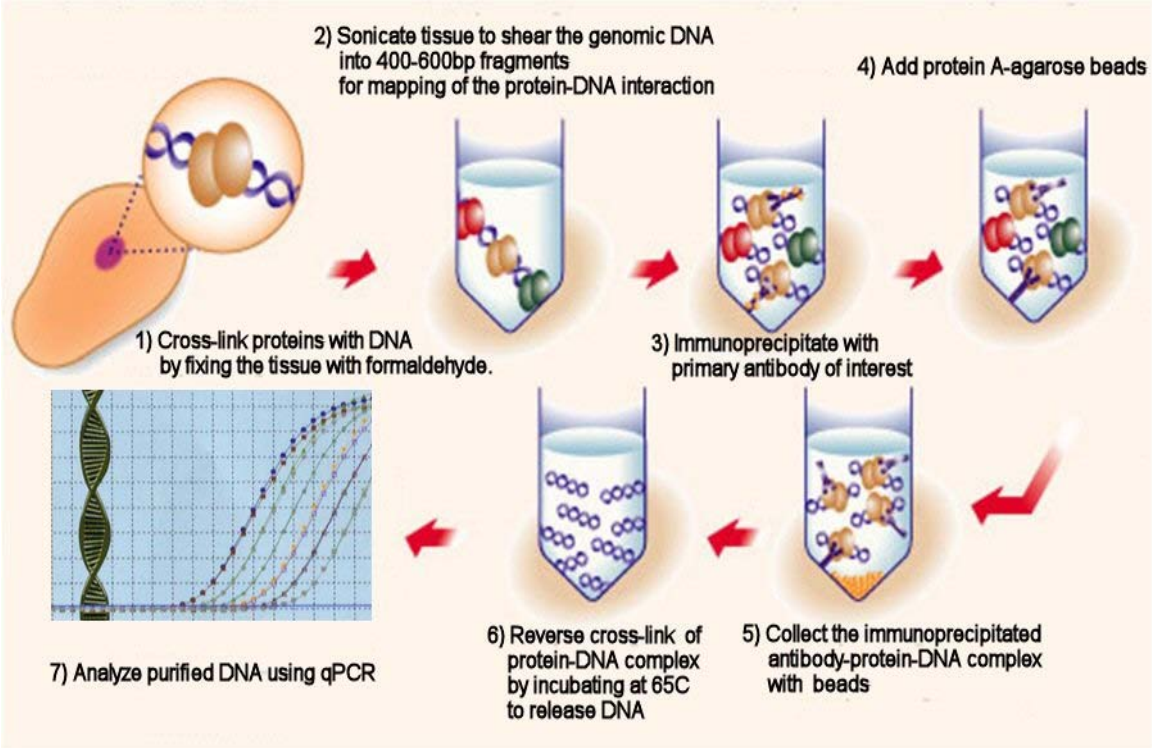
Forced swim test

The forced swim test was introduced by Porsolt et al., (1977) as a paradigm that tests behavioral state of "despair" and since then it has been widely used to assess depressive-like behavior and measure the clinical efficacy of antidepressant drugs. Therefore, depressive-like behavior in HR and LR rats was assessed using the forced swim test following exposure to chronic variable stress. Briefly, rats were placed in a 30 cm diameter cylinder filled with water (25°C). Each rat was tested for 5 min and the cylinder was cleaned and filled with fresh water following each animal. Behavior constituting immobility (defined as the absence of movement with the exception of what is necessary to keep the animal's head above water) was scored. Total amount of time spent as immobile per animal was measured by adding the duration of immobility demonstrated by the experimental animal in a single session, and percent time spent in immobility was calculated.

At the completion of behavioral testing, animals were sacrificed by rapid decapitation. For half of the animals (n=6 per experimental group), one hemisphere of each brain was harvested and snap frozen for *in situ* hybridization histochemistry, and the dorsal hippocampus from the remaining hemisphere was dissected and used for chromatin immunoprecipitation (ChIP) assay. For the second group of the animals (n=6 per experimental group), brains were harvested and snap frozen for Timm's method for silver sulfide staining and subsequent morphological analyses using design-based stereology.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed following a published protocol (Tsankova et al., 2004). Briefly, tissue punches from dorsal hippocampi were pooled and fixed with 5% formaldehyde to cross-link DNA with associated proteins. Fixed hippocampal tissue was sonicated using a sonicator (Fisher Scientific, PA) twice for 10 sec at low setting and then sonicated three more times for 15 sec at maximum power to shear the chromatin to 400-500 bp using a cell lysis buffer (1% SDS (10%),1 mM Tris-HCl (pH 8.0) and 100 mM 0.5 M EDTA). After spectrophotometer quantification, equal amounts of chromatin lysate (60 μg) were diluted with ChIP dilution buffer to a final volume of 1 ml. Samples were then immunoprecipitated overnight at 4°C with one of the following antibodies: 5 μg of anti-acetyl H3 antibody (detects acetylation on Lys9 and Lys14, Millipore, CA), 5 μg of anti-acetyl H4 antibody (detects acetylation at Lys5, Lys8, Lys12, and Lys16, Millipore, CA) and 5 μg nonimmune rabbit IgG antibody (Millipore, CA) used as a control. The immunoprecipitate was collected using Protein A beads from Thermo Scientific (Rockford, IL). The beads were washed once with low salt (NaCl), high NaCl, and LiCl and then washed twice with TE (Tris-EDTA) buffers. After washing, the DNA–histone complexes were eluted from the beads and then dissociated by incubating at 65^0C using proteinase K. DNA was extracted, purified and quantified using quantitative real time-PCR (Stratagene Mx3000P QPCR system, CA, Figure 5).



Retrieved from Vinci-Biochem, http://www.vincibiochem.it/AMChIP-IT.htm

Figure 5: Chromatin immunoprecipitation (ChIP) assay work flow diagram.

Quantitative real time-PCR (qPCR)

qPCR was performed to determine the levels of acH3 and acH4 at the P2 and P4 BDNF gene promoters, as described in previously published work (Tsankova et al., 2004). The rat BDNF gene consists of four distinct 5' exons (I-IV) which are associated with separate promoter and one common 3' exon (V), and eight distinct BDNF transcripts can be generated by use of two different polyadenylation sites in the 3'-end of exon V (Timmusk et al., 1993). BDNF P2 and P4 promoters were selected based on the reported effects of chronic stress on BDNF promoters (Tsankova et al., 2004). Experimental primer pairs were custom designed as follows for: BDNF P2: 5'-TGAGGATAGGGGTGGAGTTG-3', 5'-GCAGCAGGAGGAAAAGGTTA-3'; for BDNF P4: 5'-TGCAGGGGAATTAGGGATAC-3', 5'-TCTTCGGTTGAGCTTCGATT-3', and as an endogenous control primers for GAPDH (Applied Biosystems, CA) were used for normalization. qPCR was performed on MxPro-Mx3005P QPCR System (Agilent Technologies, CA). The reaction mixture consisted of 12.5 µl of Stratagene Brilliant II SYBR® Green Low ROX QPCR Master Mix (Agilent Technologies, CA), 2.5 µl each of forward and reverse primers, and 4 µl of DNA in a total volume of 25 µl. Cycling conditions consisted of 10 min of polymerase activation at 95°C, an amplification step consisted of 40 cycles at 95°C for 30 sec and 60°C for 1 min, followed by 1 cycle of each phase; 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec to obtain the disassociation curve. Immunoprecipitated DNA was PCR amplified in duplicate, and Ct (cycle threshold) values from each sample were obtained using the MxPro software. Ct values obtained from age-matched intermediary responders (i.e., rats that exhibited locomotor scores in the middle third of the same sample) that were handled as control

animals were used as phenotype controls to calculate the logarithmic changes in Ct values of LRHR rats exposed to chronic variable physical stress or control handling.

In situ hybridization histochemistry

Coronal brain sections were collected at 20 μm thickness throughout the dorsal hippocampus and stored at -80^{0}C. On the day of hybridization, slide mounted sections were fixed in 4% paraformaldehyde at room temperature for 1 hr, followed by three washes in 2X SSC (1X SSC is 150 mM sodium chloride, 15 mM sodium citrate). Sections were placed in a solution containing acetic anhydride (0.25%) in triethanolamine (0.1 M, pH 8) for 10 min, rinsed in distilled water, dehydrated through graded alcohols (50%, 75%, 85%, 95% and 100%) and were air dried. Antisense ^{35}S-labeled cRNA probes for rat BDNF (585 bp complementary to the mature rat coding sequence, exon V), was labeled in a reaction mixture consisting of 1 ml of linearized plasmid, 1X transcription buffer, 125 mCi [^{35}S]UTP, 125 mCi [^{35}S]CTP, 150 mM each of ATP and GTP, 12.5 mM dithiothreitol, 20 U RNAase inhibitor, and 6 U RNA polymerase. In this manner, BDNF riboprobe (kindly provided by Dr. Christine Gall, University of California, Irvine) was produced using T3 RNA polymerase as the transcription enzyme after the plasmid was linearized with XhoI. Reactions were incubated for 90 min at 37^{0}C, and unincorporated nucleotides were removed using Biorad spin columns (Biorad Laboratories, CA). Probe was diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 50 mM sodium phosphate buffer, pH 7.4, 1X Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol) to yield 10^6 dpm/70 μl. Sections were hybridized with the probe mixture inside a humidified box overnight at

55°C. The next day, sections were washed in 3X SSC for 5 min each, then incubated for 1 hr in RNAase (20 mg/ml in Tris buffer containing 0.5 M NaCl, pH 8) at 37°C. Sections were washed with 2X, 1X and 0.5X SSC, and incubated for 1 hr in 0.1X SSC at 65°C. After rinsing in distilled water, sections were dehydrated, air dried and exposed to Kodak XAR film (Eastman Kodak, NY). Section images were captured digitally from x-ray films with a CCD camera using MCID (image analysis) software, and relative optical densities were determined using the Scion imaging software. Only pixels with gray values exceeding 3.5X above background (within hippocampus or amygdala) were considered measurable signal and were included in the analyses.

Timm's method for silver sulfide staining

Coronal brain sections were collected at 160μm intervals throughout the hippocampus, and were kept at –80°C until staining. On the day of staining, sections were air-dried and immersed in a phosphate-buffered (pH 7.4) 0.5% sodium sulfide solution for 2 min, briefly rinsed in two changes of phosphate buffer, fixed in 96% ethanol and were rehydrated. Subsequently, sections were stained as described previously (Danscher, 1981; Geneser et al., 1993) by immersion in a citrate-buffered hydrochinone – silver lactate developer containing gum arabic as a protective colloid. Sections were rinsed vigorously with tap water following development and counterstained with cresyl violet and coverslipped. This protocol was successfully used on fresh frozen brain tissue in a previously published article (Isgor et al., 2004b).

Stereological estimation of mossy fiber terminal field volumes

The volumes of the two major components of the dentate gyrus MF system were estimated using the Cavalieri estimator (Stereoinvestigator, Micro-BrightField,

Colchester, VT). The tissue was viewed under brightfield illumination on a Zeiss Axiophot microscope interfaced with a CCD color video camera, and displayed on a high-resolution video monitor at a final magnification of 250X. Rostral and caudal extents of the hippocampus were determined following the convention of Paxinos and Watson (1982). A systematic, random sampling scheme was utilized such that estimates are based on every 10th section throughout the rostro-caudal extent of the hippocampus, yielding an average of 20 analyzed sections per animal. The cross-sectional areas of the (SP)-MFs, terminating on the apical dendrites of CA3 pyramidal cells, and the intra/infrapyramidal (IIP)-MFs, which terminate within or below the pyramidal cell layer (Figure 6), were estimated by an automated point-counting technique using a grid of test points displayed on the video monitor, superimposed upon the structure of interest. Volumes of different MF terminal fields were estimated from the total number of points that fell within the respective field, the sampling interval and the nominal section thickness. Values were plotted for unilateral terminal field volume estimations for SP-MF, IIP-MF, and for total (SP + IIP) mossy fiber systems.

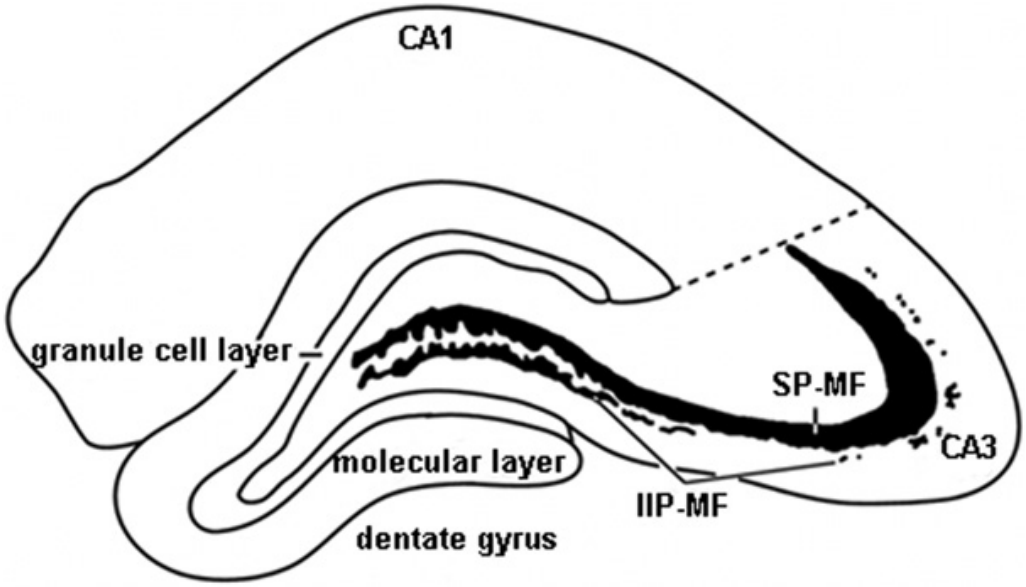


Figure 6: An illustration of a coronal hemisection of the dorsal hippocampus depicting the major hippocampal subdivisions and the two compartments of the mossy fibre system (SP-MF and IIP-MF) in which quantitative estimates were performed in the Timm-stained tissue.

Statistical analyses

Two-way ANOVAs were conducted for (1) percent time spent in social interaction (2) percent time spent in immobility on the forced swim test (3) amounts of acH3 and acH4 at the P2 and P4 promoters of the BDNF gene, (4) levels of BDNF mRNA expression following *in situ* hybridization histochemistry, and (5) the SP-MF, IIP-MF, and total MF terminal field volumes versus phenotype (LR, HR) and stress conditions (PHY, SOC, CONT). Significant interactions and main effects of ANOVAs were followed by Scheffe post-hoc tests. Furthermore, simple regression analyses were conducted between (1) percent time spent in social interaction and BDNF mRNA signal in the BLA, (2) percent time spent in immobility on the forced swim test and acH3 at the BDNF P2 promoter and acH4 at the BDNF P4 promoter, (3) percent time spent in immobility on the forced swim test and BDNF mRNA signal in CA3 and DG of the hippocampus. All significance levels were set at $p = 0.05$.

Results

Social interaction and forced swim tests

Figure 7A shows the percent time engaged in social interaction in the LRHR rats. A two-way ANOVA revealed a significant main effect of Stress (PHY, SOC, CONT) on social interaction between groups [$F_{(1,24)} = 7.816$, $p = 0.009$]. Subsequent post-hoc comparisons showed a significant decrease in percent time spent in social interaction in both phenotypes exposed to CVP compared to control animals [$ps \leq 0.015$].

Figure 7B shows the percent time spent in immobility in LRHR rats on forced swim test. A two-way ANOVA showed a significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) in immobility behavior [$F_{(1,24)} = 9.095$, $p =$

0.005]. Subsequent post-hoc comparisons showed a significant decrease in percent time spent in immobility in HRs exposed to CVP and CVS compared to control animals [P=0.002], and a significant increase in percent time spent in immobility in LRs exposed to CVP compared to handled controls [P=0.0031].

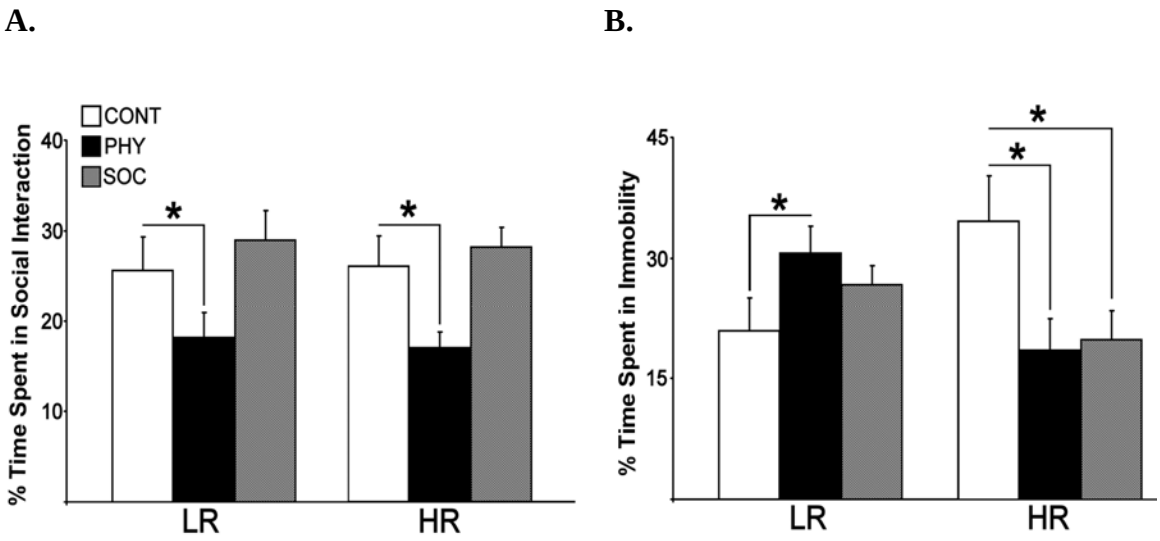


Figure 7: Anxiety- and depressive-like behaviors in LRHR rats. Percent time spent in social interaction (A) and in immobility during forced swim test (B) in LRHR rats exposed to chronic variable physical stress (PHY) and chronic variable social stress (SOC) or handled as controls (CONT). Means are plotted in bar graphs $\pm$ SEMs. *: $p \leq 0.05$.

Levels of acetylated histones at the BDNF promoters

Figures 8A and 8B show levels of acH3 at the P2 promoter of the BDNF gene and levels of acH4 at the P4 promoter of the BDNF gene respectively in the dorsal hippocampus of the LRHR rats exposed to CVP, CVS or control handling. A two-way ANOVA revealed a significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) in the acH3 levels at the BDNF P2 promoter [$F_{(2,24)} = 5.662$, $p = 0.013$]. Post-hoc comparisons showed that control HRs had lower levels of acH3 at the BDNF P2 promoter compared to control LRs [$p = 0.010$]. Furthermore, acH3 levels at the P2 promoter were increased with both CVP and CVS in HRs compared to control levels [ps ≤ 0.025]. No significant effects were detected in the acH3 levels at the P2 promoter of the BDNF gene in the dorsal hippocampus of the LR rats, or in acH3 levels at the P4 promoter of BDNF gene across phenotypes and stress conditions (data not shown). Moreover, a two-way ANOVA showed a significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT), and a significant main effect of Stress in the acH4 levels at the BDNF P4 promoter [$Fs_{(1,24)} \geq 13.058$, ps ≤ 0.002]. Post-hoc comparisons showed that control HRs had lower levels of acH4 at the BDNF P4 promoter compared to control LRs [$p < 0.001$]. Furthermore, CVP resulted in decreased levels of acH4 at the BDNF P4 promoter in LRs, while both stress conditions caused an increase in acH4 at the P4 promoter in HRs compared to handled controls [ps ≤ 0.009]. No significant effects were detected in acH4 levels at the P2 promoter of the BDNF gene between phenotypes and stress conditions (data not shown). Moreover, a significant negative correlation was detected between percent time spent in immobility on the forced swim test and acH4 levels at the P4 promoter of the BDNF gene [Figure 9B; $p = 0.0009$].

No significant correlation was detected between percent time spent in immobility on the forced swim test and acH3 levels at the P2 promoter of the BDNF gene (Figure 9A).

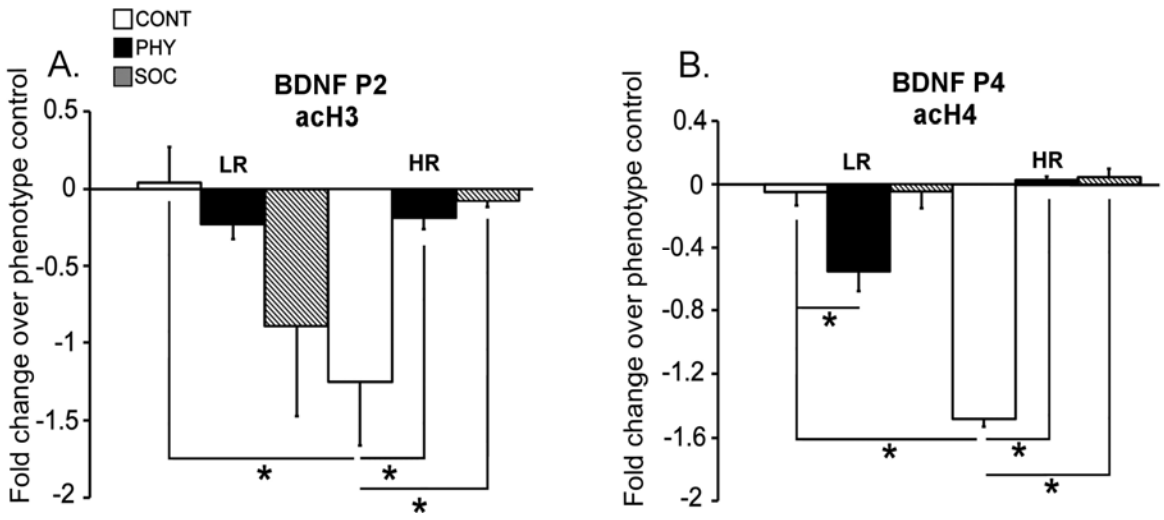


Figure 8: Levels of acetylated Histone 3 (acH3) at the BDNF P2 promoter (A), and acetylated Histone 4 (acH4) at the BDNF P4 promoter (B) in the LRHR rats exposed to chronic variable physical (PHY) and social (SOC) stress or handled as controls (CONT). Means are plotted in bar graphs $\pm$ SEMs. *: $p \leq 0.05$.

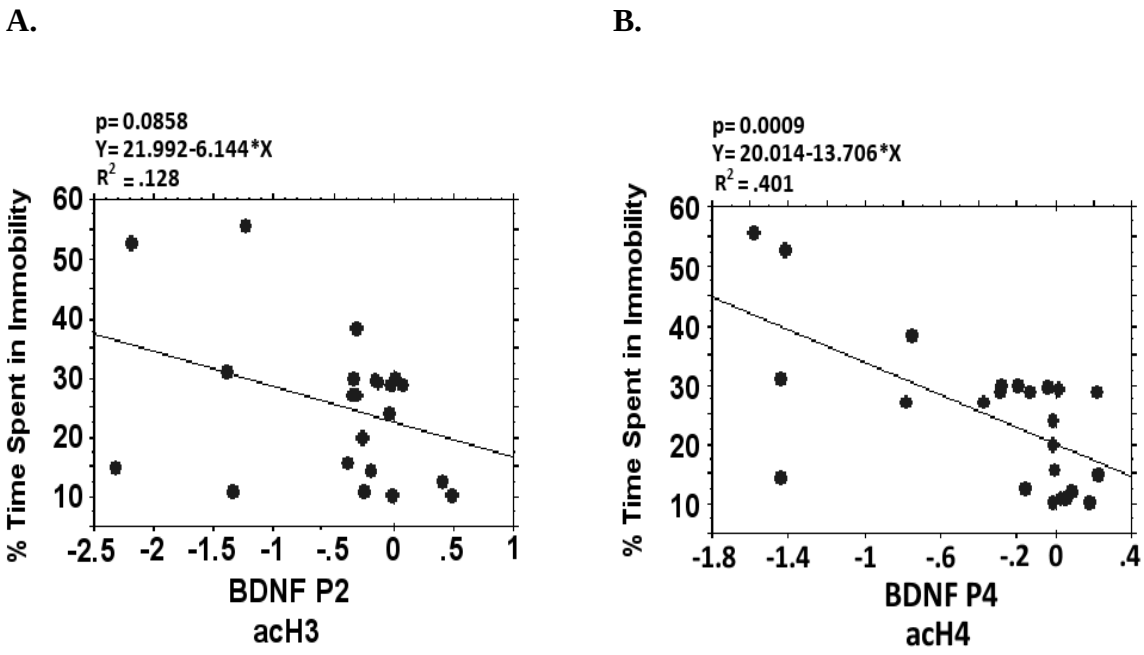


Figure 9: Regression plots between percent time spent in immobility on the forced swim test and levels of acetylated Histone 3 (acH3) at the BDNF P2 promoter (A) and acetylated Histone 4 (acH4) at the BDNF P4 promoter (B) in the dorsal hippocampus.

BDNF mRNA in the dorsal hippocampus and the basolateral amygdala

Figure 10 shows expression of BDNF mRNA in the dorsal hippocampus of the LRHR rats. Two-way ANOVAs showed significant interactions between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) in the dentate gyrus (DG) and the CA3 pyramidal field of the hippocampus [$Fs_{(2,24)} \geq 3.529$, $ps \leq 0.035$]. Post-hoc comparisons showed that BDNF mRNA was upregulated in the hippocampal DG and CA3 in HR rats exposed to CVP and CVS compared to handled controls [$ps \leq 0.034$]. However, no significant effects were observed in the hippocampal BDNF mRNA levels in LRs exposed to chronic variable stress compared to handled controls. Moreover, no significant correlations were detected between percent time spent in immobility on the forced swim test and BDNF mRNA signal in the CA3 and DG of the hippocampus (Figure 11A, B).

Figure 12 shows expression of the BDNF mRNA in the BLA of the LRHR rats. A two-way ANOVA showed a significant main effect of Stress (PHY, SOC, CONT) on BDNF mRNA in the BLA [$F_{(1,24)} = 31.771$, $p < 0.0001$]. Post-hoc comparisons showed that BDNF mRNA levels were upregulated in the BLA following the CVP exposure in both the LR and HR rats compared to their respective handled controls [$ps \leq 0.001$]. Moreover, a significant negative correlation was detected between percent time spent in social interaction and BDNF mRNA signal in the BLA [Figure 13, $p = 0.0001$].

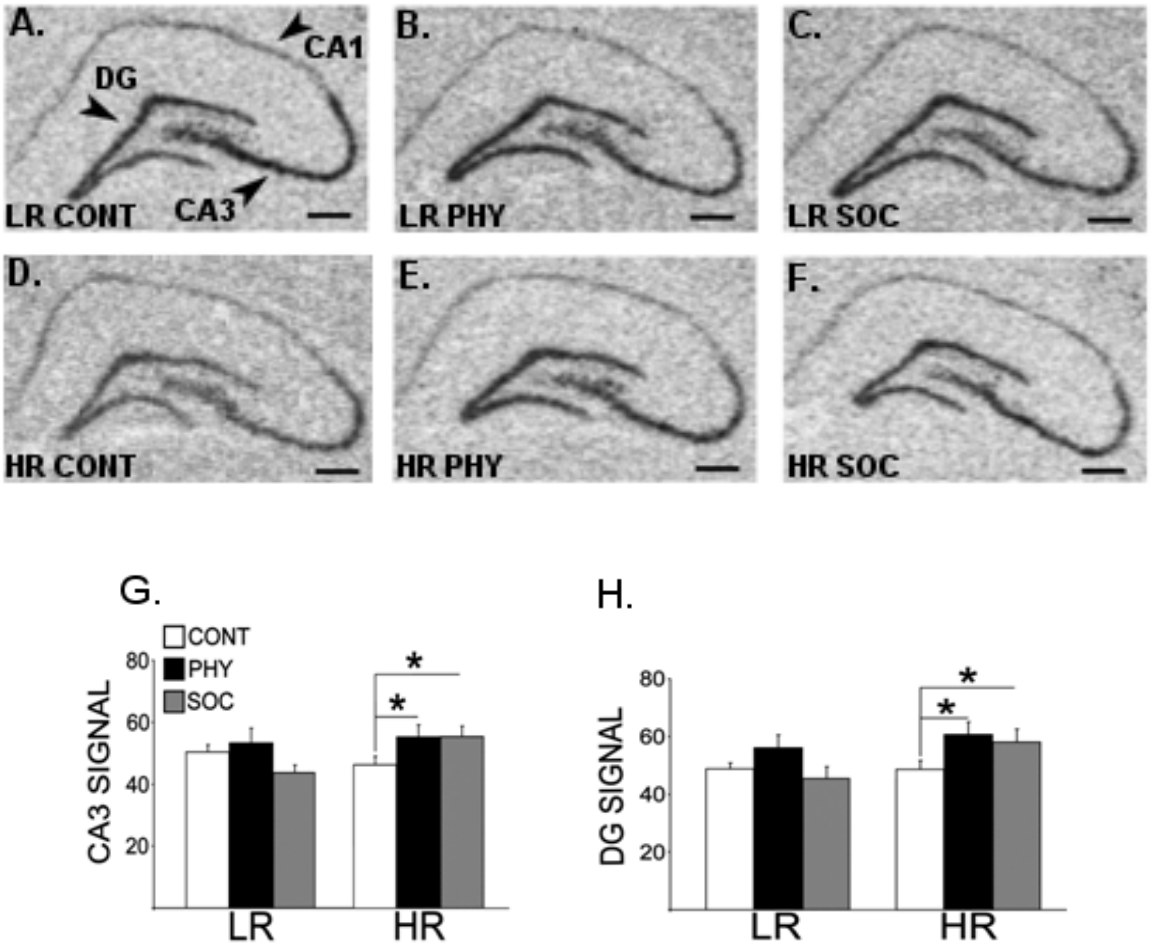


Figure 10: BDNF mRNA levels in the dorsal hippocampus. Panels A, B, C, D, E and F constitute x-ray film-exposed images of representative coronal hemisections of the hippocampus that were radioactively labeled with an antisense cRNA probe against BDNF mRNA in LRHR rats following chronic variable physical (PHY) and social stress (SOC) or control handling (CONT). Means for optical densities ± SEMs are plotted by bar graphs for signal in the CA3 pyramidal field (G) and the dentate gyrus (DG, H) of the hippocampus. *: $p \leq 0.05$. Scale bar = 250 μm.

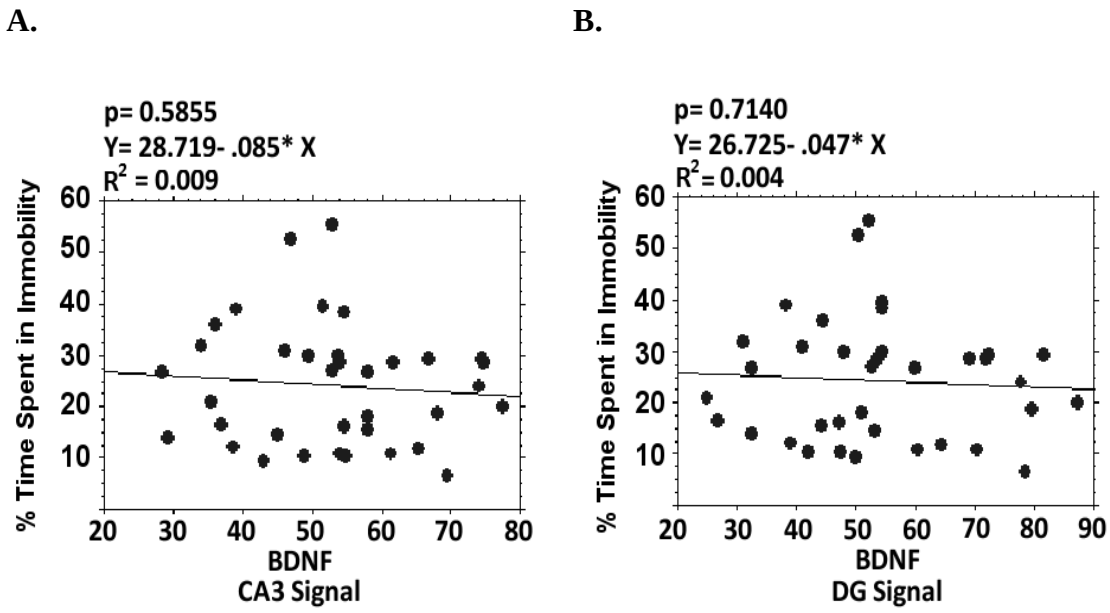


Figure 11: Regression plots between percent time spent in immobility on the forced swim test and BDNF mRNA signal in the CA3 (A) and DG (B) of the hippocampus.

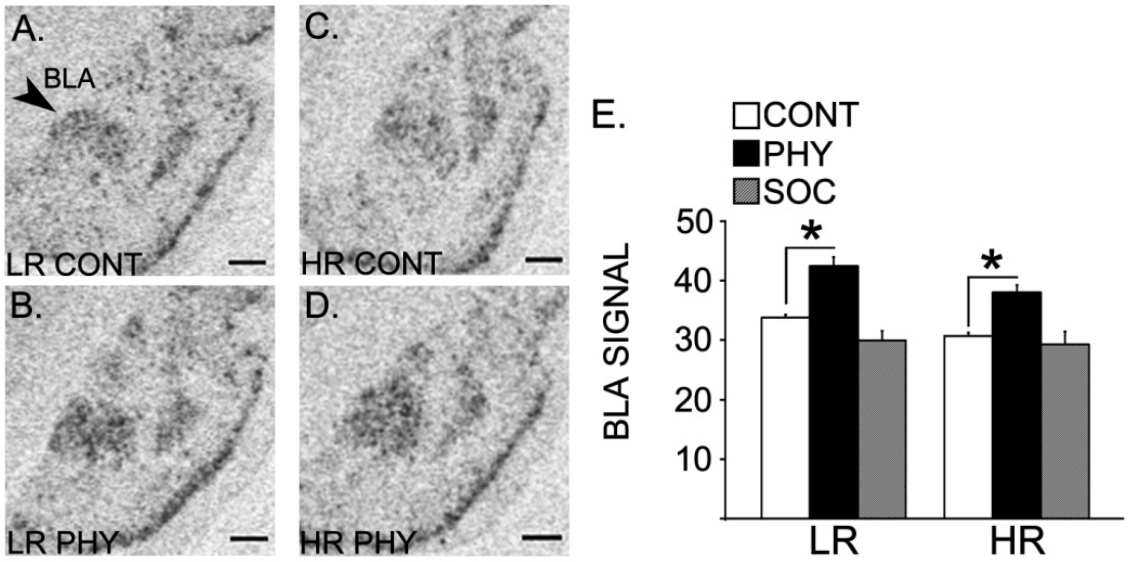


Figure 12: BDNF mRNA levels in the basolateral amygdala. Panels A, B, C and D constitute x-ray film-exposed images of representative sections showing basolateral amygdala (BLA) that were radioactively labeled with an antisense cRNA probe against the BDNF mRNA in the LRHR rats following chronic variable physical (PHY) and social (SOC) stress or control handling (CONT). Means for optical densities $\pm$ SEMs are plotted by a bar graph (E). *: $p \leq 0.05$. Scale bar = 250 μm.

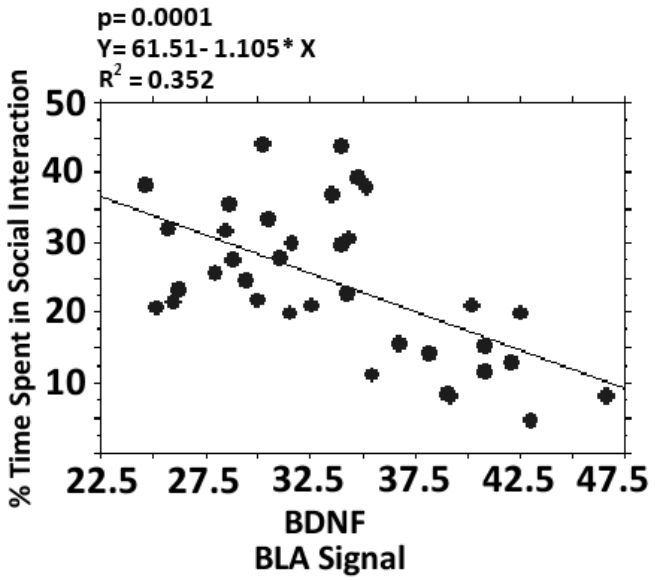


Figure 13: Regression plots between percent time spent in social interaction and BDNF mRNA signal in the BLA.

Stereological estimation of mossy fiber terminal field volumes

The two major components of the MF system (SP-MF and IIP-MF) in which quantitative estimates were performed are shown in Figure 6. Figure 14 depicts representative sections of the Timm-stained septal hippocampi from experimental groups, and estimates of terminal field volumes for SP-MF and total MF (SP-MF + IIP-MF). Two-way ANOVAs showed significant interactions between Phenotype (LR, HR) and Stress (PHY, SOC, CONT), and main effects of Phenotype in SP-MF and total MF terminal field volumes [$F\text{s}_{(2,24)} \geq 3.572$, $p\text{s} \leq 0.048$], whereas no significant effects were detected in IIP-MF terminal field volume (data not shown). Post-hoc comparisons showed a significant increase in the SP-MF and total MF terminal field volumes in HRs exposed to CVP and CVS compared to handled controls, and a significant decrease in the SP-MF volume in LRs exposed to CVP compared to handled controls [$p\text{s} \leq 0.045$].

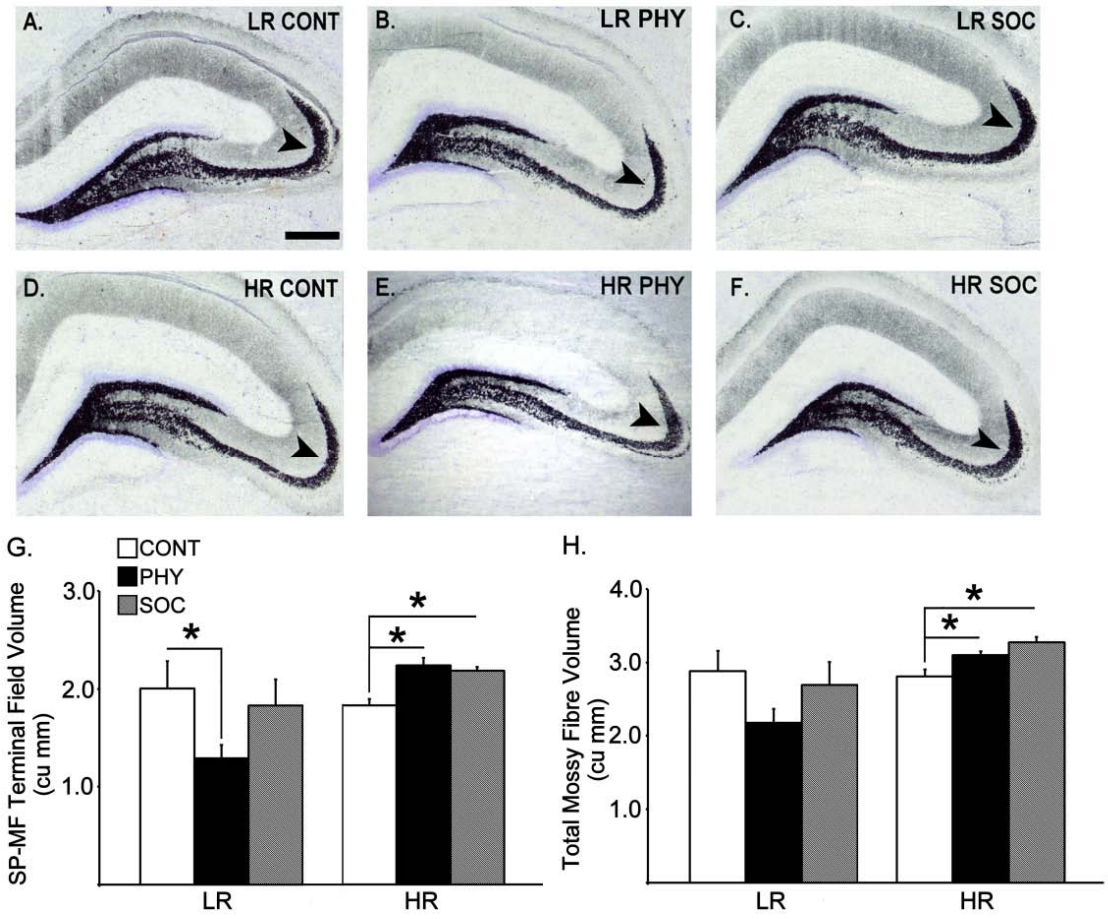


Figure 14: Mossy fibre plasticity. Coronal hemisections of the dorsal hippocampus showing Timm-stained mossy fibre terminal fields that are counterstained by cresyl violet representing LR control (A), LR exposed to chronic variable physical stress (B), LR exposed to chronic variable social stress (C), HR control (D), HR exposed to chronic variable physical stress (E) and HR exposed to chronic variable social stress (F) groups. Black arrows are pointing at the SP-MF compartment of the MF projections. Data pertaining to estimated SP-MF terminal field volume (G) and total mossy fibre terminal field volume (H, SP-MF + IIP-MF) are expressed as means ± SEMs in bar graphs. *: $p \leq 0.05$. Scale bar = 250 μm.

Discussion

These results demonstrated individual differences in stress-induced depressive-like behavior (antidepressive-like effects in HRs and depressive-like effects in LRs) in LRHR rats following peripubertal-juvenile exposure to chronic variable stress. Moreover, CVP during the peripubertal-juvenile period resulted in an opposite regulatory pattern between the depressive and social anxiety behaviors in the HR rats, in that a decrease in immobility (i.e., antidepressive-like effect) was detected on the forced swim test along with a decrease in the amount of social interaction [i.e., anxiogenic (anxiety-like) effect]. CVS also led to antidepressive behavior in HRs, however performance on the social interaction test was unaffected. In the LR phenotype, CVP led to an increase in depressive behavior while also causing an increase in social anxiety behavior. Therefore, these behavioral findings reflect a stress-specific (i.e., following CVP) but phenotype non-specific emergence of social anxiety behavior, while simultaneously revealing a phenotype-specific (i.e., LRs) emergence of depressive behavior following peripubertal-juvenile CVP exposure.

Moreover, basal levels of acH3 and acH4 at the BDNF P2 and P4 promoters, respectively, were lower in HRs hippocampus compared to LRs hippocampus. However, acetylation levels of H3 and H4 at the BDNF promoters P2 and P4, respectively, were increased with both types of stress in the HR hippocampus compared to levels observed in handled controls. In contrast, a significant decrease in acetylation levels of H4 was observed at the BDNF promoter P4 in the LR hippocampus following the CVP regimen. These findings suggest increased epigenetic activation of BDNF gene expression in the HR hippocampus with CVP and CVS, and in contrast a decreased epigenetic activation of

hippocampal BDNF gene expression in LRs with CVP. Moreover, a significant negative correlation was found between percent time spent in immobility on the forced swim test and acetylation levels of H4 at the P4 promoter of the BDNF gene, suggesting an increase in "despair" behavior associated with decreased levels of epigenetic plasticity in hippocampal BDNF gene expression. Furthermore, upregulation in BDNF mRNA levels was observed in the HR hippocampus (CA3 and DG) following both types of stress, compared to levels observed in handled controls. These effects on BDNF mRNA are in the same direction as the effects observed in acetylated histone levels at the BDNF promoters in the HR phenotype, namely both indicate increased hippocampal BDNF gene transcription.

Concurrent with findings on hippocampal BDNF levels, my results showed a significant increase in total MF volume, particularly in SP-MF terminal field size in HRs following both stress conditions. Moreover, a significant decrease in SP-MF terminal field size was observed in LRs following the CVP regimen. These findings suggest that fluctuations in the hippocampal BDNF levels induced by stressful stimulation during the peripubertal-juvenile period, particularly those that are mediated by histone modifications at the BDNF gene, may support observed structural plasticity at the MF terminals. All of which may mediate antidepressive-like effects in the HRs and depressive-like effects in LRs (see Table 2 for summary of reported results). Lastly, the emergence of social anxiety-like behavior following CVP in both phenotypes was associated with the upregulation of the BDNF mRNA in the BLA. Moreover, a significant negative correlation was detected between precent time spent in social interaction and BDNF mRNA signal in the BLA in general, suggesting that increased social anxiety-like

behavior might be associated with decreased hippocampal BDNF mRNA levels irrespective of the LRHR phenotype. These results indicate that stress-induced increase in BDNF levels in the amygdala may be mediating a general anxiogenic effect in the juvenile LRHR rats (see Table 3 for summary of reported results).

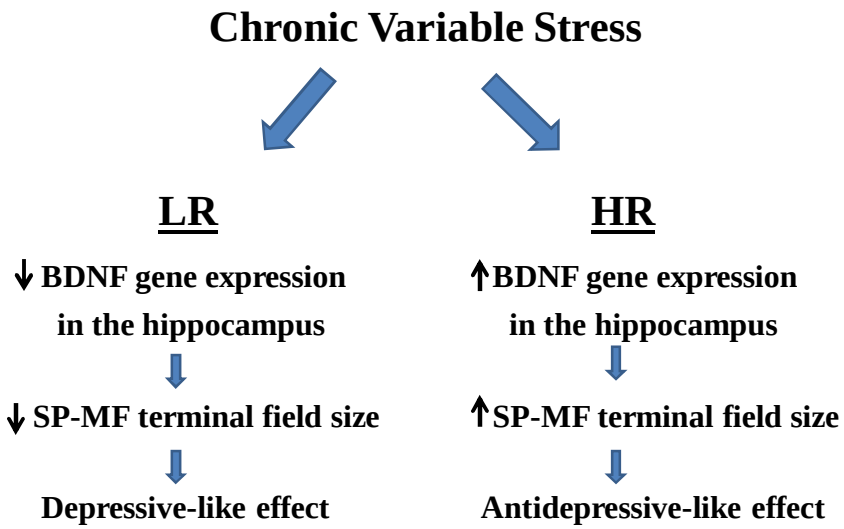


Table 2: Diagrammatic representation of chronic variable stress effects on BDNF gene expression in the hippocampus, suprapyramidal mossy fibres (SP-MF) and depressive behavior in LRHR rats.

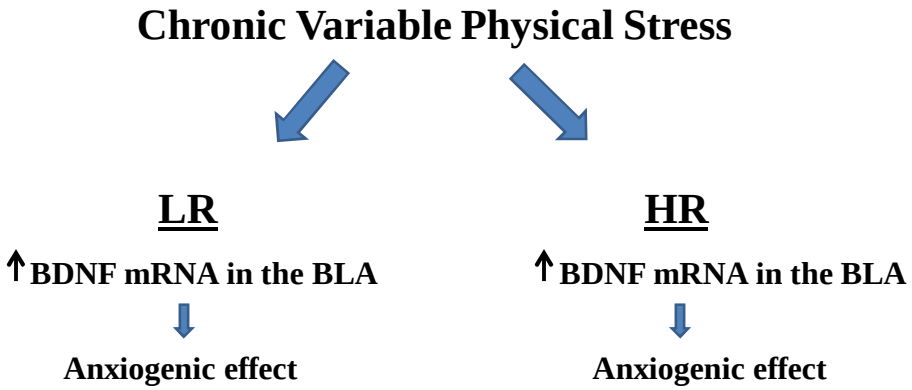


Table 3: Diagrammatic representation of chronic variable physical stress effects on BDNF mRNA levels in the basolateral amygdala (BLA) and anxiety behavior in LRHR rats.

Depressive-like behavior and hippocampal BDNF

The results showed an opposite regulatory pattern for LRHR rats in depressive-like behavior measured as immobility on the forced swim test following the peripubertal-juvenile exposure of CVP. LR rats showed increased immobility whereas HR rats showed decreased immobility compared to levels observed in handled controls. Stress-induced increases in depressive behavior observed in the juvenile LR rats is in line with numerous reports showing increased depressive behavior following chronic stress in adulthood. In fact, paradigms of chronic stress are widely accepted as animal models for studying enhanced depressive-like states (for review see; Duman, 2010). Interestingly, the same chronic stress paradigm applied during the peripubertal-juvenile period resulted in an antidepressive effect in the HR rats. It is noteworthy to mention that in our forced swim paradigm, control LRHR rats do not exhibit phenotype distinction in locomotor response to novelty. Optimally, the LRHR phenotype is manifested in locomotor reactivity to the mild stress of a novel environment in the experimentally naïve animals. Phenotypic locomotor differences are transiently neutralized in the light–dark box when HR rats are socially isolated for a week (Kabbaj et al., 2000). However, stressors that are controllable by the animal's response as opposed to experimenter-imposed are optimal for manifesting the LRHR phenotype (Kabbaj et al., 2000) and therefore the forced swim test is likely not ideal for phenotype screening per se. Moreover, novelty has been shown to induce lower glucocorticoid responses than forced swimming (Chandramohan et al., 2007; Droste et al., 2009). Hence, there is physiological evidence for higher severity of the forced swim stress compared with exposure to novelty. Therefore, distinctly different regulatory patterns for immobility are observed on the forced swim test in HR and LR

rats exposed to chronic stress during adolescence. Although individual components of our chronic variable stress regimens are commonly employed as stressors in the literature including novelty (Maccari et al., 1991), isolation, crowding (Gamallo et al., 1986; Sanchez et al., 1998) and restraint (Andrus et al., 2010), and are generally considered as "negative" experiences, a regimen of combined stressors applied intermittently at varying times of day and for varying durations during adolescence, may indeed mimic an enrichment-like effect, especially in the stress responsive HR phenotype. It is not uncommon to induce a robust physiological stress response in conjunction with rewarding environmental stimuli during adolescence. For example, rough- and tumble-play in juveniles induces positive experiences and is rewarding (Burgdorf and Panksepp, 2001), although marked by increased levels of CORT (Terranova et al., 1999). In addition to playful social interactions, positive reinforcing activities such as feeding and mating have also been shown to be associated with increased levels of CORT (Dallman et al., 1995; Frye et al., 1996). Therefore, it can be argued that environmental stimulation induced by restraint, crowding and exposure to novel environment, although highly stressful in the physiological sense, may result in an enrichment-like outcome. Moreover, a classic postweaning enrichment paradigm produces an antidepressive-like effect on the forced swim test (Brenes et al., 2009), such as the one I report here for the HR rats following CVP and CVS exposures applied from postweaning into adolescence (PN 28-42). Whether a depressive-state will emerge in the HR rats with recovery from stressful stimulation in adolescence is an empirical question that needs addressing in future studies.

In parallel with the opposite regulatory patterns seen in depressive-like behavior following chronic variable stress, accompanying changes are observed in chromatin plasticity associated with BDNF gene in the LRHR hippocampus. Namely, decreased levels of helplessness (measured as reduced immobility on the forced swim test) were associated with increased levels of acH3 at the BDNF P2 promoter and increased levels of acH4 at the BDNF P4 promoter, suggesting epigenetic induction of BDNF gene expression in the HR rats. These effects were reflected in mRNA expression levels where upregulation of BDNF mRNA in the DG and the CA3 fields of the hippocampus were detected in the HR animals following both chronic variable stress regimens. This suggests that, along with chromatin plasticity, there may be classic transcriptional and/or indirect regulatory mechanisms responsible for stress-induced changes in endogenous BDNF levels. However, simple regression analyses revealed a significant negative correlation only between percent time spent in immobility on the forced swim test and acetylation levels of H4 at the BDNF P4 promoter. No significant correlations were detected neither between percent time spent in immobility on the forced swim test and acetylation levels of H3 at the BDNF P2 promoter nor between percent time spent in immobility on the forced swim test and BDNF mRNA levels in the CA3 and DG of the hippocampus. Since HR and LR animals were pooled for simple regression analyses, the results did not reflect phenotype-specific effects, showing furthermore the importance of individual differences in the epigenetic regulation of the BDNF gene following stress exposure.

Chronic variable stress effects on epigenetic and transcriptional regulation of BDNF in HR rats are in contrast with studies showing that hippocampal BDNF levels are

reduced following chronic stress produced by social defeat (Tsankova et al., 2006) and immobilization (Smith et al., 1995; Vollmayr et al., 2001; Roceri et al., 2002; Nair et al., 2007). Furthermore, chromatin plasticity in terms of increased histone methylation at BDNF promoters has been previously linked to stress-induced suppression of hippocampal BDNF gene expression, and antidepressant treatments have been linked to hyperacetylation of H3 at the BDNF promoter (Tsankova et al., 2006). The adolescent stress exposures used in the present study appear to mimic previously reported effects of antidepressants on histone acetylation at the BDNF promoter in the HR phenotype, revealing a novel mechanism for environmental-induction of BDNF gene expression. In contrast to HRs, LRs subjected to CVP show increased depressive behavior, which is associated with decreased levels of acH4 at the BDNF P4 promoter (with no effect on total BDNF mRNA) in the hippocampus. This effect in the LR phenotype is in line with previous reports showing chronic stress-induced, epigenetic suppression of hippocampal BDNF expression and the associated emergence of depressive behavior (Tsankova et al., 2006). However, decreased levels of acetylation are confined to the H4 at the P4 promoter, and are undetectable at the level of total BDNF mRNA levels. Investigation of particular splice variants of BDNF mRNA may be useful to identify which stress-induced regulatory elements are affected in the adolescent LR phenotype. Differential responses of LRs and HRs in terms of stress-induced depressive-like behavior provide compelling evidence for detectable neurobehavioral differences in emotional behaviors following peripubertal-juvenile exposure to chronic variable stress.

Anxiety-like behavior and amgdalar BDNF

In addition to the antidepressant-like response, HR animals showed increased anxiety-like behavior following CVP, measured as reduced social contact in the social interaction test. Opposite regulatory patterns in depressive and anxiety behaviors were only apparent in the HR phenotype, whereas increased social anxiety-like behavior emerged as a phenotype non-specific effect in both LR and HR rats following CVP. Emergence of social anxiety behavior was associated with an upregulation in BDNF mRNA levels in the BLA of adolescent LRHR rats. This association was corroborated with findings from simple regression analyses where a significant negative correlation was detected between percent time spent in social interaction and BDNF mRNA signal in the BLA. Increase in anxiety-like behavior in a social context, and with traditional indices of anxiety, such as open field behavior, has been previously reported following adolescent social isolation (Lukkes et al., 2009) and with early life maternal separation, followed by chronic restraint stress (Eiland and McEwen, 2010). Furthermore, earlier studies have also linked chronic stress-induced anxiety with increased neuronal spine density in the BLA (Vyas et al., 2006; Mitra et al., 2005), together with a long-term increase in amygdalar BDNF (Fanous et al., 2010) that may mediate morphological synaptic plasticity. What is intriguing is the question of how dissociation between the depressive- and anxiety-like behaviors may develop in the HR rats, and whether there are genetic models that can mimic this effect. One study showed that transgenic mice overexpressing BDNF in forebrain display both anxiogenic and antidepressant behaviors (Govindarajan et al., 2006). Specifically these authors showed that BDNF overexpression led to increased anxiety accompanied by an increase in spine density in

the BLA, similar to chronic stress-induced effects reported in prior studies (Duman et al., 2000; Vyas et al., 2006). Also BDNF overexpression in the hippocampus was proposed as a mechanism to inhibit chronic stress-induced cellular atrophy, and to promote improved performance on the forced swim test. In conclusion, these authors stated that increased BDNF in the amygdala may facilitate the development of anxiety symptoms, whereas increased BDNF in the hippocampus may attenuate depressive symptoms (Govindarajan et al., 2006). The CVP regimen, by way of inducing BDNF expression in both the hippocampus and the BLA of the HR rats, could lead to a similar and opposing regulatory pattern in depressive and anxiety behaviors. By the same token, the chronic stress regimen, by way of inducing BDNF expression in the BLA and decreasing epigenetic induction of the BDNF in the hippocampus of the LR phenotype, could lead to emergence of both anxiety and depressive behaviors, respectively.

Mossy fibre terminal fields

Chromatin modifications in the form of histone acetylation at the BDNF promoters were closely correlated with mossy fibre remodeling in LRHR hippocampi following chronic variable stress exposure during the peripubertal-juvenile period. Namely, the results showed that increased mossy fibre terminal field size, particularly the SP-MF volume, in the HR animals following both stress regimens, was associated with increased H3 and H4 acetylation at the BDNF P2 and P4 promoters respectively. Decreased SP-MF volume in the LR animals following CVP exposure was associated with decreased H4 acetylation at the BDNF P4 promoter. These findings strongly suggest that epigenetic regulation of BDNF gene expression mediates MF synaptic plasticity in LRHR rats following peripubertal-juvenile stress.

The peripubertal-juvenile period is a critical period for MF remodeling (Mori-Kawakami et al., 2003), and the amount of MF content is highly dependent on experiential factors (Gomez-Di Cesare et al., 1997). In the face of high levels of late developmental plasticity in the MF system, maintaining normal hippocampal function would require correct MF path finding and precise formation of synaptic connections (Parent et al., 1997), which are likely to be affected by stressful environmental stimulation during adolescence. Since hippocampal BDNF is primarily stored in the axon terminals of the mossy fibres (Dieni et al., 2012), stress-induced changes in hippocampal BDNF levels could mediate structural changes in the MF-CA3 synapse. Intrahippacampal microinfusion of the BDNF has been shown to increase MF volume at the stratum oriens of CA3 (Schjetnan and Escobar, 2010). Hence, it is plausible that the increase in the hippocampal BDNF in the adolescent HR rats following stressful stimulation may lead to increased MF innervation of the CA3 neurons, and may function to rescue CA3 neurons from stress-induced atrophy latter of which will be investigated in Chapter 2 (Watanabe et al., 1992; Magarinos et al., 1996).

Although MF plasticity is linked to cognitive function (Notenboom et al., 2010), its role in depressive-like behavior is, for the most part, unexplored. Functional maturation of the dentate granule cells and their mossy fibres are implicated in pathophysiology of depression. In particular, antidepressants have been shown to increase adult granule cell neurogenesis in the DG (Malberg et al., 2000), whereas stress exposure results in decreased dentate neurogenesis (Warner-Schmidt and Duman, 2006). These findings suggest that granule neurons and their axonal projections onto CA3 may be critically involved in the pathogenesis of depression and in mediating antidepressant

effects (Kobayashi, 2009). In support of this, repeated electroconvulsive stimulation (ECS) is shown to induce MF sprouting in rodents (Vaidya et al., 1999). Similar to ECS data, increased MF terminal fields in the HR phenotype following chronic stress is accompanied by antidepressive-like effects on the forced swim test. In contrast, the stress-induced atrophy observed in the MF terminal fields in the LR animals, is accompanied by the emergence of depressive-like behavior on forced swim test. These findings implicate the MF system as a target for neuroadaptations induced by stressful stimulation during the peripubertal-juvenile period.

In conclusion, depressive and anxiogenic behavioral effects emerge with juvenile exposure to CVP in the LR rats, while antidepressive and anxiogenic behavioral effects emerge in the HR rats following juvenile exposure to CVP and CVS. These results reflect individual differences in behavioral responses to chronic variable stress in LRHR rats. Accompanying the behavioral effects, differential epigenetic regulation of hippocampal BDNF gene expression is observed, and this may mediate aspects of synaptic plasticity at the MF terminals. Unlike the phenotype-specific antidepressive effect, following CVP, an induction of social anxiety-like behavior is seen in both LR and HR rats that is accompanied by upregulation of the BDNF mRNA levels in the BLA. These findings assign differential functional significance to induction of hippocampal and amygdalar BDNF by chronic variable stress exposure during adolescence in the LRHR phenotype.

Data pertaining to Chapter 1 has been published in Neuroscience, 2011; 192:334-44; and Neuroscience Letters, 2011; 501(2): 107-11.

CHAPTER 2: CHRONIC VARIABLE STRESS-INDUCED CA3 PYRAMIDAL NEURON PLASTICITY IN THE LRHR RATS

Introduction

Chronic stress affects structural plasticity in the hippocampus, particularly remodeling of pyramidal neurons in the CA3 region. In general, chronic stress causes dendritic retraction and reduced branching on apical region of CA3 neurons in adult animals (Watanabe et al., 1992b; Magariños and McEwen, 1995; Magariños et al., 1996; Galea et al., 1997; Conrad et al., 1999; McEwen, 1999; McKittrick et al., 2000; McLaughlin et al., 2009) which is accompanied by loss of synaptic plasticity that is crucial for cognitive impairments associated with stress (Rusakov et al., 1997; McEwen, 1999; Sapolsky, 2000). Chronic stress-induced CA3 dendritic remodeling in rodents is mediated by elevated levels of CORT and activation of excitatory amino acid release (Magarinos and McEwen, 1995b). Similar to rodent studies with chronic stress, hypersecretion of cortisol is observed in patients with post-traumatic stress disorder or major depressive disorder along with reduced hippocampal volume and impaired cognitive function (Campbell et al., 2004; Wignall et al., 2004; Karl et al., 2006; Vasic et al., 2008). Chronic stress-induced dendritic retraction in CA3 neurons is known to be maladaptive since deficit in hippocampal structure and cognitive impairment are evident in both clinical and preclinical studies following chronic exposure to stress.

Although chronic stress primarily induces dendritic retraction in CA3 neurons, decrease in dendritic arbors has been reported in other hippocampal and cortical regions as well. Four weeks of chronic unpredictable stress induces dendritic retraction in CA1 and DG neurons along with CA3 neurons (Sousa et al., 2000) and repeated restraint stress (3hr/day for 21days) causes dendritic retraction in pyramidal neurons (layer II/III) of medial prefrontal cortex (Radley et al., 2004; Brown et al., 2005). These findings may provide one potential explanation for reduced volume of hippocampus and medial prefrontal cortex associated with impairment in emotionality and cognition in depressed patients (McEwen, 2007; Sacher et al., 2011).

Dendritic integrity is governed by dendritic spines which are important for synaptic function and plasticity. Reduced dendritic and/or synaptic plasticity underlies cognitive dysfunction and stress-related disorders, such as depression (for review see Pittenger and Duman, 2008). Spines are fundamental unit of neuronal synapses and they are divided into four main categories based on the relative sizes of the spine head and neck (Peters et. al., 1970; Harris et al., 1992; Hering et al., 2001). Mushroom spines have a large head and a narrow neck; thin spines have a smaller head and a narrow neck; stubby spines have no obvious constriction between the head and the attachment to the dendritic shaft and filopodium spines have hair-like morphology (Peters et. al., 1970; Harris et al., 1992; Hering et al., 2001). Types of the spines may represent different stages of spine maturation, such as thin spines may develop into more stable mushroom spines depending on synaptic activity (Bourne and Harris, 2007). Typically, mushroom-type spines are categorized as mature type of spines (Horner, 1993). Activity of spines is regulated by environmental signals. Chronic stress induces synaptic alterations including

spine loss in CA3 neurons (Magariños et al., 1997; Sousa et al., 2000; Sandi et al., 2003; Donohue et al., 2006). Therefore, stress-induced dendritic atrophy is accompanied by loss of spines and associated synaptic dysfunction which in turn affects neuronal firing properties and consequently brain plasticity.

Data from Chapter 1 show differences on stress-induced MF plasticity in LRHR rats and that is accompanied by changes on hippocampal BDNF gene expression. Since MF (the axons of dentate granule neurons) project and innervate the CA3 neurons, stress-induced remodeling of the MF terminal fields suggests potential corresponding reorganization of the CA3 dendritic receptive fields. Moreover, independent of changes in MF volume, modulation in hippocampal BDNF due to stress exposure in the LRHR rats may have resultant effects on MF-CA3 circuit, particularly the dendritic arbors of the stress-vulnerable CA3 neurons. Indeed, chronic stress-induced decrease in BDNF expression in the hippocampus is associated with atrophy of CA3 neurons (for review see Dumann et al., 1999) and BDNF is critical for providing neurotrophic support for plasticity in the mossy fibre-CA3 synapse (Gómez-Palacio-Schjetnan and Escobar, 2008). Therefore, this experiment will investigate effects of chronic variable stress on CA3 dendritic plasticity in LRHR rats. I will test the hypothesis that CVP and/or CVS will be associated with alterations in CA3 dendritic field size in the LRHR rats. Specifically, I hypothesize that stress exposure that leads to atrophy of MF and/or decrease in BDNF is likely to be associated with CA3 dendritic atrophy whereas, stress exposure that leads to hypertrophy of MF and/or increase in BDNF is expected to result in CA3 dendritic hypertrophy.

Methods

LRHR phenotype screening and chronic variable stress paradigm

Total of 54 male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (PD 22), screened for locomotor reactivity to a novel environment and then exposed to chronic variable stress as described in Chapter 1.

Histology

One day after the last stress exposure or control handling, animals were overdosed with isoflurane and then perfused transcardially with 0.9% saline. Brains were then removed and processed using a modified Golgi stain (Glaser and Van Der Loos, 1981), which allows visualization of whole neurons including processes. Brains were immersed in Golgi-Cox solution (containing 5% potassium dichromate, 5% mercuric chloride, and 5% potassium chromate in distilled water) which was kept in dark and changed every 2 days for 30 days, following that they were dehydrated and embedded in celloidin. Coronal sections through the hippocampus were cut and collected at 160µm. Tissue was further processed following the protocol described by Glaser and Van Der Loos (1981), omitting counterstain. Processing of the sections involved alkalinization in ammonium hydroxide (66%), development in Dektol (Kodak), fixation in photographic fixative (Kodak), dehydration through a graded series of ethanols and clearing in xylene. Sections were then mounted onto custom made slides to accommodate section thickness, coverslipped and air dried before analyses.

Analysis of dendritic morphology

Five animals per group were selected for dendritic and spine analyses. For each animal, 6 CA3 pyramidal neurons were reconstructed at 100X objective magnification

with motorized microscope (Zeiss, Germany) attached to a camera (Microfire by Optronics, CA) and using a three-dimensional computer-based neuron tracing system (NeuroLeucida, MicroBrightfield, Inc., Williston, VT, N= 180). To be selected for analysis, Golgi impregnated pyramidal neurons had to fulfill the following criteria: 1) relative isolation from neighboring impregnated neurons to prevent obstruction of dendritic processes, 2) dark and consistent impregnation throughout the extent of neuron, 3) clearly visible spines and 4) the location of the cell soma has to be in the middle one-third of the tissue section to trace the fully visible dendrites (Isgor and Sengelaub, 2003). CA3 pyramidal neurons were sampled systematically from all sections throughout the anterior/posterior extent of the hippocampal CA3 field. The sample equally belonged to the CA3a, CA3b, CA3c and posterior/ventral subpopulations (Figure 15) following the description of Turner et al. (1995). Measurements of dendritic morphology included dendritic length and dendritic branches for apical, basal and overall (apical + basal) dendrites per neuron. A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield).

Analysis of spine number and density

Spines were examined on apical and basal dendrites of CA3 pyramidal neurons. Spines were categorized as mushroom-shaped and total spines (mushroom-shaped + other type of spines) for analyses. Spines which were morphologically different than mushroom-shaped spines such as thin, stubby, and filopodium were considered as the other type of spines without making any distinctions among spine types. Measurements of spine morphology included spine number and spine density for apical, basal and overall (apical + basal) dendrites per neuron. Spine density was calculated by dividing

the number of total spines by the total length of dendritic arbors and was expressed as the number of spines per micrometer of dendrite. Sholl analyses were performed on CA3 pyramidal neurons to obtain distribution of mushroom-shaped and total spines by examining each 20 µm segment from the soma across the dendritic tree.

Statistical analyses

Two-way ANOVAs were conducted for dendritic (length and number of branches) and spine [mushroom-shaped and total spines (mushroom-shaped+thin, stubby, filopodia spines)] measurements, and Sholl analyses in spine (mushroom-shaped and total spines) distribution across the dendritic tree between phenotypes (LR, HR) and stress conditions (PHY, SOC, CONT). Furthermore, significant interactions and main effects of ANOVAs were followed by Scheffe post-hoc test. All significance levels were set at $p = 0.05$.

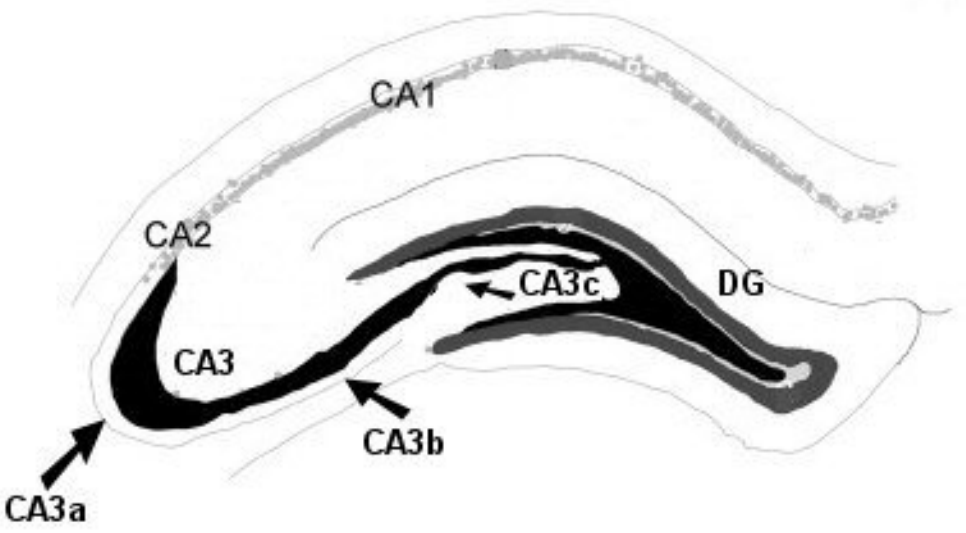


Figure 15: Schematic representation of CA3 pyramidal neuron subregions in the dorsal hippocampus. CA1: CA1 pyramidal cell layer, CA2: CA2 pyramidal cell layer, CA3: CA3 pyramidal cell layer, DG: Dentate Gyrus.

Results

Dendritic length

Reconstructions from representative neurons for control, CVP and CVS exposed LRHR rats are depicted in Figure 16. Apical, basal and overall (apical + basal) dendritic length for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 17). A two-way ANOVA revealed a significant main effect of Stress (PHY, SOC, CONT) in apical, basal and overall dendritic length between groups [$Fs_{(2,24)} \geq 5.796$, $ps \leq 0.008$]. Subsequent post-hoc comparisons showed a significant decrease in overall and apical dendritic length in LRs following CVP and CVS exposure, and a decrease in basal dendritic length in LRs following CVP exposure compared to handled controls [$ps \leq 0.016$]. However in HRs, only basal dendritic length was slightly decreased following the CVS exposure compared to handled controls [$p = 0.046$, Figure 17 (A-C)]. No significant effects were detected in overall and apical dendritic length in HRs exposed to chronic variable stress compared to handled controls.

Dendritic branches

Number of dendritic branches in apical, basal and overall dendrites (apical + basal) for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 17). A two-way ANOVA revealed a significant main effect of Stress (PHY, SOC, CONT) in number of dendritic branches for apical, basal and overall dendrites between groups [$Fs_{(2,24)} \geq 5.514$, $ps \leq 0.010$]. Post-hoc comparisons showed a significant decrease in number of dendritic branches for overall and apical

dendrites in LRs exposed to CVP and CVS, and in HRs exposed to CVS compared to handled controls [*p*s $\leq$ 0.017]. Moreover, basal number of branches were decreased in HRs exposed to CVS [$p = 0.017$] whereas in LRs no significant effects were observed compared to their respective handled controls [Figure 17 (D-F)].

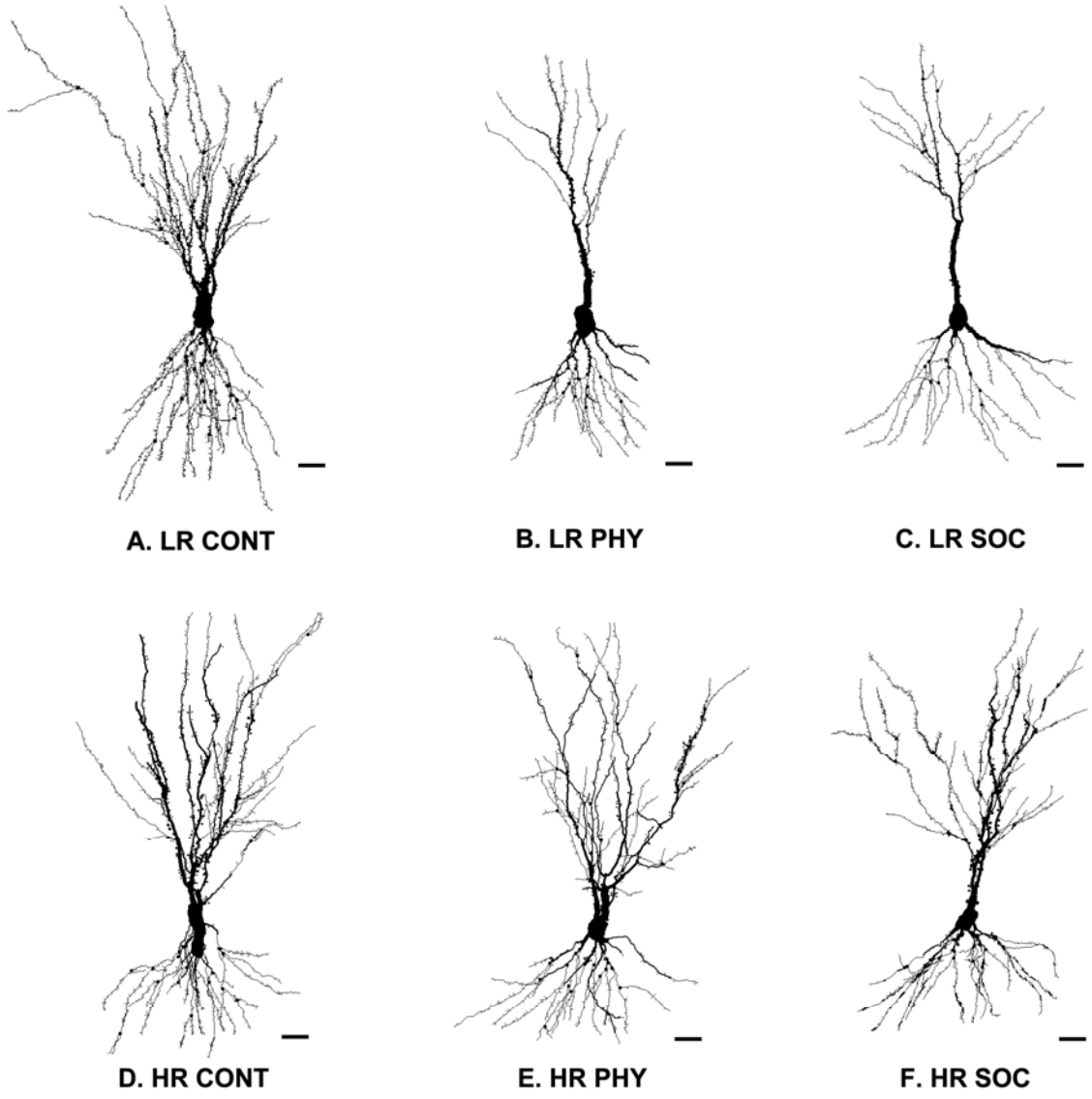


Figure 16: Representative images of reconstructed CA3 neurons from LR control (A), LR chronic variable physical stress (B), LR chronic variable social stress (C), HR control (D), HR chronic variable physical stress (E) and HR chronic variable social stress (F) groups. Scale bar = 25μm. CA3 apical and basal dendritic lengths are decreased with exposure to CVP, whereas only a decrease in the apical tree is observed with CVS exposure in LRs. The only effect detectable in dendritic length in HRs is a slight but significant decrease in the basal tree with CVS exposure. Both stress regimens resulted in a marked decrease in the number of dendritic branches in the apical arbors in LRs. This effect is only seen with CVS exposure in HRs, and reflected in both apical and basal dendritic trees.

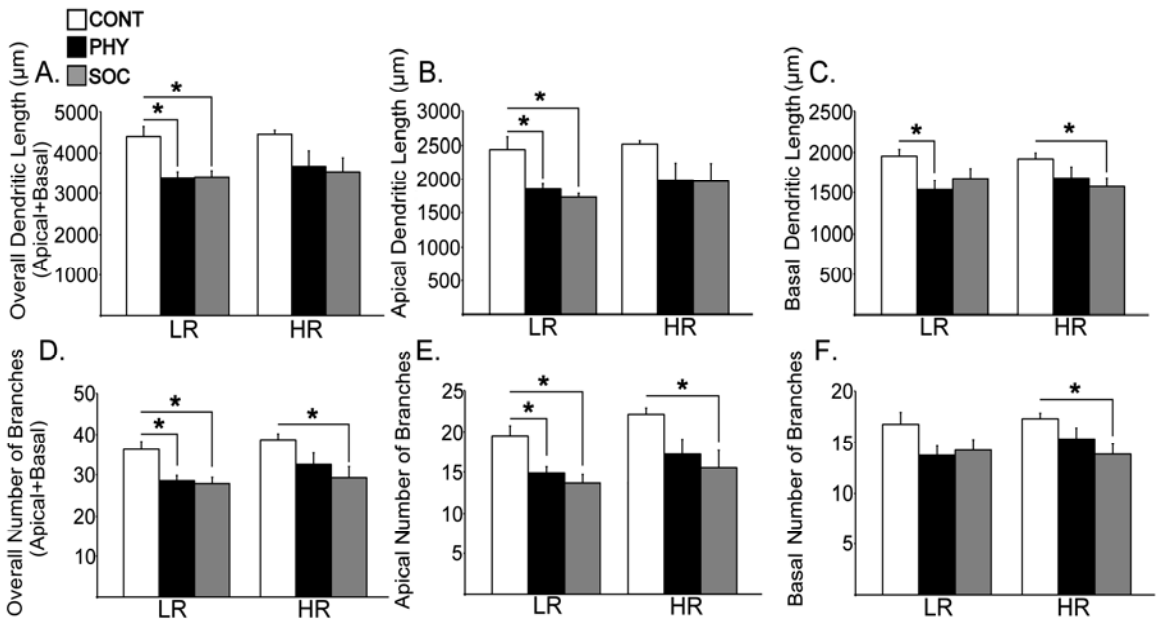


Figure 17: CA3 dendritic length and number of branches in the LRHR rats following chronic variable physical (PHY) and social stress (SOC) or control handling (CONT). Mean dendritic length of CA3 neuron $\pm$ SEMs are plotted by bar graphs for overall dendritic length (apical+basal, A), apical dendritic length (B) and basal dendritic length (C); and mean number of dendritic branches $\pm$ SEMs are plotted by bar graphs for overall dendrites (apical+basal, D), apical dendrites (E) and basal dendrites (F). *: $p \leq 0.05$.

Number of total spines and densities

A representative dendritic segment imaged at two different Z-planes is shown in Figure 18 to exhibit different types of spines that are visible using the Golgi-Cox technique. Number of total spines irrespective of type residing in apical, basal and overall dendrites (apical + basal) for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 19). A two-way ANOVA showed a significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) and significant main effect of Stress in number of total spines for apical, basal and overall dendrites [$Fs_{(2,24)} \geq 9.046$, $ps \leq 0.001$]. A two-way ANOVA also showed significant main effect of Phenotype in number of total spines in overall and basal dendrites [$Fs_{(1,24)} \geq 8.118$, $ps \leq 0.008$]. Post-hoc comparisons showed that number of total spines in apical, basal and overall dendrites was lower in control HRs compared to control LRs [$ps \leq 0.007$]. Moreover, number of total spines in apical, basal and overall dendrites was decreased in HRs exposed to CVP, and in LRs exposed to CVP and CVS compared to handled controls [$ps \leq 0.043$, Figure 19 (A-C)]. Additionally, Sholl analysis showed that number of total spines was lower in control HRs compared to control LRs between 60 and 440 µm segment of apical dendritic tree; and between 40 and 240 µm segment of basal dendritic tree [$ps \leq 0.045$, Figure 20A, B]. Furthermore, Sholl analyses showed decreased number of total spines (1) in HRs exposed to CVP, between 120 and 340 µm segment of apical dendritic tree and between 60 and 140 µm segment of basal dendritic tree [$ps \leq 0.023$, Figure 20A, B]; (2) in LRs exposed to CVP and CVS, between 60 and

440 µm segment of apical dendritic tree and between 40 and 240 µm segment of basal dendritic tree [*p*s $\leq$ 0.031, Figure 20A, B].

Density of total spines in apical, basal and overall (apical + basal) dendrites for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 19). A two-way ANOVA revealed a significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) [$Fs_{(2,24)} \geq 7.265$, *p*s $\leq$ 0.003], significant main effect of Phenotype [$Fs_{(1,24)} \geq 6.345$, *p*s $\leq$ 0.018] and Stress [$Fs_{(2,24)} \geq 9.236$, *p*s $\leq$ 0.001] in density of total spines in apical, basal and overall dendrites. Post-hoc comparisons showed that density of total spines in apical, basal and overall dendrites was lower in control HRs compared to control LRs [*p*s $\leq$ 0.018]. CVP and CVS resulted in decreased levels of total spine density in apical, basal and overall dendrites in LRs, whereas in HRs no significant effects were detected compared to their respective handled controls [*p*s $\leq$ 0.0003, Figure 19 (D-F)].

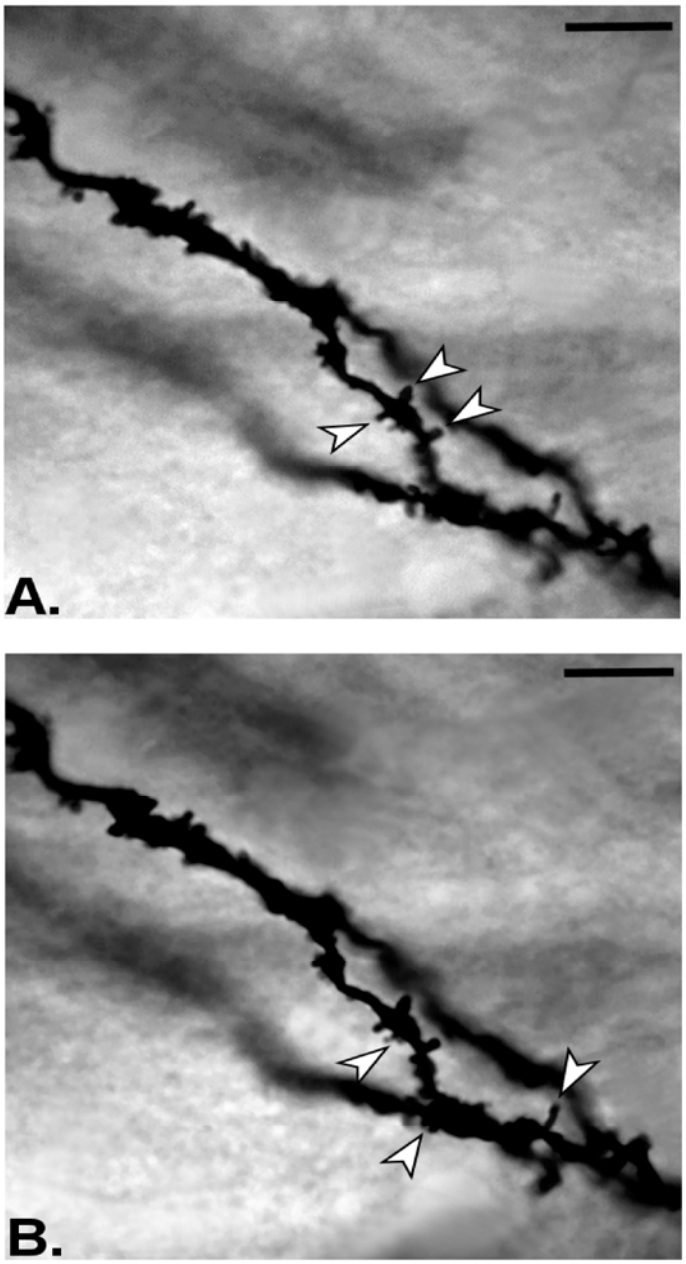


Figure 18: Golgi-stained CA3 pyramidal neuron, distal segment of a basal dendrite. A) Arrows point to mushroom spines that are in focus in this z-position. B) Z-axis is dropped down by 5 µm compared to A. Arrows point to other types of spines (e.g., stubby, filopodia) that are in focus in this z-position. Scale bar = 5 µm.

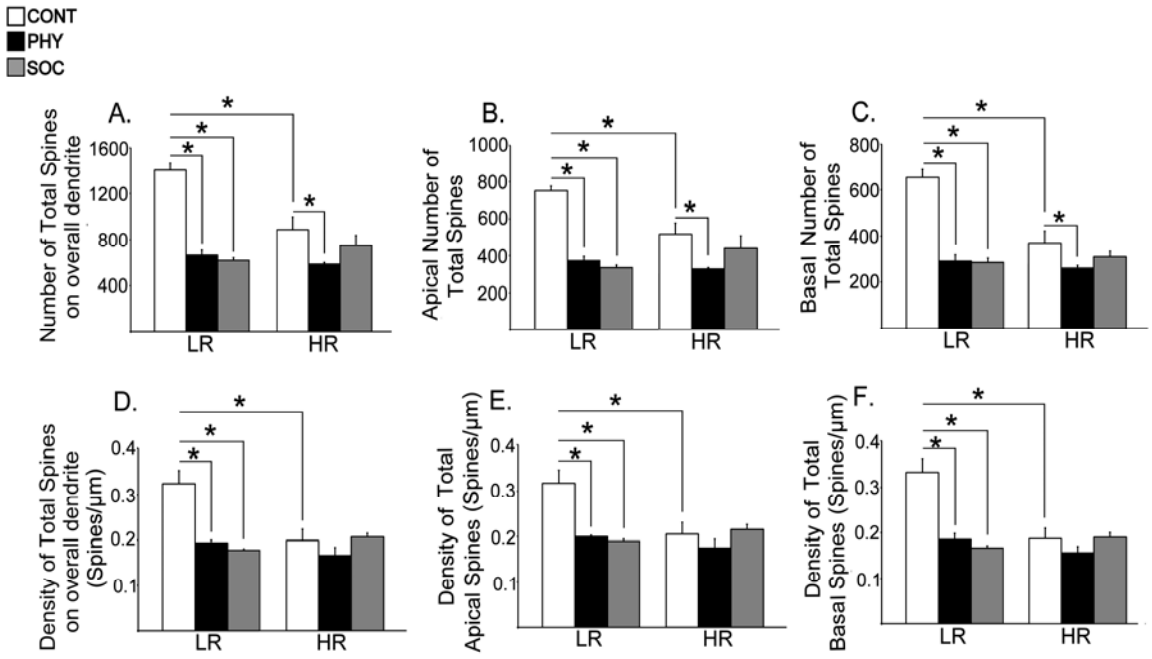


Figure 19: Number of total spines (mushroom-shaped spines+ thin, stubby, filopodia spines) and densities in LRHR rats following chronic variable physical (PHY) and social stress (SOC) or control handling (CONT). Mean number of total spines $\pm$ SEMs are plotted by bar graphs for overall dendrites (apical+basal, A), apical dendrites (B) and basal dendrites (C); and mean density of total spines $\pm$ SEMs are plotted by bar graphs for overall dendritic field (apical+basal, D), apical dendritic field (E) and basal dendritic field (F). *: $p \leq 0.05$.

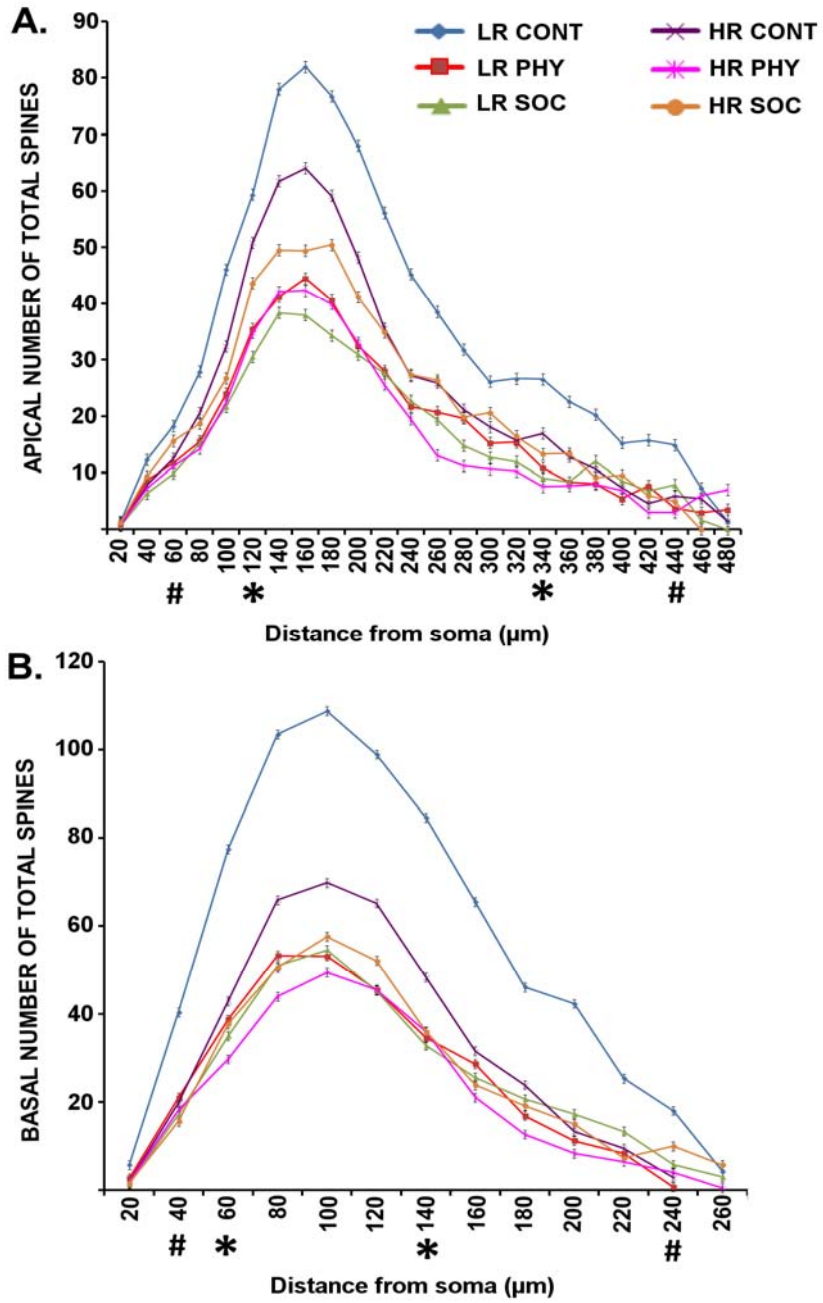


Figure 20: Sholl plots illustrate the distribution of number of total spines in apical dendritic tree (A) and basal dendritic tree (B) against distance from soma. Chronic variable physical stress (PHY), chronic variable social stress (SOC), control handling (CONT). *:PHY effect (120-340 µm segment of apical tree and 60-140 µm segment of basal tree) in HR rats. #: PHY and SOC effects (60-440 µm segment of apical tree and 40-240 µm segment of basal tree) in LR rats. *, #: $p \leq 0.05$.

Number of mushroom spines and densities

Number of mushroom spines in apical, basal and overall (apical + basal) dendritic fields for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 21). A two-way ANOVA showed significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT), a significant main effect of Stress in number of mushroom spines for apical, basal and overall dendrites [$Fs_{(2,24)} \geq 10.337$, $ps \leq 0.0006$]. A two-way ANOVA also showed significant main effect of Phenotype in number of mushroom spines in overall and basal dendrites [$Fs_{(1,24)} \geq 3.322$, $ps \leq 0.014$]. Post-hoc comparisons showed that number of mushroom spines in apical, basal and overall dendrites was lower in control HRs compared to control LRs [$ps \leq 0.008$]. Moreover, CVP and CVS resulted in decreased number of mushroom spines in apical, basal and overall dendrites in both LR and HR rats compared to their respective handled controls [$ps \leq 0.0003$, Figure 21 (A-C)]. Additionally, Sholl analyses showed that number of mushroom spines was lower in control HRs compared to control LRs between 60 and 440 µm segment of apical dendritic tree; and between 40 and 240 µm segment of basal dendritic tree [$ps \leq 0.046$, Figure 22A, B]. Furthermore, Sholl analyses showed decreased number of mushroom spines in (1) HR rats exposed to CVS and CVP, between 80 and 280 µm; and between 80 and 360 µm segments of apical dendritic trees respectively [$ps \leq 0.026$, Figure 22A]; and between 60 and 160 µm and between 60 and 200 µm segments of basal dendritic trees respectively [$ps \leq 0.017$, Figure 22B]; (2) LR rats exposed to CVS and CVP, between 60 and 440 µm segment of apical dendritic tree and between 40 and 220 µm segment of basal dendritic tree [$ps \leq 0.0004$, Figure 22A, B].

Density of mushroom spines in apical, basal and overall (apical + basal) dendrites for each CA3 pyramidal neuron was computed from three-dimensional reconstructions, averaged and plotted in bar graphs for control and stress (CVP and CVS) groups in LRHR rats (Figure 21). A two-way ANOVA revealed significant interaction between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) [$Fs_{(2,24)} \geq$, ps < 0.0001] and a significant main effect of Phenotype [$Fs_{(1,24)} \geq 5.427$, ps ≤ 0.028] and Stress [$Fs_{(2,24)} \geq 38.452$, ps ≤ 0.001] in density of mushroom spines in apical, basal and overall dendrites. Post-hoc comparisons showed that density of mushroom spines in apical, basal and overall dendrites was lower in control HRs compared to control LRs [ps ≤ 0.018]. Moreover, density of mushroom spines in apical, basal and overall dendrites was decreased with CVP and CVS in both LR and HR rats compared to their respective handled controls [ps ≤ 0.016, Figure 21 (D-F)].

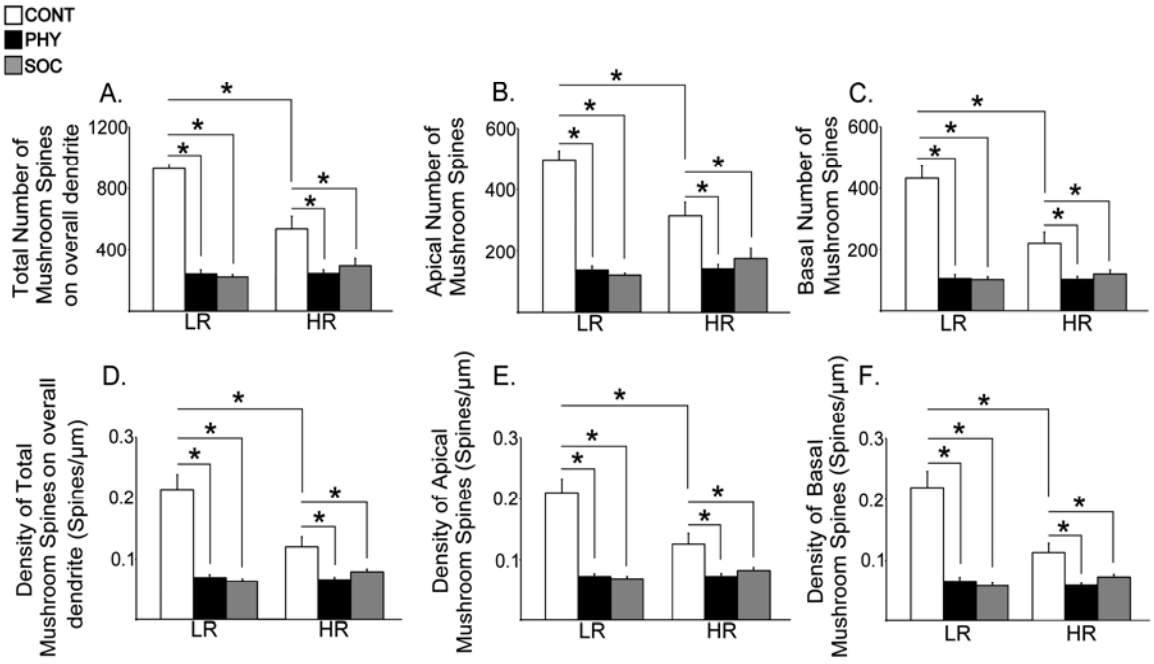


Figure 21: Number of mushroom spines and densities in LRHR rats following chronic variable physical (PHY) and social stress (SOC) or control handling (CONT). Mean number of mushroom spines $\pm$ SEMs are plotted by bar graphs for overall dendrites (apical+basal, A), apical dendrites (B) and basal dendrites (C); and mean density of mushroom spines $\pm$ SEMs are plotted by bar graphs for overall dendritic field (apical+basal, D), apical dendritic field (E) and basal dendritic field (F). *: $p \leq 0.05$.

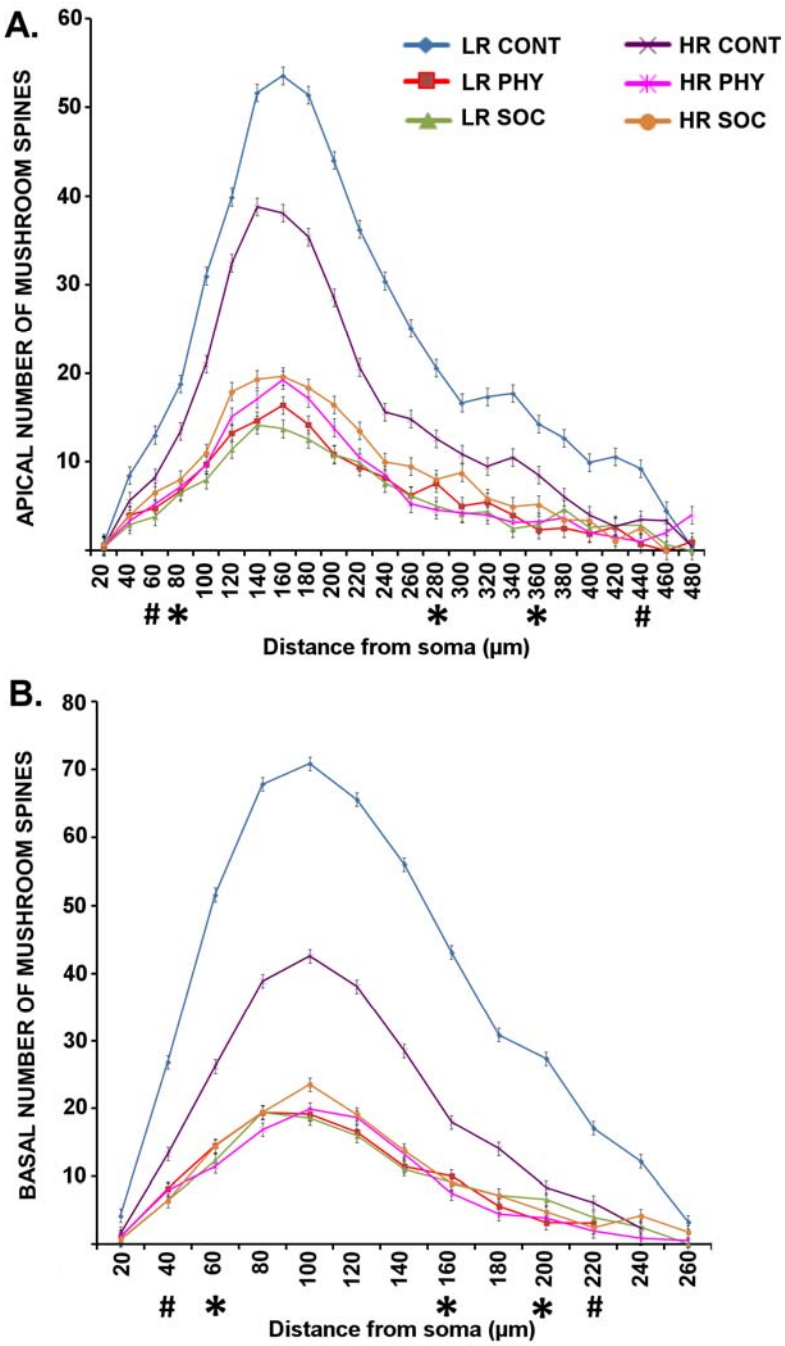


Figure 22: Sholl plots illustrate the distribution of number of mushroom spines in apical dendritic tree (A) and basal dendritic tree (B) against distance from soma. Chronic variable physical stress (PHY), chronic variable social stress (SOC), control handling (CONT). *:PHY effect (80-360 µm segment of apical tree and 60-200 µm segment of basal tree) and SOC effect (80-280 µm segment of apical tree and 60-160 µm segment of basal tree) in HR rats. #: PHY and SOC effects (60-440 µm segment of apical tree and 40-220 µm segment of basal tree) in LR rats. *, #: $p \leq 0.05$.

Discussion

An extensive decrease in dendritic length, number of branches (termed as dendritic complexity) and overall spine density were observed in LRs with both CVP and CVS exposures albeit effects were more widespread with the CVP exposure. However, stress-induced effects in dendritic length and complexity were much smaller and only with CVS in HRs compared to LRs. Moreover in HRs in contrast to LRs, decrease in spine density was restricted to mushroom spines without altering total spine density (mushroom spines + other type of spines) in both apical and basal dendrites and this effect was observed with both type of stress exposures. These results show that although chronic variable stress paradigm induces CA3 dendritic remodeling in both LR and HR rats, the intensity of stress-induced CA3 dendritic remodeling in HRs is not as severe as in LRs. Moreover, stress and/or phenotype effects in dendritic spine plasticity are equally distributed across the apical and basal dendritic trees, suggesting no particular clustering of changes in mushroom and/or total spines along the dendritic tree as indicated by Sholl analyses. Thereby, these results emphasize exclusion of changes in MF innervations volume as a sole regulatory mechanism observed in CA3 dendritic plasticity.

Current results showed resilience of CA3 neurons against deleterious effects of CVP in HR rats indicated by a lack of dendritic atrophy and maintained number of branches. Interestingly, a slight but significant decrease in dendritic length and complexity in CA3 neurons was observed in HR rats with CVS only. In the light of widely present CVP- and CVS- induced atrophy in dendritic length in the LRs, lack of such effects with CVP and only limited changes with CVS in the HR phenotype may underline fundamental difference in neurobiological mechanisms affected by stress. In

agreement with data from Chapter 1, it is plausible that stress-induced increase in BDNF and/or MF sprouting in the HR rats could account synergistically or independently for protection of CA3 neuron from stress-induced dendritic atrophy. Although mossy fibre sprouting is likely not over emphasized since data also show homogenous effects in spine measurements along the dendritic extent stretching from proximal to distal dendrites. Nonetheless, the hypothesized "rescue" of CA3 dendritic material from stress-induced atrophy in the HR animals is full in response to CVP and partial in response to CVS. These results suggest an overall resilience to stress-induced attenuation in CA3 dendritic complexity in the HR phenotype.

It is possible that stress-induced atrophy in CA3 dendritic length and/or branch number may lead to a compensatory increase in spine number and/or density as it was shown in a previous report. In this report, chronic restraint (6 hr/day for 21 days) stress-induced decrease in CA3 dendritic complexity is coupled with increased number of thorny excrescences where mossy fibres form synapses on CA3 cells in adult rats (Sunanda et al., 1995), implicating compensatory adjustments in mossy fibre innervations. However in the LR phenotype where I observed dendritic atrophy in response to CVP and CVS, I note no compensatory spinogenesis. Indeed in agreement with published studies, my findings indicate a widespread decrease in mushroom spine number/density and overall spine number/density in both apical and basal trees together with atrophy in dendritic length (Vyas et al., 2002, Kleen et al., 2006; Pillai et al., 2012). This emphasizes that CVP or CVS exposure in peripubertal period has global and widespread detrimental effect on CA3 neurons of LRs without any observable compensatory dendritic response. Similar to LRs, HRs also showed decreased mushroom

spine number and density in response to both stress types and in both apical and basal dendritic arbors. However in HRs, total (mushroom+ other type) spine number decreased only in response to CVP and did not accompany a corresponding decrease in total spine density. These results indicate that although number of mature spines decrease in HRs with stress, total spine density does not change probably due to an increase in immature type of spines on dendritic surfaces. This is noteworthy because overall dendritic length of CA3 neuron does not change with CVP exposure in HRs and hence a sharp decline in mushroom spine number most likely be reflected as decrease in total spine density with stable dendritic length which is clearly not the case here. Therefore, HR rats in contrast to LR rats may attempt to compensate for substraction of mature spines from CA3 dendritic fields in response to stress at least partially via generating new synapses that are in the process of maturation.

An intriguing question is the timeline of morphological changes that accompany chronic stress in LRHR phenotype. Isgor and colleagues (2004a) showed that following 3 weeks of recovery from juvenile CVS exposure into young adulthood (PD 77), atrophy of hippocampal volume in the CA1, DG and CA3 cell layers were reversed in a phenotype non-specific population (Isgor et al., 2004). However, CVP effects on hippocampal gross morphology were more robust and resistant to recovery (Isgor et al., 2004). Therefore, it is plausible to expect that these stress exposures in adolescence, primarily CVS, could produce dynamic and reversible effects on CA3 dendrites. Indeed in a previously published report, chronic restraint (6hr/day for 21 days) stress-induced CA3 dendritic atrophy emphasized as reduced number of branch points and shortened total length of apical dendritic tree was reversed to control levels by 10 days after the last

stress session (Conrad et al., 1999). In light of these reports, CVS-induced changes in dendritic length, branch number and alterations in spine number and density may possibly have a time course of reversal with recovery from stress in LRHR rats that will be investigated in future studies.

An intriguing phenotype effect was observed in stress-free conditions where control HRs showed lower number of mushroom spines and spine density in CA3 apical and basal dendrites compared to control LRs. This effect was reflected in total spine number and density irrespective of spine type. In parallel with these findings, data from Chapter 1 showed that in control HRs, levels of acH3 and acH4 at BDNF promoters P2 and P4 were lower, respectively compared to control LRs. BDNF has been shown to play an important role for maintaining dendritic and spine morphology of hippocampal neurons (Magariños et al., 2011). Therefore, lower levels of chromatin plasticity on hippocampal BDNF gene expression in control handled HRs compared to LRs may be a factor in markedly lower number of CA3 spines and spine density in the HR rats. This finding is also in line with previous reports showing elavated levels of CORT in experimentally naïve HR rats compared to LR rats as part of initial description of the LRHR phenotype (Piazza et al., 1989). Excess basal levels of CORT, albeit in small magnitude, may contribute to lower levels of CA3 spines in HRs compared to LRs. Additionally, data from Chapter 1 showed stress-induced decrease in MF terminal fields and epigenetic suppression of hippocampal BDNF gene expression in LRs that were accompanied with decreased mushroom and overall spine numbers and densities in CA3 neurons reported here. Moreover, Sholl analyses revealed that CVS- and CVP-induced decrease in mushroom and overall spine numbers in LR and HR rats is uniform on both

proximal and distal dendritic trees, and these effects in LR rats are much higher in magnitude through apical and basal dendrites than HR rats exposed to same stressors. In addition, number of mushroom and overall spines in control and stress groups in both LR and HR rats reaches a peak at 160 µm from the soma, the segment falls inside the stratum lucidum region on apical dendrite, (Henze et al., 1996) possibly due to mossy fibre innervations to stratum lucidum region of CA3 neurons. However, stress effects remain the same on mushroom and overall spine numbers in both LR and HR rats in stratum lucidum region, proximal to the CA3 soma. In LRs, CVP and CVS-induced decrease in MF terminal fields coupled with drastic reduction in length and complexity of CA3 dendritic arbors, and mushroom and overall spines on proximal and distal dendrites. The commissural fibers from the contralateral hippocampus, recurrent axon collaterals from CA3 neurons and the perforant path from the entorhinal cortex are distal afferents on CA3 neuron (Brown and Zador, 1990; Martinez and Barea-Rodriguez, 1997; Witter and Amaral, 2004) and this drastic remodeling within CA3 dendritic tree in LRs suggest that commissural-associational collaterals and perforant path inputs contribute to CA3 dendritic retraction along with MF. However in HRs, CVP- and CVS-induced increase in MF terminal fields and epigenetic induction of hippocampal BDNF gene expression were accompanied by a decrease in CA3 mushroom spine number and density without alterations in overall spine density. This finding indicates that loss of mushroom spines might be an adaptation to limit the increased excitatory input from commissural-associational collaterals from CA3 neurons since no reduction in MF terminal fields is observed in stress-exposed HR rats. Moreover, loss of mushroom spines may lead to increase in other type of spines with CVP and CVS that result in as no alterations in

overall spine density. In present experiment, thin, stubby and filopodium spines were considered as other type of spines and analyzed together without any differentiation between spine types. Additionally, number of other type of spines was similar to control levels in HRs with both stress exposure (data not shown). However, chronic variable stress may induce a decrease in the one type of spine (e.g., stubby) and simultaneously it may promote the induction of another type of spine (thin, filopodium or vice versa), resulting in no change as a final effect. Other type of spines are highly dynamic spines and they hold transient morphological stage that maintains structural flexibility, leading either to enlargement and stabilization (e.g., mushroom spines), or to shrinkage, depending on strength of synaptic activity (Bourne and Harris, 2007). Therefore, blunted increase in other type of spine/spines with chronic variable stress in HRs that is supported by newly sprouted axon terminals of dentate gyrus granule neurons, might be a compensatory mechanism for stress-induced loss of mushroom spines. Moreover, it is important to keep in mind that some synapses may turn into shaft synapses, as spines are lost, as a compensatory mechanism to replace the loss of spines (for review see Yuste and Bonhoeffer, 2001). Three-dimensional morphometric analyses also revealed that, repeated restraint stress (6h/day for 3 weeks) induces a decrease in large spines (e.g., mushroom spines) and shift towards small spines (e.g., thin, stubby) in pyramidal neurons of medial prefrontal cortex (Radley et al., 2008). These results suggest that synaptic plasticity is modulated by chronic stress via changing the balance among spine subtypes. Thus, chronic variable stress-induced decrease in the mushroom spines in HRs can be substituted with thin, stubby or filopodium spines in the hippocampus compared to LRs. These are possible hypotheses to be investigated in future studies.

Since BDNF has been shown to play a role on neurogenesis in the dentate gyrus (Lee et al., 2002; Sairanen et al., 2005), increased levels of hippocampal BDNF with chronic variable stress in HRs may induce generation of new granule neurons which may also stimulate formation of new synapses with CA3 pyramidal neurons. Although our 15 days of chronic variable stress paradigm may not be long enough for newborn neurons to fully differentiate and become mature neurons, the function of immature granule neurons should not be overlooked. Even though neuronal maturation takes 3 to 4 weeks for new neurons to be functionally integrated into existing circuits (Esposito et al., 2005), it has been shown that immature granule neurons could participate in hippocampal information processing before maturation (Mongiat et al., 2009). Immature granule neurons may also provide neurotrophic support by secreting neurotrophic factors such as BDNF for mature neurons to form their synaptic connection, which is corroborated by findings from neural stem cell studies (Lu et al., 2003; Llado et al., 2004). These studies showed that in addition to differentiation into neuron-like cells, neural stem cells also produce various trophic factors which play an important role to support other cell types (Lu et al., 2003; Llado et al., 2004). Additionally, immature granule neurons have been shown to form functional glutamatergic synapses onto dentate gyrus interneurons and CA3 pyramidal neurons before they are fully matured (Faulkner et al., 2008; Toni et al., 2008). Therefore, stress-induced induction of hippocampal BDNF in HRs may lead to generation of new granule neurons that in turn establishes synaptic connections with CA3 neurons. These newly formed synaptic connections may be governed by an increase in other type of spines in dendrites of CA3 pyramidal neurons. Later on, these spines may differentiate into more stable mushroom spines depending on the functional significance

of neuronal activity. Taken together, these suggest that compensatory mechanisms at work during chronic variable stress exposure in juvenile HRs that partially protects CA3 neurons from adverse effects of stress.

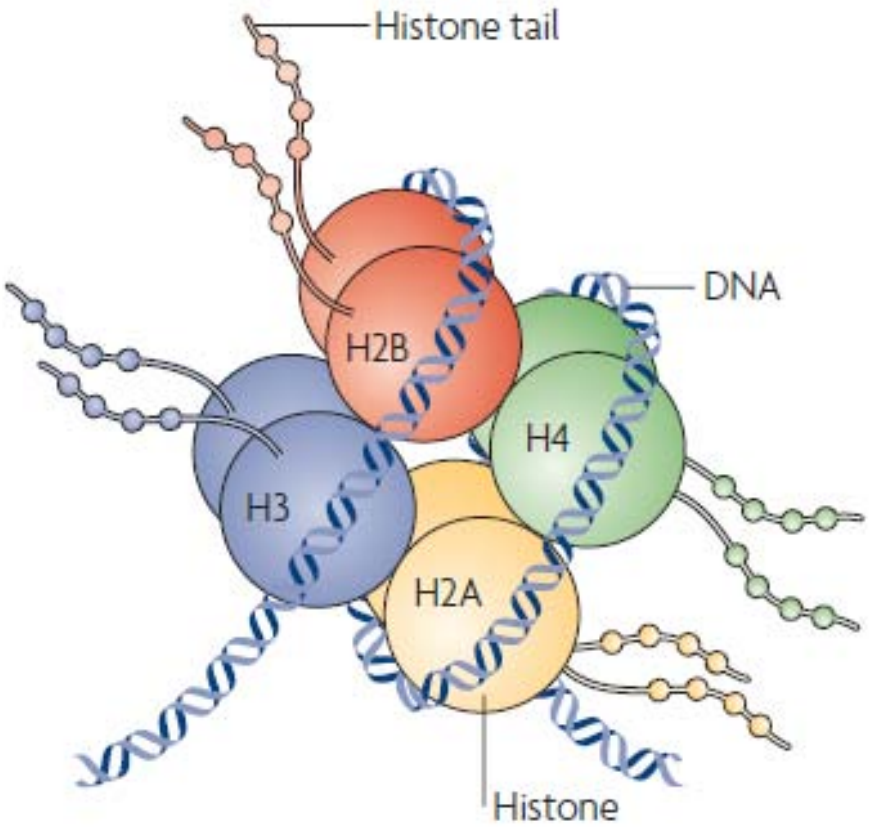
In conclusion, CVP-induced increase in MF terminal fields and/or hippocampal BDNF either partially or fully could account for protection of CA3 neurons from deleterious effects of same stress in HRs. In LRs however, stress-induced extensive CA3 dendritic retraction and accompanied loss of spines are coupled with decrease in MF terminal fields and hippocampal BDNF without any compensatory dendritic response. Moreover, CA3 dendritic remodeling is detectable with CVS exposure in HRs but it is not as severe as in LRs, indicating that CA3 neurons in HRs are more resilient to effects of chronic variable stress compared to LRs. These findings highlight individual differences in neuromorphology of stress responsiveness during peripubertal-juvenile period in LRHR rats and validate that these two types of stress regimens can produce differential effects in an individual difference model of stress hyperactivity.

CHAPTER 3: CHRONIC VARIABLE STRESS DURING PERIPUBERTAL-JUVENILE PERIOD ALTERS EPIGENETIC REGULATION OF THE HIPPOCAMPAL BDNF GENE AND DEPRESSIVE-LIKE BEHAVIOR IN LRHR RATS: MODULATION BY POTENTIAL THERAPEUTIC, HISTONE DEACETYLASE INHIBITOR

Introduction

Epigenetic regulation is defined as reversible chromatin modifications at gene promoter regions that affect gene expression without changing the DNA sequence (Verdone et al., 2005; Santini et al., 2007). DNA methylation and histone modifications (acetylation, methylation, and phosphorylation) are widely studied epigenetic mechanisms in animal models of stress-induced depression (Tsankova et al., 2004, 2006; Schroeder et al., 2007; Covington et al., 2009). The fundamental unit of chromatin is the nucleosome, which contains 147 base pairs of DNA wrapped around the octamer of core histones, consisting of two copies of each histones H2A, H2B, H3 and H4 (for review see Tsankova et al., 2007, Figure 23). Histone tails are composed of lysine amino acids, which play an important role for regulation of chromatin structure and gene transcription (Wolffe, 1994). Histones can be acetylated at lysine residues by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs), thereby leading to gene expression or repression, respectively (Grunstein, 1997; Hsieh and Gage, 2005). Thus, interplay between HAT and HDAC function is important for epigenetic regulation of genes and dysregulation of this delicate function by environmental factors,

such as stress, may contribute to the molecular mechanisms that play important role in regulation of stress-related disorders, including depression.



Adapted from Tsankova et al., 2007

Figure 23: Schematic of a nucleosome showing a DNA strand wrapped around a histone octamer composed of two copies of each histones H2A, H2B, H3 and H4 (Tsankova et al., 2007).

Investigation of the role of chromatin remodeling in mechanisms of stress-induced depressive-like behavior *in vivo* in animal studies has involved the use of HDAC inhibitors. CNS-penetrable HDAC inhibitors have been shown to prevent histone deacetylation by selectively deactivating the class I and/or II HDACs, thereby causing increased levels of histone acetylation. Rodent studies found that systemic administration of sodium butyrate, a nonspecific class I and II HDAC inhibitor, results in antidepressant-like effects (Tsankova et al., 2006; Schroeder et al., 2007). Similarly, infusion of MS-275, a selective class I HDAC inhibitor, and SAHA, a selective class I and II HDAC inhibitor into the mouse nucleus accumbens (NAc) also induced antidepressant-like effects in animal models of depression (Covington et al., 2009). This line of work has highlighted HDAC inhibitors as potential therapeutics for the treatment of depression, and has initiated the investigation of target genes in particular brain regions implicated in depressive-like symptoms.

In the present study, the effects of the class I and II HDAC inhibitor, Trichostatin A (TSA), in conjunction with chronic variable stress will be assessed for its affects on depressive-like behavior and epigenetic regulation of hippocampal BDNF gene expression in juvenile LRHR rats. Systemic administration of TSA has been used in animal studies including cocaine and alcohol addiction paradigms to investigate epigenetic modifications associated with these disorders and this drug has been shown to alter acetylation levels of histones in the brain (Kumar et al., 2005; Pandey et al., 2008; Romieu et al., 2008; Host et al., 2011). Moreover, TSA has been shown to induce transcriptional activation of BDNF promoter 1, which is correlated with increased occupancy of the promoter by acH3 and subsequent exon 1-specific increases in BDNF

mRNA levels in cultures of hippocampal neurons (Tian et al., 2009, 2010). However, TSA has not been used *in vivo* in a chronic stress paradigm to investigate its effects in depressive-like behavior and specifically the regulation of the BDNF gene, which is the focus of the present study using the LRHR phenotype. Data from Chapter 1 showed phenotype-specific (i.e., LRs) emergence of depressive-like behavior following peripubertal-juvenile CVP exposure. Accompanying the behavioral effect, CVP resulted in decreased epigenetic activation of hippocampal BDNF gene expression in LRs. However in HRs, decreased depressive behavior was accompanied by epigenetic induction of hippocampal BDNF gene expression. If stress-induced epigenetic regulation of hippocampal BDNF gene expression is a molecular mechanism that contributes to observed behaviors in LRHR rats, then it would be expected the depressive-like state in LRs can be reversed by an increase in histone acetylation at BDNF promoters. Therefore, this experiment will test the hypothesis that chronic TSA administration during chronic variable stress exposure will induce the HR-like phenotype in LR rats by way of inducing increased acetylation of histone 3 and/or 4 at the BDNF promoters, to a degree similar to that observed in stress-exposed HRs with resulting antidepressant effects.

Methods

LRHR phenotype screening

A total of 108 male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (PD 22) and were screened for locomotor reactivity to a novel environment as described in Chapter 1.

Chronic variable stress paradigm and TSA administration

Animals were exposed to chronic variable stress (as described in Chapter 1) in conjunction with systemic TSA injections between PD 28 and PD 42 (Table 4).

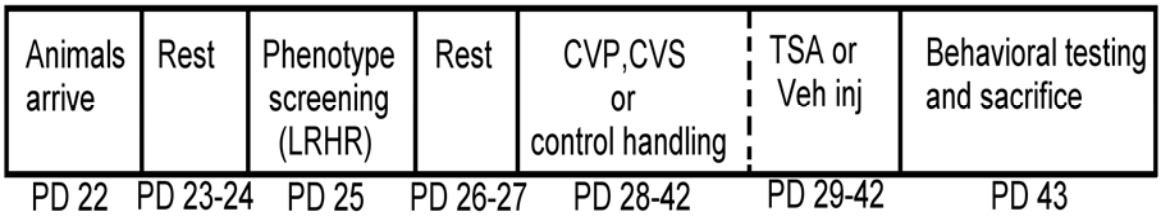
TSA treatment

TSA (Sigma-Aldrich, MO, USA), a commercially available pan-HDAC inhibitor, was administrated via intraperitoneal (i.p.) injection at a dose of 1mg/kg dissolved in a vehicle solution consisting of DMSO and 0.9% NaCl (1:4) and 30 min before stress exposure or control handling. This dose of TSA does not produce any toxicity, as neither cell death nor neurotoxic effects have been shown with higher doses (7.5 mg/kg; chronic treatment) of TSA in the mouse brain (Camelo et al., 2005). TSA or vehicle injections occurred every other day during stress or handling regimen starting from PD 29, for a total of 7 doses.

Behavioral assessments

Social interaction and forced swim tests were conducted as described in Chapter 1.

Table 4: Behavioral procedure for phenotype screening, chronic variable stress exposure and TSA injections.



CVP: Chronic Variable Physical Stress, CVS: Chronic Variable Social Stress, TSA: Trichostatin A, Veh: Vehicle, inj: injection, PD: Postnatal day

Molecular assessments

1- *In situ* hybridization histochemistry was conducted using BDNF riboprobe as described in Chapter 1.

2- Chromatin immunoprecipitation (ChIP) assay was conducted using anti-acetyl histone 3 (H3) and anti-acetyl histone 4 (H4) antibodies as described in Chapter 1.

3- Quantitative real time-PCR (qPCR) was conducted using custom designed primers for BDNF promoters P2 and P4 as described in Chapter 1. Input or total DNA (nonimmunoprecipitated) and immunoprecipitated DNA were PCR amplified in duplicate, and Ct values from each sample were obtained using Stratagene MxPro software. Percent changes in levels of acetylated H3 and H4 at the hippocampal BDNF P2 and P4 promoters in experimental groups relative to corresponding inputs were plotted in bar graphs.

Statistical analyses

Three-way ANOVAs were conducted for (1) percent time spent in social interaction (2) percent time spent in immobility on the forced swim test (3) amounts of acH3 and acH4 at the P2 and P4 promoters of the BDNF gene in the hippocampus, (4) levels of BDNF mRNA expression in the hippocampus following *in situ* hybridization histochemistry between phenotypes (LR, HR), stress (PHY, SOC, CONT) and drug conditions (TSA, VEH). Furthermore, significant interactions and main effects of ANOVAs were followed by Scheffe post-hoc tests. All significance levels were set at $p = 0.05$.

Results

Depressive- and social anxiety-like behaviors in LRHR rats

Figure 24A shows the percent time spent in social interaction in the LRHR rats. A three-way ANOVA revealed significant interaction between Stress (PHY, SOC, CONT) and Drug (TSA, VEH) and a significant main effect of Stress on percent time spent engaged in social behaviors [Fs $_{(2, 60)} \geq 4.668$, ps ≤ 0.036]. Subsequent post-hoc comparisons showed a significant decrease in percent time spent in social interaction in vehicle-injected LR and HR rats exposed to CVP compared to vehicle-injected handled controls [ps ≤ 0.017]. Moreover, TSA administration led to a decrease in percent time spent in social interaction in LRs that received control handling compared to vehicle-injected counterparts [$p = 0.001$].

Figure 24B shows the percent time spent in immobility in LRHR rats tested on the forced swim test. A three-way ANOVA showed significant interactions between Phenotype (LR, HR) and Stress (PHY, SOC, CONT), between Stress and Drug (TSA, VEH) and amongst Phenotype, Stress and Drug [Fs $_{(2, 60)} \geq 4.950$, ps ≤ 0.010]; and significant main effects of Phenotype and Drug [Fs $_{(1, 60)} \geq 10.010$, ps ≤ 0.002] on immobility behavior. Subsequent post-hoc comparisons showed a significant increase in percent time spent in immobility in both vehicle- and TSA-treated LR animals exposed to CVP compared to vehicle- and TSA-treated handled controls, respectively [ps ≤ 0.032]. However, TSA injections resulted in decreased immobility in LRs exposed to CVP compared to vehicle-injected counterparts [$p = 0.002$]. In contrast, vehicle-treated HR animals exposed to CVP or CVS showed a significant decrease in percent time spent in immobility compared to vehicle-treated handled controls [$ps \leq 0.021$]. TSA injections

resulted in a significant decrease in immobility in both control LR and control HR rats compared to vehicle-injected control counterparts [$ps \leq 0.008$].

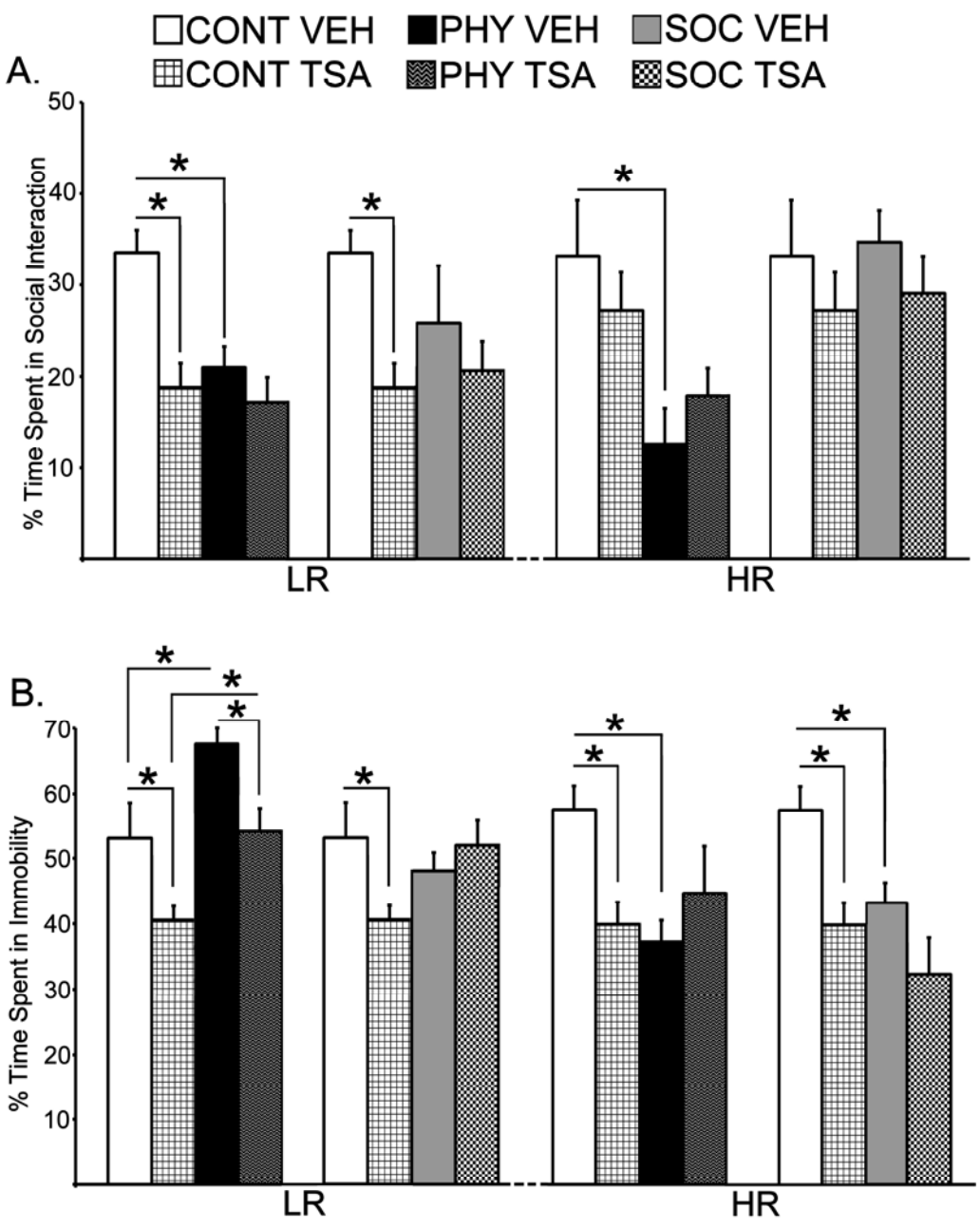


Figure 24: Anxiety- and depressive-like behaviors in LRHR rats. Percent time spent in social interaction (A) and in immobility in the forced swim test (B) in LRHR rats exposed to chronic variable physical stress (PHY) and chronic variable social stress (SOC) or handled as controls (CONT) along with Trichostatin A (TSA) or vehicle (VEH) injections. Means are plotted in bar graphs $\pm$ SEMs. *: $p \leq 0.05$.

Acetylation levels of histone 3 and 4 at the BDNF promoters in LRHR rats

Levels of acH3 at the BDNF P2 promoter and acH4 at the BDNF P2 and P4 promoters in the dorsal hippocampus of the TSA or vehicle-injected LRHR rats exposed to CVP, CVS or control handling are depicted in Figure 25A, 25B and 25C respectively. A three-way ANOVA revealed significant interaction between Stress (PHY, SOC, CONT) and Drug (TSA, VEH) and a significant main effect of Drug in the acH3 levels at the BDNF P2 promoter [Fs $_{(1, 60)} \geq 5.918$, ps ≤ 0.022]. Post-hoc comparisons showed that acH3 and acH4 levels at the BDNF P2 promoter were increased following TSA administration in both LR and HR rats exposed to CVP, CVS or control handling compared to levels observed in vehicle-injected counterparts [ps ≤ 0.024]. Moreover, TSA administration resulted in higher levels of acH3 at the BDNF P2 promoter in LR animals exposed to CVP compared to vehicle-injected handled controls [$p = 0.0001$]. No significant effects were detected in the acH3 levels at the P4 promoter of the BDNF gene in the dorsal hippocampus of the LRHR rats between drug and stress conditions (data not shown). Moreover, a three-way ANOVA revealed significant interaction between Phenotype (LR, HR), Stress (PHY, SOC CONT) and Drug (TSA, VEH) and a significant main effect of Drug in the acH4 levels at the BDNF P4 promoter [Fs $_{(2, 60)} \geq 10.300$, ps $\leq$ 0.003]. Subsequent post-hoc comparisons showed that CVP and CVS resulted in decreased levels of acH4 at the BDNF P4 promoter in vehicle injected-LRs [ps ≤ 0.003]. In contrast, CVP and CVS resulted in increased levels of acH4 at the BDNF P4 promoter in vehicle injected-HRs compared to respective handled controls [ps ≤ 0.011]. Furthermore, TSA administration could fully reverse the decrease in the levels of acH4 at the BDNF P4 promoter observed in LR animals following CVP and CVS [ps ≤ 0012]. In

control handled HRs, TSA administration led to increased levels of acH4 at the BDNF P4 promoter compared to vehicle-injected handled controls **[***p* = 0.028**]**.

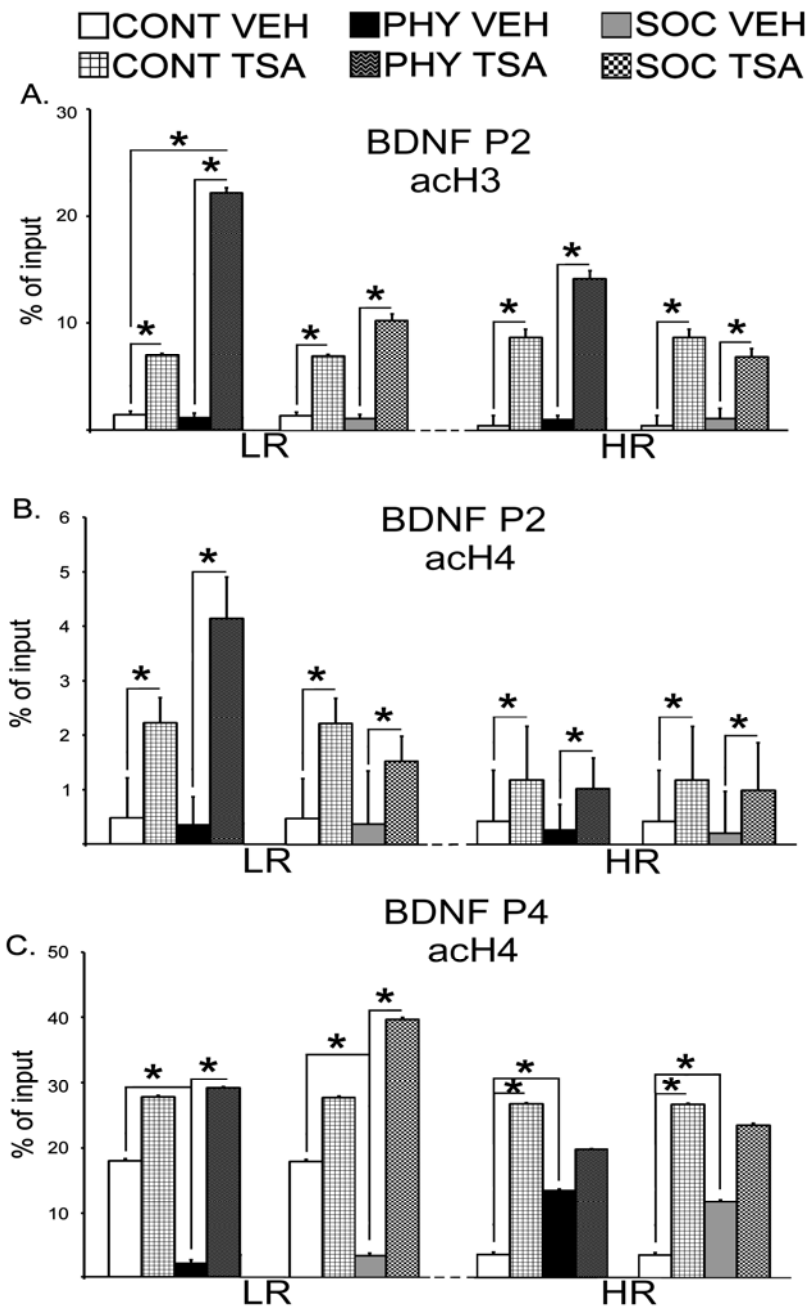


Figure 25: Levels of acetylated Histone3 (acH3) at the BDNF P2 promoter (A), acetylated Histone4 (acH4) at the BDNF P2 promoter (B) and acH4 at the BDNF P4 promoter (C) in the LRHR rats exposed to chronic variable physical stress (PHY) and chronic variable social stress (SOC) or handled as controls (CONT), along with Trichostatin A (TSA) or vehicle (VEH) injections. Means are plotted in bar graphs $\pm$ SEMs. *: $p \leq 0.05$.

BDNF mRNA in the dorsal hippocampus

Figure 26 show levels of the BDNF mRNA in the dorsal hippocampus of the LRHR rats. Three-way ANOVAs showed significant interactions between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) and significant main effects of Stress and Drug (TSA, VEH) in BDNF mRNA levels in the DG and the CA3 field of the hippocampus and in the CA3 field of the hippocampus [Fs $_{(2,\ 60)} \geq 5.414$, ps ≤ 0.026]. Post-hoc comparisons showed that in vehicle-injected HRs, CVP and CVS resulted in upregulated levels of BDNF mRNA in CA3 and DG compared to vehicle-injected handled controls [$ps \leq 0.030$]. Moreover, BDNF mRNA levels were upregulated in the LR hippocampal CA3 field and DG following TSA administration, along with CVP or control handling compared to levels observed in vehicle-injected counterparts [$ps \leq 0.036$]. Furthermore, in control handled HRs, TSA administration resulted in an upregulation of BDNF mRNA levels in both CA3 and DG compared to vehicle-injected counterparts [$ps \leq 0.013$]. In DG, TSA administration together with CVP resulted in augmented BDNF mRNA levels above those observed with CVP in vehicle-injected HRs [$p = 0.04$].

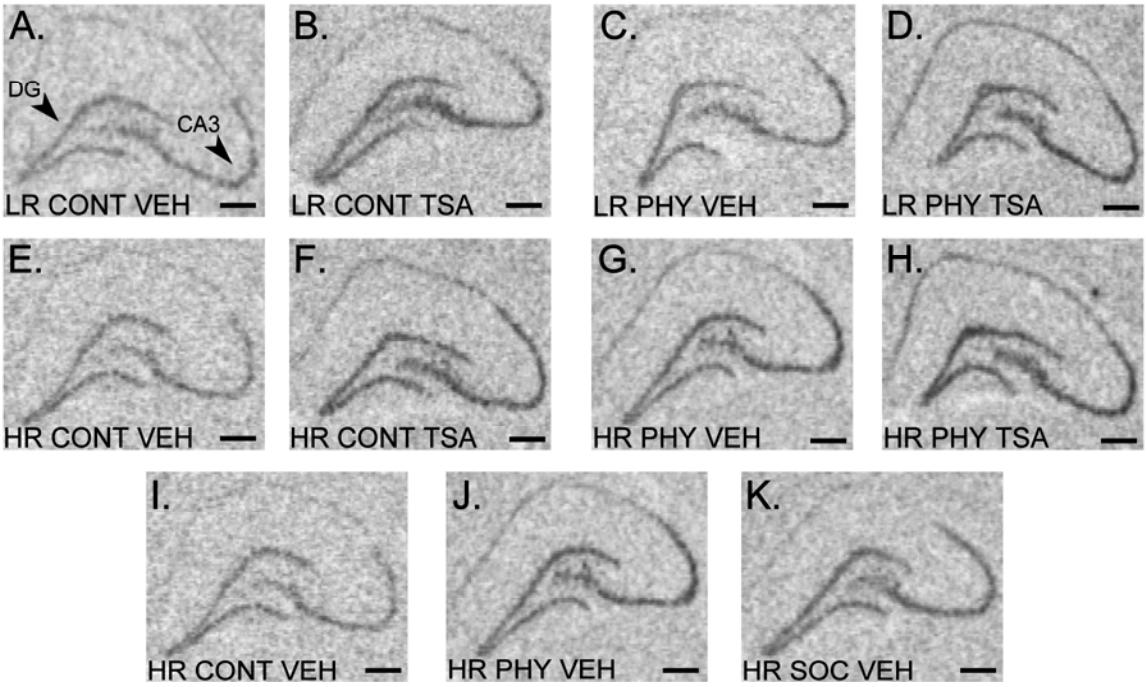


Figure 26: BDNF mRNA expression in the dorsal hippocampus. Panels A, B, C, D, E, F, G, H, I, J and K constitute x-ray film-exposed images of representative coronal hemisections of the hippocampus that were radioactively labeled with an antisense cRNA probe against the BDNF mRNA in the LRHR rats following chronic variable physical (PHY) and social stress (SOC) or control handling (CONT), in conjunction with Trichostatin A (TSA) or vehicle (VEH) injections. Scale bar = 250 μm.

(Figure continues)

(Figure 26 continued)

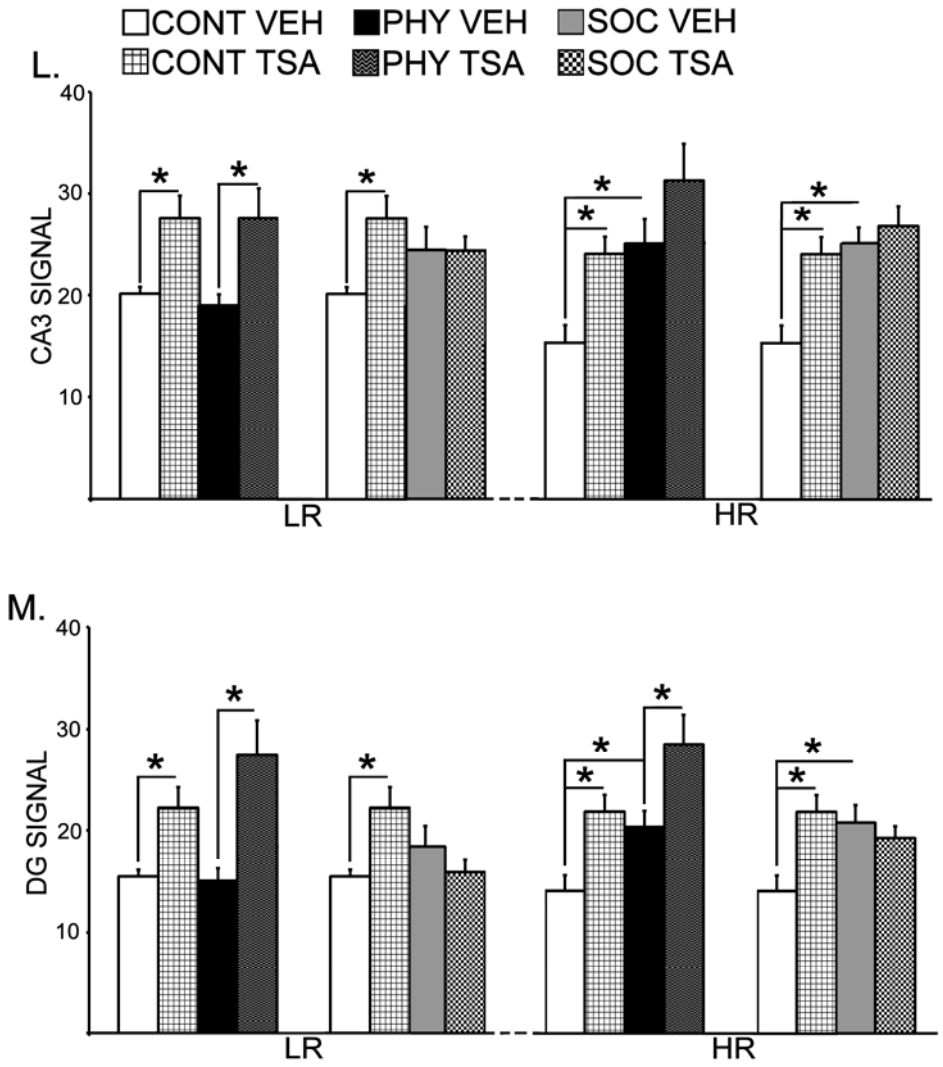


Figure 26: BDNF mRNA levels in the dorsal hippocampus. Means for optical densities ± SEMs are plotted by bar graphs for signal in the CA3 field (L) and the DG (M) of the hippocampus. *: $p \leq 0.05$.

Discussion

The results demonstrate emergence of depressive-like behavior in LRs with CVP, and emergence of antidepressive-like behavior in HRs following both CVP and CVS exposure. These results are in agreement with behavioral findings in Chapter 1, confirming observed behavioral effects in LRHR rats with chronic variable stress exposure. Moreover, TSA treatment reversed the stress-induced increase in depressive-like behavior in LRs and the same treatment mimicked the antidepressive-like behavior observed with CVP and CVS in HRs. These behavioral findings suggest that CVP exposure induces phenotype specific effects on depressive-like behavior in LRHR rats, and that TSA treatment may hold promise for reversing stress-induced depressive-like behavior.

Moreover, in both LRs and HRs, TSA administration either alone or with CVP and CVS resulted in increased levels of acH3 and acH4 at the BDNF promoter P2 in the hippocampus. These findings confirm that the TSA administration regimen is effective at increasing acetylation levels of histones at hippocampal BDNF promoters in LRHR rats. Although levels of acH4 at the BDNF promoter P4 were decreased following CVP and CVS in LRs, TSA treatment reversed these effects in the same phenotype. In control handled HRs, TSA treatment mimicked CVP- and CVS-induced increases in acH4 at the BDNF promoter P4. Furthermore, an upregulation in the BDNF mRNA levels was observed in the LR hippocampal CA3 field and DG following TSA administration along with CVP exposure or control handling compared to levels observed in vehicle injected counterparts. In control handled HRs, TSA administration resulted in upregulation of BDNF mRNA levels in both DG and CA3, in a way similar to effects observed with CVP

and CVS exposure in the same phenotype. These effects on BDNF mRNA are in the same direction as the effects observed in acH3 and acH4 levels at the BDNF promoter P2 in the LRHR rats. Namely, both indicate increased hippocampal BDNF gene transcription, confirming the antidepressive-like effects of the TSA in LR and HR rats.

The results also showed phenotype non-specific emergence of social anxiety-like behavior in LRHR rats with CVP exposure in agreement with behavioral results in Chapter 1. Additionally, TSA treatment induced an increase in social anxiety-like behavior in control-handled LRs in a way similar to effects observed with CVP exposed-LRs. Moreover, CVP-induced anxiety-like behavior remained unaffected in HRs following the TSA treatment, suggesting that anxiogenic effect of CVP may not be mediated by epigenetic plasticity in HR rats.

Antidepressant-like effects of histone deacetylase inhibitors

In agreement with findings in Chapter 1, the present study revealed that CVP has a different impact on the regulation of depressive-like behavior in LRHR rats during peripubertal-juvenile period. Namely, LR rats showed increased depressive-like behavior (measured as increased immobility behavior on the forced swim test) following CVP in agreement with widely reported results on stress-induced depression in adult animals. However, the same stress regimen resulted in decreased depressive-like behavior in HRs showing a stress-induced enrichment-like effect. Moreover, TSA administration reversed depressive-like behavior in LRs and same treatment mimicked the stress-induced antidepressive-like behavior in HRs. Interestingly, both vehicle-treated HR and LR control animals spent more time in immobility on the forced swim test when compared to their respective counterparts (handled controls) in Chapter 1. It is noteworthy to mention

that HR and LR control animals in Chapter 3 studies were not only handled daily, but also received intra peritoneal vehicle injections. Repeated injections have been shown to be stressful since they are perceived as aversive by the animal (Gartner et al., 1980) and affect animals' performance in behavioral tests (Davis and Perusse, 1988). Vehicle injections therefore could be responsible for increased immobility on the forced swim test in HR and LR control animals in Chapter 3.

Antidepressant-like effects of HDAC inhibitors have been shown in other animal models of stress-induced depression (Schroeder et al., 2007; Covington et al., 2009). Sodium butyrate, a pan-HDAC inhibitor, was shown to induce antidepressant effects in a mouse model of depression, when chronically administrated either alone or together with the selective serotonin reuptake inhibitor fluoxetine (Schroeder et al., 2007). Continuous infusion of MS-275, a selective class I HDAC inhibitor, and SAHA, a selective class I and II HDAC inhibitor, in the nucleus accumbens (NAc) of mice exposed to chronic social defeat stress showed an increase in the levels of acetylated H3 and induced antidepressant effects (Covington et al., 2009). Moreover, treatment with MS-275 showed social defeat-induced gene expression modifications that were similar to the effects of systemic fluoxetine, showing common antidepressant effects of both drugs (Covington et al., 2009). These studies show that chromatin modifications play important role in the regulation of depressive-like behaviors in animal models of depression, and that HDAC inhibitors are potential therapeutics to reverse depressive-like states.

Currently, not much information is available for stress-induced alterations in histone acetylation at specific gene promoters linked to depressive-like behavior. Tsankova and colleagues (2006) have shown that chronic social defeat stress results in

downregulation of BDNF transcripts III and IV due to increased repressive histone methylation at the promoter regions. However, antidepressant imipramine treatment reverses downregulation of these transcripts by hyperacetylation of histone 3 at the BDNF promoter, an effect that seems to be mediated at least in part by downregulation of HDAC5 in the hippocampus (Tsankova et al., 2006). Similar to these findings, CVP- and CVS-induced decrease in acetylated Histone 4 at the BDNF promoter P4 was reversed by TSA treatment in LRs. This effect was accompanied by decrease in depressive-like behavior. Moreover, increased acetylation levels of histone 3 and histone 4 at the BDNF promoter P2 in LR and HR rats by TSA treatment were corroborated by upregulation in BDNF mRNA levels in the hippocampus. Since TSA is generalized HDAC inhibitor, it deactivates both class I and II HDACs with no specificity to any members of the HDAC family. Therefore investigation of particular HDAC types and many other genes with genome wide analysis that plays important role on mediating antidepressant-like effects of HDAC inhibitors in the brain need addressing in future studies.

Taken together, these results show that *in vivo* administration of TSA is a potent antidepressant treatment that can reverse stress-induced depressive-like state and current study is the first to report this effect of TSA. However, since TSA was administered systemically, effects of drug were not restricted only to hippocampus. TSA also induces potent anxiogenic effect in LR rats. Therefore, increased social anxiety-like behavior in control handled LRs following TSA administration may be due to increased epigenetic induction of the BDNF gene expression as a consequence of HDAC inhibition in amygdala. Correlation between increased BDNF levels in BLA and elevated anxiety behavior has been shown with transgenic mice overexpressing BDNF study

(Govindarajan et al., 2006). Therefore, TSA-induced increase in amygdalar BDNF levels may mediate increase in anxiety behavior in control handled LRs while simultaneously increase in hippocampal BDNF may induce antidepressant-like effects in the same phenotype. It is plausible to expect that TSA administration may also induce epigenetic regulation of genes different than BDNF in other brain regions that may affect behavioral parameters in terms of depressive- and social anxiety-like behaviors in LRHR rats. Moreover, infusion of TSA in hippocampus may induce avoidance of unwanted anxiogenic effects of drug, all of which need testing in future studies.

In conclusion, CVP-induced depressive behavior and accompanying decrease in BDNF gene expression in LRs are reversed by TSA administration. TSA exerts its effects by inducing increased levels of acH3 and acH4 at the hippocampal BDNF promoters in a degree similar to levels observed in stress-exposed HRs thereby resulting in phenotypic switch in the LR rats. Although TSA induces anxiety behavior, these findings provide evidence that HDAC inhibitor treatment is promising for reversing stress-induced depressive behavior in juvenile LR rats.

GENERAL DISCUSSION

A. Behavioral effects of chronic variable stress during adolescence in the LRHR phenotype.

In this dissertation, LRHR rat model was utilized to study behavioral responses to chronic variable stress applied during the peripubertal-juvenile period as an attempt to model neurobehavioral differences in stress responsiveness among humans. Although as with any other animal model, I do not propose that the LRHR model to match perfectly the human condition, there are sufficient overlaps between the novelty-seeking phenotype and human emotional reactivity to make this model useful in understanding molecular, structural and behavioral differences in stress responsiveness. Results obtained from Chapters 1 and 3 showed striking individual differences in stress-induced depressive-like behavior measured as immobility on the forced swim test in LRHR rats. In agreement with a vast body of literature showing enhanced depressive-like behavior following chronic stress in adulthood (reviewed in General Introduction), LR rats exposed to CVP during peripubertal-juvenile period showed increased depressive-like behavior (increased immobility on the forced swim test). However in HR rats, the same stress paradigm resulted in antidepressive-like behavior (decreased immobility on the forced swim test). Moreover, data from Chapter 3 showed that TSA treatment reversed the stress-induced depressive-like behavior in LR rats, inducing a phenotypic switch in this phenotype towards HR-like behavioral response. In HR rats, TSA treatment mimicked chronic

variable stress-induced antidepressive-like behavior. These results demonstrate that CVP exposure during adolescence induces opposite effects on depressive-like behavior in HR and LR rats, and that TSA is an effective antidepressant treatment to reverse stress-induced depressive-like behavior in LR rats.

B. Role of epigenetic regulation of hippocampal BDNF gene in actions of chronic variable stress and TSA treatment in LRHR phenotype.

In parallel with depressive-like behavior, decreased levels of acetylated H4 at the hippocampal BDNF P4 promoter was observed in CVP-exposed LR rats (Chapters 1 and 3). This decreased epigenetic induction of BDNF gene was reversed by TSA treatment and associated with antidepressive behavior (Chapter 3). Current findings are in agreement with published studies showing antidepressant-like effects of HDAC inhibitors upon systemic administration in animal models of stress-induced depression (Tsankova et al., 2006; Schroeder et al., 2007) specifically by reversing decreased BDNF gene expression in the hippocampus (Tsankova et al., 2006). These studies thus suggest that HDAC inhibitors are potential agents to reverse depressive-like behavior in animal models of depression. These effects are consistent with the neurotrophic hypothesis of depression (for review see Duman and Monteggia, 2006), which states that decreased expression of BDNF contributes to depression, and that upregulation of BNDF is associated with antidepressive behavior as shown by antidepressant treatment-induced increases in BDNF levels. Indeed, preclinical data has shown that BDNF expression in the hippocampus is decreased in response to chronic stress and this effect can be reversed with antidepressant treatments in adult animal models of depression (for review see

Duman and Monteggia, 2006). These effects in animal studies are in line with clinical studies showing that hippocampal BDNF expression is decreased in depressed suicide patients, and that antidepressant treatment at the time of death induced an increase in BDNF expression (Dwivedi et al 2003; Karege et al 2005). Thus, both clinical and preclinical studies suggest that BDNF regulation may be a key mechanism for behavioral effects of chronic stress and could be targeted for antidepressant action. Interestingly, chronic variable stress in HR rats did not induce depressive behavior and reduction in hippocampal BDNF levels (Chapter 1 and 3). However, the association between chronic variable stress-induced antidepressive behavior and increased hippocampal BDNF expression (measured as increased acetylation levels of H3 and H4 at the hippocampal BDNF P2 and P4 promoters, respectively and upregulated BDNF mRNA in the DG and CA3 fields of the hippocampus) in HR rats strengthen neurotrophic hypothesis of depression by mimicking previously reported effects of antidepressants on BDNF levels. Furthermore, the current data are in agreement with a recent report showing that while four weeks of unpredictable mild stress applied during adulthood induces anhedonia, a major symptom of depression, and decreases hippocampal BDNF expression, the same chronic stress applied at PD 30 results in resilience to depression and increased hippocampal BDNF expression (Toth et al., 2008). This suggests age-dependent effects of stress on both depressive symptoms and hippocampal BDNF. In addition, a recent study showed that classic environmental enrichment during adolescence can stimulate expression of hippocampal BDNF through chromatin-specific events (Kuzumaki et al., 2010), providing further support for the enriching quality of the stress paradigm in HR rats. Therefore, it is possible that increased hippocampal BDNF expression in adolescent

HR rats exposed to chronic variable stress is part of a mechanism that mediates the cellular effects of chronic stress on depressive-like behavior and enables establishment of resilience to depression during adolescence. Additionally, it should be noted that depression is not a homogeneous disorder in either adults or adolescent individuals, and other neurochemicals or neurotrophic factors, may also mediate the cellular effects of chronic stress in LRHR rats that need addressing in future studies. However, the current results showed that epigenetic regulation of BDNF gene expression was a target mechanism for actions of both chronic variable stress and TSA treatment on regulation of depressive-like behavior in HR and LR rats (Chapters 1 and 3). These findings thus assigned a functionally significant role for BDNF in the actions of stress and TSA treatment, specifically at the epigenetic level. Eventually, the opposite regulatory patterns in depressive-like behavior seen with chronic variable stress and accompanying epigenetic regulation of hippocampal BDNF gene in adolescent LRHR rats provide compelling evidence for individual differences in neurobiology of stress-induced depression.

The link between chronic stress exposure and BDNF regulation, both at the transcriptional and epigenetic levels, deserves discussion since the animal model used in this dissertation is a stress responsiveness model and downstream effects of chronic stress exposure converge on the hippocampal BDNF expression. Depression is associated with hyperactive HPA function (Nemeroff et al., 1992; Holsboer, 2000; Pariante, 2009). Namely, depression is accompanied by long term elevation of cortisol (Parker et al., 2003) and chronic CORT treatments in preclinical studies mimic hyperactive HPA physiology in the human condition by way of inducing depressive-like behavior (Murray

et al., 2008; David et al., 2009). Moreover, different types of chronic stress paradigms have been shown to induce elevated CORT levels in rodents (Mathews et al., 2008; Toth et al., 2008). Increased levels of CORT generally decrease BDNF expression in the hippocampus (Nibuya et al., 1995; Smith et al., 1995) and is associated with depressive-like behavior in adult animals (Toth et al., 2008). CORT has been shown to regulate hippocampal BDNF expression via the exon 4 promoter, in that administration of CORT significantly decreased BDNF exon 4 mRNA levels in the hippocampus (Hansson et al., 2006). It is noteworthy to mention that the putative glucocorticoid response element (GRE) is located in the rodent BDNF promoter region 4 (Benraiss et al., 2001) which could allow for the possibility of direct genomic effects of CORT on BDNF transcription through this specific BDNF promoter (Suri and Vaidya, in press). Therefore, decreased levels of acetylated H4 at the BDNF promoter P4 in LR rats exposed to CVP could be reflected by changes in exon 4 mRNA levels and mediated by actions of CORT on exon specific BDNF transcript variants (Chapters 1 and 3). Interestingly, levels of acetylated H4 at the BDNF promoter P4 were increased in HR rats exposed to both types of stressors and were associated with antidepressive behavior, suggesting resilience to deleterious effects of CORT (Chapters 1 and 3). These findings in HR rats are in agreement with a previously published study showing antidepressive behavior (measured with sucrose preference test) and increased hippocampal BDNF expression in chronically stressed adolescent rats despite increased CORT release (Toth et al., 2008). Moreover, it is important to note that CORT may also have some protective effects in the nervous system. Jeanneteau and colleagues (2008) showed that in vivo administration of synthetic CORT, dexamethasone (Dex), increased hippocampal TrkB (BDNF receptor)

phosphorylation independent of BDNF release in rats (Jeanneteau et al., 2008). Moreover, in vitro Dex treatment in cortical brain slices induced slow but long-lasting increase in TrkB, Akt, PLC, and Erk phosphorylation (Jeanneteau et al., 2008). These findings suggest that CORT selectively enhance phosphorylation of the TrkB receptor and down-stream signaling proteins independent of BDNF release. Therefore, it is plausible that chronic variable stress-induced CORT may activate the BDNF receptor, TrkB via non-ligand activated pathways, and its down-stream signaling cascades, rather than repressing BDNF expression in stress-exposed HR rats. These stress-induced cellular effects could be a part of mechanism that mediates antidepressive behavior in HR rats. These are valid hypotheses to be investigated in future studies.

Both HR and LR animals showed increased anxiety-like behavior following CVP, measured as reduced social contact in the social interaction test (Chapter 1 and 3). Emergence of social anxiety-like behavior in response to CVP was marked by upregulation of BDNF mRNA levels in the BLA of juvenile LRHR rats (Chapter 1). These findings are consistent with previous studies showing emergence of anxiety-like behavior following chronic stress, and an associated increase in spine density in the BLA (Vyas et al., 2006; Mitra et al., 2005), together with increased levels of amygdalar BDNF in rodents (Fanous et al., 2010). Anxiety disorders are highly comorbid with depression in human population (Hoffman et al., 2008). Similar to the clinical data, both depressive- and anxiety-like behaviors were evident in LR rats exposed to CVP (Chapter 1 and 3). However, depressive- and anxiety-like behaviors were oppositely regulated in HR rats following CVP exposure (Chapters 1 and 3). This interesting disassociation in the emergence of depressive- and anxiety-like behaviors, and associated increases in

hippocampal and amygdalar BDNF levels in CVP-exposed HR rats is corroborated, at least in part, by a previously published study. In this report, transgenic overexpression of forebrain BDNF has been shown to induce anxiety-like behavior concomitant with increased spinogenesis in the BLA and to cause antidepressant effects as shown by improved performance on the forced-swim test together with an absence of chronic stress-induced hippocampal atrophy in mice (Govindarajan et al., 2006). In conclusion, these authors hypothesized that increased BDNF in the amygdala may act to facilitate the development of anxiety symptoms, whereas increased BDNF in the hippocampus may paradoxically attenuate depressive symptoms (Govindarajan et al., 2006). Therefore, CVP-induced BDNF expression in both hippocampus and amygdala could lead to contrasting effects on depressive- and anxiety-like behaviors in HR rats whereas in LR rats, CVP-induced decrease in epigenetic induction of the hippocampal BDNF and increased BDNF mRNA in the BLA could lead to co-emergence of both depressive- and anxiety-like behaviors.

Systemic administration of TSA was successful to reverse depressive-like behavior in LR rats. However, TSA treatment also induced anxiogenic effect in the same phenotype (Chapter 3). Since effects of TSA were clearly not restricted to hippocampus, decreased HDAC activity in the BLA might also induce increased BDNF gene expression, and consequently result in anxiety-like behavior in LR rats. As noted, such correlation between increased BDNF levels in BLA and elevated anxiety behavior has been shown in transgenic mice overexpressing BDNF (Govindarajan et al., 2006). Therefore, direct TSA infusion into hippocampus may eliminate unwanted anxiogenic effects of the drug, which will need to be addressed in future studies. Taken together,

these results suggest that even though TSA treatment induces social anxiety-like behavior in LR rats, the observed molecular and behavioral results confirm that TSA is an effective antidepressant treatment to counteract stress-induced depressive behavior in vivo. Treatment and diagnosis of depression vary at the individual level and not all patients respond well to antidepressant treatments. In current study, investigating the individual differences in stress responsiveness and effects of TSA treatment in depressive-like behavior could shed light to future studies for development of effective antidepressants, which may overcome personality trait-induced resistance to some current treatments for depression.

C. Mossy fibre-CA3 plasticity in chronic variable stress-exposed LRHR rats.

Chronic variable stress-induced mossy fibre remodeling was closely correlated with histone modifications in some BDNF gene promoters in LRHR rats (Chapter 1). Indeed, increased MF terminal field size in HR rats following both CVP and CVS was associated with increased acetylation levels of H3 and H4 at the BDNF P2 and P4 promoters respectively. These findings accompanied antidepressant-like effects of stress in HR rats. However in LR rats with depressive-like behavior, decreased MF terminal field size following CVP was associated with decreased H4 acetylation at the BDNF P4 promoter, implicating a potential role for BDNF in the regulation of MF plasticity in response to chronic variable stress. Similar to stress-induced hippocampal morphological changes in LR rats reported here, chronic stress is shown to result in hippocampal atrophy, which is also associated with decreased BDNF expression (for review see Duman et al., 1999). These findings may provide an explanation for the observed hippocampal atrophy of depressed patients based on postmortem and structural imaging

studies (MacQueen et al, 2003; Campbell et al., 2004; Stockmeier et al, 2004). Although CVP-induced decrease in levels of H4 acetylation at the BDNF P4 promoter in LR rats was not reflected to total BDNF mRNA levels, it could be reflected as a decrease in BDNF exon 4 mRNA levels, which is associated with P4 promoter (Chapters 1 and 3). However, in situ hybridization specific for BDNF exon 4 was not analyzed in the current work to directly address this question.

Decreases in MF plasticity could also be regulated via CVP-induced precursor form of BDNF (proBDNF). CVP may increase proBDNF levels because our BDNF riboprobe detects all BDNF splice variants containing exon 5 as well as proBDNF. High levels of proBDNF exist in the postnatal CNS, suggesting that proBDNF actions may be most robust during postnatal development when axonal extension, dendritic spine pruning and synaptic maturation are prevalent, whereas proBDNF effects are more regionally restricted, but are maintained in adulthood (Yang et al., 2009). BDNF and proBDNF activate different receptors to regulate their neuronal actions. BDNF activates its high affinity receptor TrkB to promote cell survival and synaptic plasticity, whereas proBDNF induces neuronal apoptosis via activation of a p75 receptor (Lee et al., 2001; Huang and Reichardt, 2003). It has been shown that hippocampal proBDNF levels were increased following chronic stress and associated with altered hippocampal synaptic plasticity in rats (Yeh et al., 2012). Therefore, it is plausible that increased proBDNF expression following CVP in LR rats could mediate MF synaptic plasticity by way of inducing decrease in MF terminal field volume. In summary, specifics of how stress-induced BDNF effects could be related to structural changes in MF terminals, if at all in the LRHR rats, remains to be resolved in future work.

Mossy fibres, a traditionally-accepted excitatory projection at the second synapse of the trisynaptic hippocampal circuitry, consist of axons originating from the dentate granule neurons and synapsing onto the CA3 field. Despite the traditional role, it has been shown that inhibitory interneurons receive approximately 10 times more synapses from mossy fibres than do principal neurons (Acsady et al., 1998), generating a strong feedforward inhibition at physiological firing frequencies of dentate granule cells (Lawrence and McBain, 2003). The mossy fibre-CA3 network dynamics under pathophysiological circumstances such as chronic stress exposure may depend on the net innervation ratio between the excitatory primary neurons and inhibitory interneurons. Moreover, it has also been shown that mossy fibre synapses onto CA3 can switch from inhibition to excitation depending on the stimulation frequency of the dentate granule neurons (Mori et al., 2004), and can be selectively muted (Losonczy et al., 2003) providing a state-dependent switch in the network. Therefore, the newly-formed MF synapses in response to chronic variable stress in the HR rats are likely to have different physiological properties than those formed under basal, stress-free conditions and CVP-induced decrease in MF terminal field volume in LR rats may induce, at least in part, disruption in hippocampal circuit and enables estabilishment of vulnerability to depression. Critical properties of the MF-CA3 synapses in terms of their excitation/inhibition state and/or neurotransmitter content need addressing in future studies. Although the role of MF plasticity in depressive-like behavior is, for the most part, unexplored, generation of new dentate granule cells and output mossy fibres are implicated in pathophysiology of depression. Specifically, antidepressant treatments have been shown to increase adult granule cell neurogenesis in the DG (Malberg et al.,

2000) and similar to antidepressant treatments, repeated ECS induced MF sprouting in rodents (Vaidya et al., 1999), suggesting that granule neurons and their axonal projections onto CA3 may be critically involved in the pathogenesis of depression and in actions of antidepressants. Therefore, chronic variable stress-induced increase in MF terminal fields in HR rats showing antidepressive-like effects and decrease in LR rats with depressive-like effects may constitute a morphological adaptation for resilience or vulnerability to stress-induced depression respectively.

Since hippocampal BDNF is primarily stored in the axon terminals of mossy fibres (Dieni et al., 2012), alterations in hippocampal BDNF levels in response to chronic stress during peripubertal-juvenile period could, at least in part, mediate structural changes in the MF-CA3 synapse. In Chapter 2, lack of CVP-induced dendritic atrophy in CA3 neurons could be interpreted as resilience to the deleterious effects of CVP in HR rats. In the same phenotype, a partial protection from CVS-induced effects was also noted by a slight but significant decrease in dendritic length and complexity. These effects were coupled with increased hippocampal BDNF expression and MF terminal field size in HR rats as shown in data obtained from Chapter 1. In LR rats, significant dendritic retraction was evident in both apical and basal regions of CA3 neurons following both CVP and CVS in agreement with published studies (Vyas et al., 2002, Kleen et al., 2006; Pillai et al., 2012) and associated with CVP-induced decrease in hippocampal BDNF expression and MF terminal field size. CVP-induced increase in hippocampal BDNF and/or MF sprouting may therefore contribute to the protection of CA3 neurons from the detrimental effects of stress in HR rats, whereas LR rats show vulnerability to adverse effects of stress by way of similar cellular/molecular

mechanisms.

Moreover, CVP- and CVS-induced extensive decrease in dendritic length and complexity were accompanied by widespread decrease in mushroom spine number/density and overall spine number/density in both apical and basal trees in LR rats, implicating absence of a compensatory mechanism (i.e., spinogenesis) to rescue CA3 neurons from dendritic atrophy (Chapter 2). It has been shown that endogenous CORT has a role in stress-induced dendritic atrophy in the hippocampus (McEwen et al., 1995) by way of inducing glutamate release (Moghaddam et al., 1994). MF terminals in the stratum lucidum region of CA3 neuron contain presynaptic kainate receptors that postively regulate glutamate release (Chittajallu et al., 1996) under the control of CORT. Namely, density of these presynaptic kainate receptors is decreased by adrenalectomy and is restored to normal by CORT replacement (Watanabe et al., 1995). Therefore, increased glutamatergic stimulation from MFs as well as from other distal afferents (commissural/associational collaterals and perforant path projections) in response to chronic variable stress may induce increase in intracellular calcium levels, leading to dendritic cytoskeleton proteolysis (McEwen et al., 1995) and result in massive CA3 dendritic atrophy and consequently loss of these synaptic inputs in LR rats. In HR rats, even though small and CVS specific effects were observed in dendritic length and branch number, equally widespread effects in mushroom spine number/density were observed in response to both types of stress and in both apical and basal dendritic arbors as they were observed in LR rats. However, CVP- and CVS-induced decrease in mushroom spine number/density in HR rats was not accompanied by a corresponding decrease in total spine density. These findings suggest that loss of mushroom spines could be

compensated for by addition of more immature type spines in stress-exposed HR rats. Indeed, repeated restraint stress (6h/day for 3 weeks) has been shown to induce a decrease in large spines (e.g., mushroom spines) with a shift towards small spines (e.g., thin, stubby) in pyramidal neurons of medial prefrontal cortex (Radley et al., 2008). Moreover, immature type of spines are highly dynamic spines subject to structural flexibility, either enlarging and stabilizing (e.g., mushroom spines), or shrinking, depending on the level of synaptic activity (Bourne and Harris, 2007). Therefore, increased immature type of spines may differentiate into more stable mushroom spines if functional presynaptic elements can establish contacts in HR rats. These findings implicate compensatory mechanisms at work to protect CA3 neurons, at least in part, from deleterious effects of stress in adolescent HR rats unlike LR rats. Taken together, these findings underline individual differences in neuromorphological changes in response to stress which may play an important role for development of stress-induced mood disorders.

In conclusion, HR and LR rats displayed differential vulnerability to chronic variable stress induced depressive-like behavior but similar vulnerability to the emergence of anxiety-like behavior. Indeed, LR rats possess higher vulnerability to development of depression along with decreased epigenetic induction of the hippocampal BDNF gene, MF and CA3 atrophy whereas HR rats exhibit somewhat resilience to depressive-like behavior with increased BDNF expression (at both epigenetic and transcriptional levels), MF sprouting and protected (partially) CA3 neurons. However, both HR and LR rats exhibit high vulnerability to social anxiety-like behavior together with increased BDNF mRNA levels in the BLA. Moreover, whilst systemic HDAC

inhibitor, TSA, treatment induced anxiety-like behavior in LR rats, same treatment reversed stress-induced depressive-like behavior together with decreased acetylation levels of H4 at the BDNF P4 promoter in LR rats. In HRs, TSA treatment resulted in identical effects that were observed with chronic variable stress exposure on acetylation levels of histones at BDNF promoters and corresponding antidepressive behavior. The current work showed individual differences in stress responsiveness during adolescence at molecular, structural and behavioral parameters, and investigated a new possible treatment method. These results provide valuable evidence for development of new treatments for depression to overcome personality trait-induced resistances to current treatments. Moreover, major changes in neural, hormonal and behavioral systems during adolescence may render individuals either vulnerable (e.g., LR rats in current study) or resistant (e.g., HR rats in current study) to the adverse effects of stress during this critical developmental period. Current findings therefore provide further evidence for the importance of adolescent brain plasticity in stress responsiveness. Taken together, these findings show that LRHR animal model is a valid model to study individual differences in resilience and/or vulnerability to stress-induced mood disorders during adolescence.

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