CHRONIC VARIABLE STRESS AFFECTS HIPPOCAMPAL NEUROTROPHIC FACTOR GENE EXPRESSION IN THE NOVELTY-SEEKING PHENOTYPE: EPIGENETIC REGULATION

by

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ABSTRACT

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Experimentally naïve rats exhibit varying degrees of novelty exploration. Some rats display high rates of locomotor reactivity to novelty (high responders; HR), and others display low rates (low responders; LR). The novelty-seeking phenotype (LRHR) is introduced as a model of stress responsiveness. In this thesis I examined effects of chronic variable physical and social stress or control handling on the levels of various neurotrophins in the hippocampus, and changes in mossy fibre terminal fields in LRHR rats. A positive correlation is seen between histone deacetylase 2 and brain-derived neurotrophic factor (BDNF) levels both of which are oppositely regulated in LRHR CA3 fields in response to chronic social stress. Increase in BDNF levels in CA3 field accompanied increase in supra-pyramidal mossy fibre terminal field size (SP-MF) in HRs, and decrease in

BDNF levels accompanied decrease in SP-MF volume in LRs. Epigenetic regulation of neurotrophic support underlying these structural changes is discussed.

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INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

5HT – Serotonin

Bdnf – Brain derived neurotrophic factor

CAMK – Ca^{2+} / calmodulin kinase

DA – Dopamine

DG – Dentate gyrus

FGF2 – Fibroblast grouth factor 2

GABA – Gamma-aminobutyric acid

GR – Glucocorticoid R

GC – Glucocorticoid

HDA – Hypothalamic pituitary-adrend axis

HDAC – Histone deaceytylase

HR – High responder

IIP-MF – Intra-infra pyramidal

IR – Intermediate responders

LR – Low responder

LHPA – Limbic hypothalamic axis pituitary adrenal axis

LSD – Least significance difference

MF – Mossy fiber

mRNA – Messenger ribonucleic acid

NAC – Nucleus accumbens

NE – Norepinephrine

NGF – Nerve grouth factor

NT – Neurotrophin

NT-3 – Neurotrophin-3

PN – Postnatal day

SP-MF – Suprapyramidal mossy fiber

GENERAL INTRODUCTION

Neurobiological differences between individuals contribute an observable variance in human behavior and vulnerability to psychopathology (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 later in life (Zuckerman and Neeb, 1979). To study the neurobiological mechanisms underlying risk-taking behavior in humans, an outbred rat model of novelty-seeking is developed (Piazza *et al*., 1989). When experimentally naïve rats are exposed to mild stress of a novel environment, some rats display high rates of locomotor reactivity to novelty [high responders (HR)], whereas others display low rates of locomotor reactivity to novelty [low responders (LR)]. HR rats repeatedly choose novel environments over familiar ones (Piazza *et al*., 1996), appear less anxious in the light–dark box and the elevated plus maze (Akil *et al*., 2000), and demonstrate prolonged stress-induced secretion of corticosterone when compared to LR rats (Piazza *et al.,* 1996), suggesting a hyperactive hypothalamic pituitary adrenal axis (HPA) in the HR phenotype. Furthermore, HR

animals acquire self-administration of psychostimulants such as amphetamine and cocaine quicker and at lower drug doses when compared to LR rats (Piazza *et al.,* 1989; Hooks *et al.,* 1991; Piazza *et al.,* 2000). Moreover LRHR phenotype is reported useful for predicting propensity to self-administer nicotine (Suto *et al*., 2001). We have recently shown that HR animals are more vulnerable to behaviorally sensitizing effects of nicotine (Bhatti *et al*., 2007), all of which suggest that HR animals may be more vulnerable to psychostimulant-taking behavior. HR and LR rats also have distinct patterns of basal gene expression concerning stress-related molecules in regions of the brain critical for the control of stress responsiveness (Kabbaj *et al*., 2000). Specifically, a GR deficit is implicated in the hippocampus of HR rats compared to LRs. Moreover, microinjection of a GR antagonist into hippocampus of previously screened LR rats resulted in a phenotype switch, in that following GR antagonist treatment locomotor reactivity to novelty in LRs is increased in a dose-dependent manner, suggesting that deficit in GR activation may promote the novelty-seeking behavior (Kabbaj *et al*., 2000). HR rats also express lower levels of basal corticotrophin releasing hormone (CRH) mRNA in the central nucleus of the amygdala (CeA) compared to LR rats, and show higher levels of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) compared to LR counterparts (Kabbaj *et al*., 2000). These findings all together suggest that the LRHR rat model may be a model for individual differences in vulnerability to emotional reactivity in response to stress and psychostimulants.

Adolescence is a critical time period where brain continues to develop.

There appears to be both progressive (i.e., axonal growth, myelination) as well as regressive (i.e., axonal and synaptic pruning) mechanisms at work during adolescence, suggesting that this period marks the maturation of neuronal connectivity (Pffeferbaum *et al*., 1994; Caviness *et al*., 1996; Reiss *et al*., 1996). Especially brain areas involved in emotional behavior continue to grow across peripubertal-juvenile period into young adulthood (Bayer *et al.,* 1982; Sousa *et al.,* 1998; Isgor *et al.,* 2004a). Adolescence is also a period of human development where accumulation of stressful life events of varying magnitudes is shown to have predictive value for the onset and severity of drug dependence and psychopathologies later on (Hoffmann and Su, 1998; Cerbone and Larison, 2000; Hoffman *et al.,* 2000). For example, human clinical data suggest that adolescence is a particularly vulnerable period for nicotine dependence. Even though adolescents smoke fewer cigarettes than adults, they develop higher rates of dependence over shorter period of time (DiFranza *et al.,* 2000; Kandel and Chen, 2000). Moreover, adolescence is also the period when sensation-seeking/risk-taking trait first emerges (Zuckerman, 2004). Both heightened responsiveness to environmental perturbations and emerge of phenotypic disposition during adolescence are likely consequences of a phase of rapid neural growth. All of these suggest that adolescence is a critical developmental period where brain is responsive to emotionally salient stimuli. In this thesis, I will use the LRHR rat model to assess individual differences in the neurobiology of stress responsiveness during the peripubertal-juvenile period (defined as the period between weaning and postnatal day 60).

Chronic stress, a condition mediated by elevated concentrations of glucocorticoids (GC) over an extended period of time, has been shown to be unfavorable for neurons and to cause damage and neuronal loss in certain brain areas such as the hippocampus (Sapolsky *et al*., 1990). Due to high levels of both type I and II glucocorticoid receptors, hippocampus is one of the primary targets of stress and adrenal steroids in the brain. Pyramidal cells and dentate gyrus are differentially affected by stress and adrenal-glucocorticoid treatments. Severe chronic stress and prolonged exposure to GCs have been shown to cause cell loss and atrophy of the pyramidal neurons in the CA fields, prominently in the CA3 region, whereas granule cells of dentate gyrus are relatively resistance to damage by these treatments (Uno *et al*., 1989; Sapolsky *et al*., 1990). Ultrastructural plasticity has also been reported such as decrease in total length of apical dendrites of CA3 pyramidal neurons in addition to loss of CA3 neurons in response to repeated restraint stress or glucocorticoid treatment (Wooley *et al*., 1990; Watanabe *et al*., 1992). Prolonged exposure to stress or glucocorticoids could be sufficient to produce cell damage via glutamate excitoxicity and a reduction in metabolic capacity, the latter resulting from a reduction in glucose uptake (Sapolsky 1996; McEwen 1999). Another possibility is that hippocampal neurons may become more vulnerable to other insults, such as hypoxia–ischemia, hypoglycemia, or viral infection in response to stress and glucocorticoid treatments (McEwen *et al*., 1990; Sapolsky *et al*., 1992; Stein-Behrens *et al*., 1994). Although, stress and glucocorticiods lead to neuronal atrophy of CA3 neurons, glucocorticoids are necessary for survival and function

of other neurons in the hippocampus, such as dentate gyrus granular neurons. It has been shown that adrenalectomy leads to degeneration and death of granule cells (Sloviter *et al*., 1989; Gould *et al*., 1991a; Gould *et al*., 1991b; Sapolsky *et al*., 1991). These findings indicate that balance of glucocorticoid levels is important for cell survival and function, and chronic stress can induce morphological changes especially in the hippocampus. In this thesis, I will use chronic stress exposure during adolescence to ascertain individual differences in downstream morphological effects in the hippocampus, specifically concentrating on the axonal projections of the dentate granular neurons on the CA3 field using the LRHR phenotype.

In parallel with morphological effects, stress is also reported to decrease the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (Smith *et al*., 1995). BDNF is a major neurotrophic factor in the brain that is important for the growth and differentiation of new neurons and synapses during development, but is also required for the survival of existing neurons in the adult brain (McAllister *et al*., 1999; Thoenen, 1995). The potential role of neurotrophins in the actions of stress has been examined by acute or chronic stress paradigms. For example, it has been shown that chronic restraint stress (7d) decreases BDNF mRNA in the CA1 field of the hippocampus (Smith *et al*., 1995a; Smith *et al.,* 1995b). Acute restraint (2h) also causes a rapid decrease in BDNF mRNA most prominently in the CA3 and dentate gyrus (DG) of the hippocampus (Smith *et al*., 1995; Duman *et al*., 1995). Tsankova and colleagues (2006) have showed that chronic social defeat stress (daily exposure for 10 min over a total of 10

days) also downregulates the BDNF mRNA in the hippocampus of the mice. These animals also showed depressive-like behavior in the social interaction test coinciding with the downregulation of the BDNF mRNA in the hippocampus. Using these animal models, BDNF has been introduced as a critical neurotrophin for the molecular effects of chronic stress. Specifically, autocrine and target derived BDNF is shown to contribute to protection of CA3 neurons against chronic stress-induced atrophy (Duman *et al*., 1995). Using the findings from a set of chronic stress experiments, Duman and colleagues (2000) have proposed a model for neurotrophic factor-mitogen-activated protein (MAP) kinase cascade in the regulation of cell survival. The influence of neurotrophic factors, such as BDNF on cell survival is mediated by activation of the MAP kinase cascade. Activation of neurotrophic factor receptors also referred to as Trks, results in activation of the MAP kinase cascade via several intermediate steps. One target of the MAP kinase cascade is Rsk, which influences cell survival in at least two ways. Rsk phosphorylates and inactivates the pro-apoptotic factor BAD. Rsk also phosphorylates cyclic adenosine monophosphate response element-binding protein (CREB) and thereby increases the expression of the anti-apoptotic factor bcl-2 to promote cell survival. The identification of this pathway shed light to molecular mechanisms underlying chronic stress-induced morphological changes in the hippocampus as well as chronic stress-induced changes in depressive-like behavior in animal models, with common denominator being changes in the BDNF gene expression.

Mechanisms underlying decrease in BDNF levels in the hippocampus

following acute or chronic stress have been investigated. Although, Duman and colleagues (1995) have proposed several mechanisms, such as elevated glucocorticoids causing a loss of Ca^{2+} homeostasis, inhibition of glucose uptake and generation of free radicals, none of them could fully explain the decrease in BDNF levels following chronic stress. Stress may activate inhibitory neurotransmitter, such as GABA. Depolarization and increased intracellular Ca^{2+} mediates the induction of BDNF, probably via the activation of Ca^{2+}/calmodulin kinase (CAMK) and phosphorylation of CREB. Activation of $GABA_A$ receptors could lead to decreased levels of intracellular Ca^{2+} and inhibition of BDNF gene expression. Another possibility is that stress can also activate some unknown factor(s) that decreases the expression of BDNF via the Ca^{2+} pathway or via another intracellular pathway and transcription factor (Table 1).

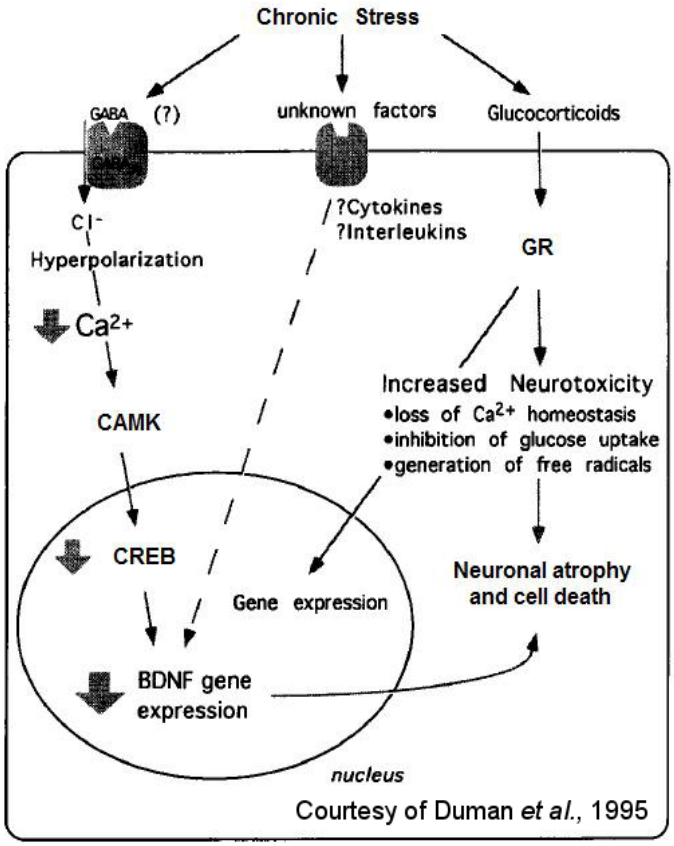

Table 1: Schematic drawing showing molecular pathways activated via chronic stress in the regulation of BDNF.

Same team of researchers also showed that inhibition of BDNF gene expression in response to chronic stress can be reversed by antidepressant treatments (Duman *et al.*, 2000; Table 2). Antidepressant treatment increases synaptic levels of norepinephrine (NE) and serotonin (5-HT) via blocking the reuptake or breakdown of these monoamines. This results in activation of intracellular signal transduction cascades, one of which is the cAMP-CREB cascade. Chronic antidepressant treatment increases Gs coupling to adenylyl cyclase (AC), levels of cAMP-dependent protein kinase (PKA) and CREB. CREB

can also be phosphorylated by Ca^{2+}-dependent protein kinases, which can be activated by the phosphatidylinositol pathway (not shown) or by glutamate ionotropic receptors (e.g., *N*-methyl-D-aspartate [NMDA]). Glutamate receptors and Ca^{2+}-dependent protein kinases are also involved in neural plasticity. All of these studies led to identification of a critical gene target for antidepressant treatment via the cAMP-CREB cascade, namely BDNF that is simultaneously shown to contribute to the cellular processes underlying neuronal plasticity and cell survival (Duman *et al*., 2000). This is also proposed as the neurotrophic hypothesis of depression. In this thesis, I will focus on neurotrophic factors, particularly BDNF expression in the hippocampus following chronic stress exposure in adolescence in the LRHR rats.

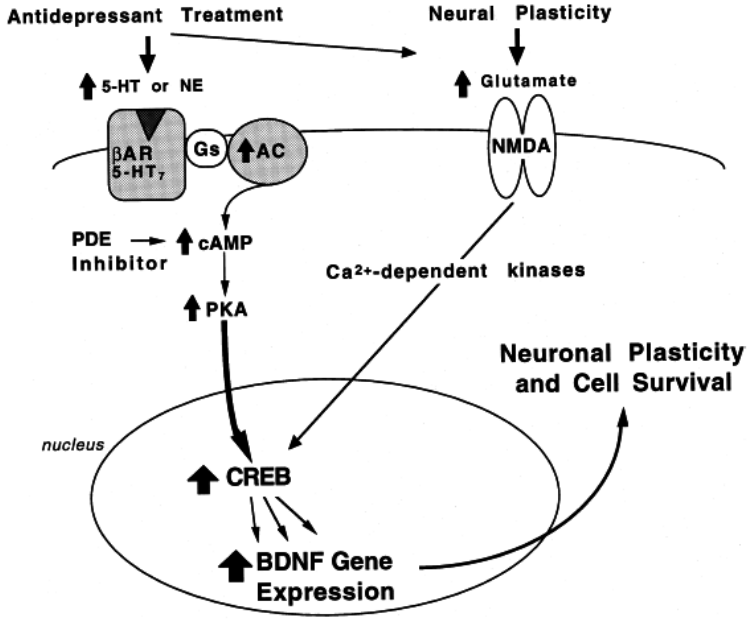

Table 2: Schematic drawing showing molecular substrates of antidepressant action in the regulation of BDNF.

Smith and colleagues (1995) have shown that chronic restraint stress causes a rapid decrease in BDNF levels in the CA1, CA3 and DG of the hippocampus. But there are other neurotrophins that are also implicated in effects of chronic stress. In contrast to downregulation of BDNF, chronic restraint stress increases neurotrophin 3 (NT3) mRNA most prominently in the DG. Other reports have shown that cold stress can decrease the number of NGF binding sites in the hippocampus possibly as a result of increased NGF release (Taglialatela *et al*., 1990). And indeed, 1 h of cold stress increases the NGF mRNA in the hippocampus as measured by Northern blotting (Foreman *et al*., 1993). Stress-induced changes in hippocampal NGF and NT-3 are proposed to be compensatory mechanisms working to protect cells from adverse effects of stress (Smith, 1996). Smith proposed that enhanced expression of NT3 could replace the loss of BDNF in response to chronic stress and could thereby act in an autocrine fashion to help protect those neurons expressing NT3. Another group of researchers most specifically studied chronic stress exposure during the early developmental period and showed that neonatal isolation attenuates the induction of NGF mRNA in the CA3 and DG following immobilization stress (Kawano *et al*., 2008). Opposite effects in NT3 levels have also been reported following chronic stress. For example, chronic intermittent stress exposure during the peripubertal-juvenile period causes a decrease in NT3 and BDNF in the dorsal and ventral hippocampus of the rats as well as resulting in cell loss in the same regions (Uys *et al*., 2006). In the following experiments I will employ two kinds of chronic variable stress paradigms during adolescence using the

LRHR rat model (see Experiment 1, Introduction for detailed description of the stress paradigm) and subsequently investigate changes in the neurotrophin levels (i.e., BDNF, NT3, NGF) to see if chronic stress induces differential regulation of neurotrophic factors in the hippocampus of the LRHR phenotype.

In addition to direct regulation of the BDNF gene by transcriptional factors such as CREB that I have outlined above in the neurotrophic hypothesis of depression, 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 genes that can be expressed in a cell and is originally proposed as a key process in brain development (Jaenisch and Bird, 2003; Margueron *et al*., 2005). This posttranslational modification of the N-terminal domains of core histone proteins, including methylation and acetylation, regulate chromatin structure and gene expression (Strahl and Allis, 2000). Histone deacetylation is catalyzed by a family of histone deacetylase (HDAC) proteins which remove acetyl groups from N-terminal domains of histones and resulting in increased chromatin compaction and diminished accessibility to transcription factors (Hong *et al*., 1993; Strahl and Allis, 2000). 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). Histone acetylation is catalyzed by histone acetyltransferases (HATs). Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. This makes RNA polymerase and transcription factors easier

to access the promoter region (Sterner and Berger, 2000). DNA methylation is the addition of a methyl group (CH_3) to the DNA's cytosine base. It may affect gene transcription through several different mechanisms. The methylation pattern is heritable after cell division. Therefore, DNA methylation plays an important role in cell differentiation during development (Singal and Ginder, 1999). Specifically BNDF gene regulation has been linked to HDAC activity in psychostimulant addiction paradigms in the nucleus accumbens and striatum (Kumar *et al.,* 2005; Renthal *et al.,* 2007). H3 is hyperacetylated at the BDNF promoter leading to increased BDNF gene transcription following chronic cocaine in the striatum, and HDAC4 is implicated in this regulation (Kumar *et al.,* 2005). Apart from psychostimulants, alcohol withdrawal is also linked to epigenetic regulation of gene activity. It has been shown that acute alcohol causes a decrease in histone deacetylase (HDAC) activity and increases in acetylation of histones (H3 and H4), levels of CREB in the amygdala, however, HDAC activity is increased in the amygdala of alcohol-fed animals during withdrawal (Pandey *et al.,* 2008). By blocking HDAC activity with an HDAC inhibitor known as trichostatin A, they prevented the development of alcohol withdrawal-related anxiety, and restored levels of histone acetylation and neuropeptide Y (Pandey *et al*., 2008). In addition to the drug addiction literature, epigenetic regulation of gene transcription is also reported following chronic stress. Tsankova and colleagues (2006) have shown that social defeat stress causes decrease in BDNF levels in the hippocampus of mice. Prolonged exposure to an aggressor induces lasting changes in mouse behaviour such as social avoidance, which are reversed by imipramine treatment

which downregulates HDAC5 and subsequently increases acetylation of H4 in the hippocampus. In this thesis I will assess regulation of two class I (i.e., HDAC2, 3) and one class 2 (i.e, HDAC5) histone deacetylases in the hippocampus following chronic stress exposure in the LRHR rats to study individual differences in chromatin plasticity.

The hippocampus is a late developing brain region which has been implicated in the novelty-seeking phenotype. The hippocampal mossy fibres comprise the axons of the dentate gyrus granule neurons that innervate the CA3 field (Gaarskjaer, 1986). The hippocampus, specifically mossy fibre system, has been critically involved in detection of novelty (Vinogradova, 2001). The mossy fibre projection relays multimodel sensory information from the entorhinal cortex to the hippocampus proper, which upon receipt is compared with previously stored information (Amaral and Witter, 1989; Witter *et al*., 1989; Vinogradova, 2001). Studies using Timm's method of silver sulfide staining for visualization of mossy fiber pathway showed that the intra- and infra-pyramidal mossy fibre (IIP-MF) and supra-pyramidal mossy fibre (SP-MF) may regulate different behavioral functions (Crusio *et al*., 1987; 1989; Prior *et al*., 1997). It is previously shown that in drug naïve, basal conditions, the SP-MF content shows phenotypic differences in the LRHR animals (Isgor *et al*., 2004b). In this research plan we propose to study hippocampal mossy fibre neuroplasticity in the novelty-seeking phenotype in response chronic variable stress exposure during adolescence when this fibre pathway normally goes under massive remodeling. The amount of mossy fibre content is highly dependent on experiential factors which can induce sprouting

and/or pruning (Gomez-Di Cesare *et al*., 1997). In the face of the high level of late development plasticity in the mossy fibre system, maintaining normal hippocampal function would require correct mossy fibre path finding and precise synaptic connections (Parent *et al*., 1997), all of which may be affected by chronic variable stress exposure.

In summary, the LRHR model is a model of emotional reactivity and especially adolescence is a critical time period for emotional development. We will use this model in **Experiment 1** to test how chronic variable stressors, especially chronic variable social stress, affect neurotrophic factor mRNA expression in the hippocampus. Since epigenetic regulators are recently shown to be very important mechanism to regulate neurotrophic gene transcription, specifically BDNF gene, we will study transcript levels of representatives of class I and II HDACs in the hippocampus following chronic variable stress exposures in **Experiment 2** and will link them to BDNF gene transcription. Since chronic stress has been shown to change hippocampal morphology, specifically in the CA3 field of the hippocampus, and since neurotrophic factors are also involved in this neuroplasticity, we will further investigate the effect of chronic variable stress on mossy fibre terminal field size in LRHR animals in **Experiment 3**.

EXPERIMENT 1: Chronic variable stress and neurotrophic factor messenger RNA regulation in the LRHR hippocampus

INTRODUCTION

In this experiment, I will use the novelty-seeking phenotype in the rat as a model of individual differences in vulnerability to chronic stress. Briefly, outbred rats display variance in their exploration of novel environments. In our laboratory, the upper 1/3 rd of the population of rats with highest locomotor reactivity scores to novelty is identified as high responders, HR; lower 1/3 rd of the population of rats with lowest locomotor reactivity to novelty is identified as low responders, LR. The LRHR phenotype has a predictive value in identifying vulnerability to both psychostimulant addiction (Suto *et al*., 2001; Bhatti *et al*., 2008) and to chronic stress (Kabbaj *et al*., 2000). Hence, a simple behavioral screening procedure (i.e, LRHR locomotor activity screening) provides a powerful tool to study neurobiological differences underlying vulnerability to emotional stimuli.

Isgor and colleagues (2004a) have previously described two chronic variable stress regimens (chronic, variable physical stress, CVP; chronic, variable social stress, CVS), although they were equivalent based on their acute activation of the physiological response to stress (i.e, plasma corticosterone, CORT), when applied chronically during the peripubertal-juvenile period, they led

to differential alterations in stress related gene expression and hippocampal morphology (Isgor *et al.,* 2004a). Even though the two stress regimens showed immediate deficits in the central and peripheral indices of the LHPA axis function, remarkably they differ in sustainability of deficits lasting into recovery from stress. The CVP regimen was capable of inducing delayed neuromorphological alterations in the hippocampus that led to emergence of delayed cognitive deficits. The CVS regimen during the peripubertal-juvenile period on the other hand resulted in inhibition of the expression of behavioral sensitization to amphetamine, whereas the CVP exposure caused a robust sensitization to amphetamine (Kabbaj *et al*., 2002), suggesting that these two stress paradigms induce different neuroplastic changes in the brain reward/emotional circuitry. These findings suggest that the physical and social stress exposures during the adolescence differentially modulate the HPA axis function, hippocampal morphology as well as brain reward circuitry. I will use these two chronic stress paradigms to investigate hippocampal gene expression particularly pertaining to neurotrophic factor regulation in the LRHR animals.

The hippocampus is one of several limbic brain structures implicated in the pathophysiology and treatment of mood disorders. Chronic stress is widely used as a model for mood disorders in experimental animals (Fuchs *et al*., 2004), and results in stress-induced morphological changes (decreased neurogenesis and neuronal atrophy) in the hippocampus, which are reversed by antidepressant treatments (Sapolsky *et al*., 2000; Duman *et al*., 2004). Several neurotrophic factors are regulated with acute and chronic stress and are associated with

depression. BDNF may have an important role in chronic stress-induced adaptations in the hippocampus and how antidepressants may reverse them (Nestler *et al*., 2002; Duman *et al*., 2004). When mice is previously subjected to chronic social defeat stress, they spend less time in the interaction zone with an aggressor in a social interaction test, suggesting that chronic social defeat stress leads to increased anxiety-like behavior. Moreover, these mice showed downregulated BDNF mRNA levels in the hippocampus and this effect of stress is reversed by a chronic tricyclic antidepressant treatment (Tsankova *et al*., 2006). These findings suggest that BDNF regulation may be a key mechanism for behavioral effects of chronic stress and could be targeted for antidepressant action. Furthermore, NGF is known to be involved in the regulation of the survival and differentiation of developing sympathetic and sensory neurons, and stress can cause alterations on function of NGF. For example, it has been shown that single neonatal isolation on PN 9 or PN 16 causes increase in NGF mRNA levels in the DG of rats, whereas, chronic neonatal isolation decreases this early time induction of NGF mRNA in the CA3 and DG of juvenile and adult rodents in response to immobilization stress (Kawano *et al*., 2008). These results collectively implicate neurotrophic factor gene regulation, specifically neurotrophins, in chronic stress-induced molecular and structural plasticity in the hippocampus. Uys and colleagues described a developmental trauma stress regimen using three stressors (i.e., restraint stress, swim stress, and ether inhalation) applied intermittently between PN 28 and PN 60. At the completion of the stress application, they showed a significant increase in acute restraint

stress-induced CORT levels and a corresponding decrease in NT3 in the dorsal and ventral hippocampus in adulthood (Uys *et al*., 2006). Since NT3 is a key player in neuronal differentiation, synaptic plasticity and neuronal survival, these findings suggest that chronic stress during a time corresponding to the peripubertal-juvenile period (PN 28-60) could lead to structural compromise in the hippocampus along with hyperactivity in the physiological response to stress.

Central purpose of this experiment is to test if there are robust individual differences in neurotrophic factor mRNA expression levels between LR and HR hippocampi following chronic variable physical and social stress exposures in the peripubertal-juvenile period (defined as period between postnatal days 28 and 60). To test this hypothesis, animals will be subjected to two weeks of chronic variable stress or control handled. After the last stress exposure, animals will be sacrificed via rapid decapitation and *in situ* hybridization histochemistry will be conducted to assess BDNF, NGF, NT3, fibroblast growth factor 2 (FGF2) and HDAC2 mRNA expression levels. FGF2 is assessed in the hippocampus following chronic stress as a neurotrophic factor outside of the neurotrophin family which is also implicated in human depression (Perez *et al.,* 2009). We will expect the vulnerable HR phenotype to show widespread changes in neurotrophic mRNA expression levels in the hippocampus along with changes in HDAC2 mRNA. Changes in histone acetylation via modulation of histone deacetylase activity are implicated in neurotrophic gene expression in paradigms of chronic stress before (Feng *et al*., 2007). In addition to profiling mRNA expression of neurotrophins (e.g., NGF, BDNF, NT3) and FGF2 we will also

assess HDAC2 regulation following the two chronic stress regimens during adolescence in the LRHR rats.

METHODS

LRHR phenotype screening: Animals were treated in accordance with the National Institute of Health guidelines on laboratory animal use and care. A total of 54 of male Sprague-Dawley rats (Charles River, Wilmington, MA) arrived at weaning (postnatal day, PN 22) and were housed three per cage in 43 X 21.5 X 25 cm Plexiglas (high) clear acrylic cages and allowed ad libitum access to food and water. Animals were kept on a 12-h 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 (high) 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 located 2.5 cm above the grid floor. Each frame was located 14.3 cm from the end of the cage. Horizontal locomotion was monitored by the lower bank of photocells. Each time a locomotor response was recorded on one of these lower photocells, that photocell was inactive until a response was recorded on the other lower photocell. Thus, each locomotor count recorded a minimum 14.3-cm traversing of the cage. Additional upper frame of photocells were located 11.5 cm above the grid floor. Rearing was monitored by this 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, *n*=18) or LRs (i.e., rats that exhibited locomotor scores in the lowest third of the sample, *n*=18). Intermediary responders (IRs) were eliminated from the the experiment.

Chronic variable stress exposure: Per each chronic stress regimen, we used three stressors selected from the literature, and utilized in the previously published work (Isgor *et al.,* 2004a). Stressors were applied in a systematic random order and at varying times of the day (one or two stressors per day) for a total of 14 stress exposures to avoid habituation.

Physical stressors: 1) Forced swim: Animals were placed in a water tank, 70 X 50 X 40 cm, filled with water at room temperature, and forced to swim for 15 min. 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 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: Animals (n = 16) were placed in one cage similar to the home cage for 2-hr in the home colony. As animals matured, the total number placed in a cage during crowding session was decreased to n = 8.

After the last stress exposure animals were killed by rapid decapitation. One half of the dorsal hippocampi were snap frozen for *in situ* hybridization histochemistry procedure and Timm's method for silver sulfide staining (see Experiment 3) and one half of the dorsal hippocampi were used for RNA extraction (see Experiment 2).

***In situ* hybridization histochemistry:** Coronal brain sections were collected at 16 mm thickness throughout the dorsal hippocampus. On the day of hybridization, 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 air dried. Antisense ^{35}S-labeled cRNA probes for rat BDNF, NGF, NT3, FGF2 and HDAC2 were separately 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 polymerase. BDNF and FGF2 riboprobes (kindly provided by Dr. Stanley Watson, University of Michigan) were produced using T3 RNA polymerase as the transcription enzyme after the plasmids were antisense linearized with XhoI and HindIII respectively. BDNF cRNA probe consisted of 385 bases complementary to the mature rat BDNF mRNA (Gall *et al*., 1991). FGF2 cRNA probe consisted of 280 bases complementary to the mature rat FGF2 mRNA (Turner *et al*., 2008). Beta-NGF riboprobe (kindly provided by Dr.

Kathleen Guthrie, Florida Atlantic University) was produced using T3 RNA polymerase after antisense linearization with EcoR1. The resulting riboprobe is complementary to the full rat preproNGF mRNA sequence (Whittemore *et al*., 1988). NT3 riboprobe (commercially available from Invitrogen, CA) was generated using T7 RNA polymerase following antisense linearization with XhoI. This riboprobe consisted of 1439 bases complementary to the mature rat NT3 mRNA (accession #: BC070504, NCBI). HDAC2 riboprobe (cloned in our laboratory in pBluescript II SK (+), accession #: XM-001061582, NCBI) was produced with T3 RNA polymerase following antisense linearization with KpnI. This cRNA consisted of 867 bases complementary to mature rat HDAC2 mRNA. Reactions were incubated for 90-min at 37^0C, and separated from unincorporated nucleotides over Biorad columns (Biorad laboratories, CA). Probes were 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 ml. Sections were hybridized with probe mixture inside a humidified box over night at 55°C. 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 a Kodak XAR film (Eastman Kodak, NY). Section images were captured digitally from x-ray films with a CCD camera, and relative optical densities were determined. Only pixels with gray values exceeding 3.5X above

background were included in the analyses.

Statistical analysis: Two-way ANOVAs (Phenotype X Stress) were conducted for levels of mRNA expression for all riboprobes investigated. Furthermore, significant interactions and main effects of ANOVAs were followed by post-hoc comparisons using Fisher's Least Significant Difference (LSD) test. Simple regression analyses were conducted between HDAC2 and BDNF mRNA signal and integrated density in the CA3 region of the hippocampus. All significance levels were set at $p = 0.05$.

RESULTS

Figure 1 depicts expression of the BDNF mRNA in the dorsal hippocampus in the LRHR rats. Autoradiography film-exposed images of riboprobe-hybridized representative sections for LR control (Figure 1A), LR social stress (Figure 1B), HR control (Figure 1C) and HR social stress (Figure 1D) are displayed. Two-way ANOVA results showed significant interactions between Phenotype (LR, HR) and Stress (PHY, SOC, CONT) in BDNF mRNA in the CA1 signal (Figure 1E) and integrated density [Figure 1F; $Fs \geq 3.632$, $ps \leq 0.0419$], and in the CA3 signal (Figure 1G) and integrated density [Figure 1H; $Fs \geq 5.227$, $ps \leq 0.0127$] between groups. Specific post-hoc comparisons showed that BDNF mRNA expressions in hippocampal CA1 and CA3 were significantly decreased in LR rats exposed to both physical and social stress compared to control groups [Figure 1E, F; CA1 signal and integrated density respectively; $ps \leq 0.0407$]. Whereas, BDNF mRNA levels in the CA3 were increased in HR rats exposed to social stress compared to those observed in control HRs [Figure 1G, H; CA3

signal and integrated density respectively, *ps* ≤ 0.0412]. No significant effects were observed in BDNF mRNA levels in the DG between experimental groups. (Figure 1I, J).

FIGURE 1.

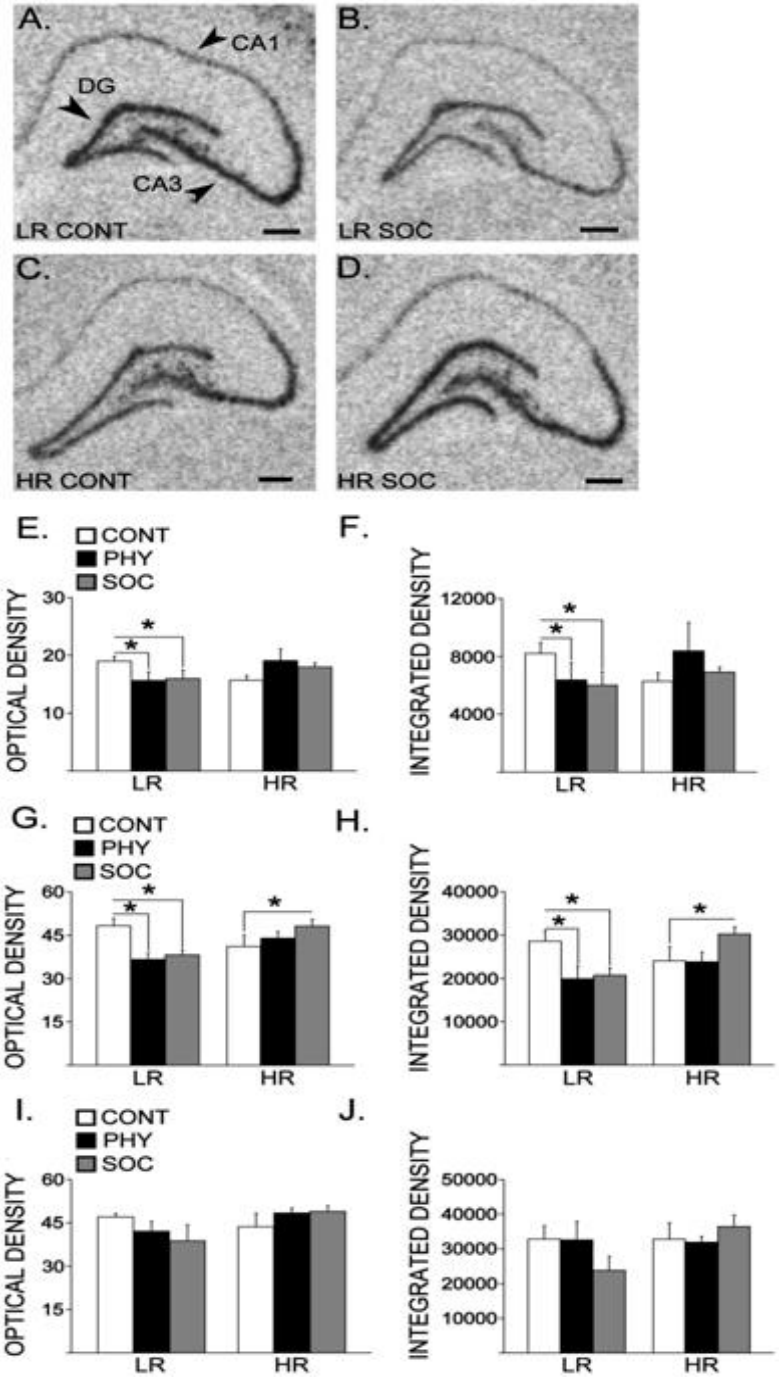

Figure 1: X-ray film images of representative coronal hemisections of the dorsal hippocampus that were radioactively labeled with an antisense cRNA probe against the BDNF mRNA in LR and HR animals following control handling (A, C respectively), chronic variable physical stress (not shown) or chronic variable social stress (B, D respectively) are shown. Alterations in the BDNF mRNA expression in the CA1 signal (E) and integrated density (F), CA3 signal (G) and integrated density (H), DG signal (I) and integrated density (J) are depicted in bar graphs following chronic variable stress between experimental groups. Means for optical and integrated densities ± SEMs are plotted; (*: $p \leq 0.05$). Scale bar = 250 μm.

Figure 2 shows expression of the NGF mRNA in the dorsal hippocampus of the LRHR rats. Autoradiography film-exposed images of riboprobe-hybridized representative sections for LR control (Figure 2A), LR social stress (Figure 2B), HR control (Figure 2C) and HR social stress (Figure 2D) are displayed. Two-way ANOVA revealed a significant main effect of stress in the CA1 signal and integrated density, CA3 signal and integrated density, and the DG integrated density between groups [$Fs \geq 4.746$, ps ≤ 0.0175]. Subsequent post-hoc comparisons showed that social stress exposure led to downregulation of the NGF mRNA expression in the CA1 in HRs compared to control levels [Figure 2E, F; CA1 signal and integrated density respectively, $ps \leq 0.0140$]; while both stress regimens led to downregulation of the NGF mRNA expression in LRs compared to control levels in the CA1 field of the hippocampus [$ps \leq 0.0119$]. Both physical and social stress exposure caused downregulation of the NGF mRNA expression

in the CA3 in LR rats compared to control levels [Figure 2G, H; CA3 signal and integrated density respectively, *ps* ≤ 0.0222]; whereas, only social stress regimen led to downregulation of the NGF mRNA expression in both CA3 and DG in HR rats [Figure 2H, J; CA3 and DG integrated density respectively, *ps* ≤ 0.0294].

FIGURE 2.

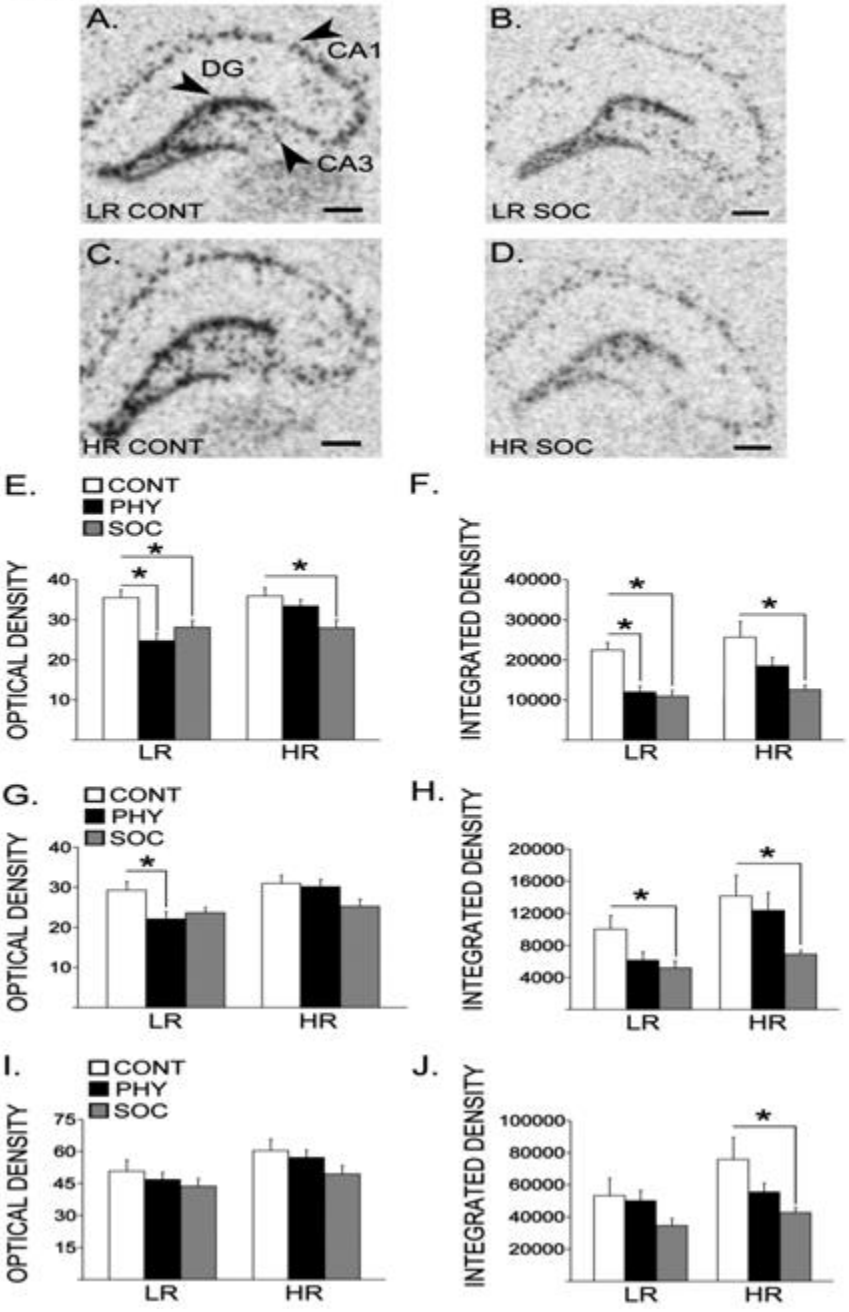

Figure 2: Panel A, B, C and D show x-ray film-exposed images of representative coronal hemisections of the dorsal hippocampus that were radioactively labeled with an antisense cRNA probe against the NGF mRNA in the LR and HR animals following control handling (A, C respectively), chronic variable physical stress (not shown) or chronic variable social stress (B, D respectively). Means for optical and integrated densities ± SEMs are plotted by bar graphs. (E), (G) and (I); CA1, CA3 and DG signals respectively*;* (F), (H) and (J); CA1, CA3 and DG integrated densities respectively; (*: $p \leq 0.05$). Scale bar = 250 μm.

Figure 3 shows the expression of the NT3 mRNA in the dorsal hippocampus of the LRHR rats. Autoradiography film exposed-images of riboprobe-hybridized representative sections for LR control (Figure 3A), LR physical stress (Figure 3B), LR social stress (Figure 3C), HR control (Figure 3D), HR physical stress (Figure 3E) and HR social stress (Figure 3F) are displayed. Two-way ANOVA revealed a significant main effect of stress in the DG signal [$F = 7.443$, $p = 0.0115$]. Specific post-hoc comparisons revealed that chronic physical stress exposure resulted in a downregulation of the NT3 mRNA expression in the DG of HRs compared to control levels [Figure 3G, H; DG signal and integrated density respectively, $ps \leq 0.0352$]; whereas in LRs, chronic social stress exposure resulted in a downregulation of the NT3 integrated density in the same region compared to control levels [$p = 0.202$].

FIGURE 3.

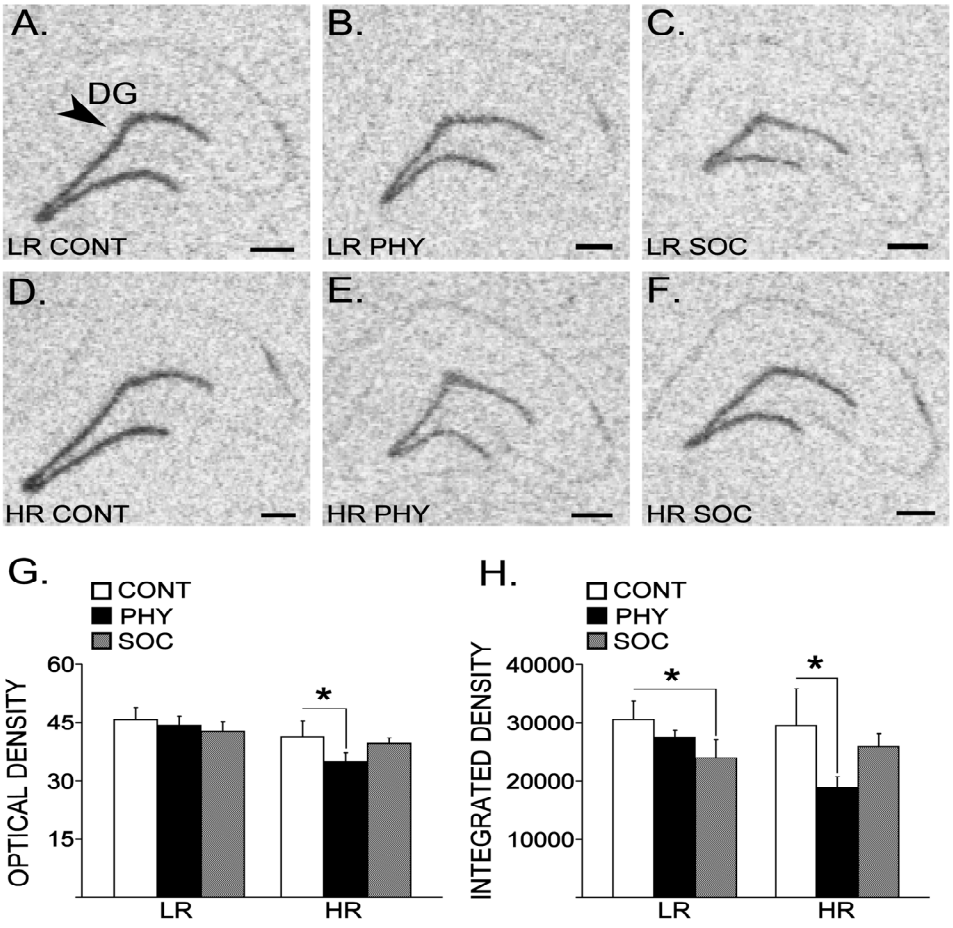

Figure 3: Panel A, B, C, D, E and F show x-ray film-exposed images of representative coronal hemisections of the hippocampus that was radioactively labeled with an antisense cRNA probe against the NT3 mRNA in the LR and HR animals following control handling (A, D respectively), chronic variable physical stress (B, E respectively) or chronic variable social stress (C, F respectively). Alterations in the NT3 mRNA expression in the DG signal (G) and integrated density (H) are depicted in bar graphs following chronic variable stress between experimental groups. Means for optical and integrated densities ± SEMs are plotted; (*: $p \leq 0.05$). Scale bar = 250 μm.

Figure 4 depicts expression of HDAC2 mRNA in the dorsal hippocampus of LRHR rats following chronic variable stress exposures. X-ray film images of representative hippocampal sections that are hybridized with radioactively labeled HDAC2 riboprobe for LR control (Figure 4A), LR social stress (Figure 4B), HR control (Figure 4C) and HR social stress (Figure 4D) are shown. Two-way ANOVAs were conducted for Phenotype (LR, HR) across Stress (PHY, SOC, CONT), and showed significant interactions in the HDAC2 signal and integrated density in the CA3 and DG [$Fs \geq 3.413$; $ps \leq 0.0483$]. Specific post-hoc comparisons showed that chronic variable social stress exposure led to significant downregulation of HDAC2 mRNA expression in the CA3 and DG in LRs compared to control levels [Figure 4G, H; CA3 signal and integrated density respectively; Figure 4I, J; DG signal and integrated density respectively, $ps \leq 0.0402$]. A strong trend toward an increase in HDAC2 mRNA expression was observed in the CA3 region of the hippocampus in HR rats exposed to social stress compared to control levels [$p = 0.1003$]. Interestingly, significant positive correlations were detected between HDAC2 and BDNF mRNAs in both measures of optical and integrated densities in the CA3 region of the hippocampus [Figure 4K, L; CA3 signal and integrated density respectively, $ps \leq 0.0493$]. No significant results were observed in the FGF2 mRNA levels in any subregions of the hippocampus between experimental groups (data not shown).

FIGURE 4.

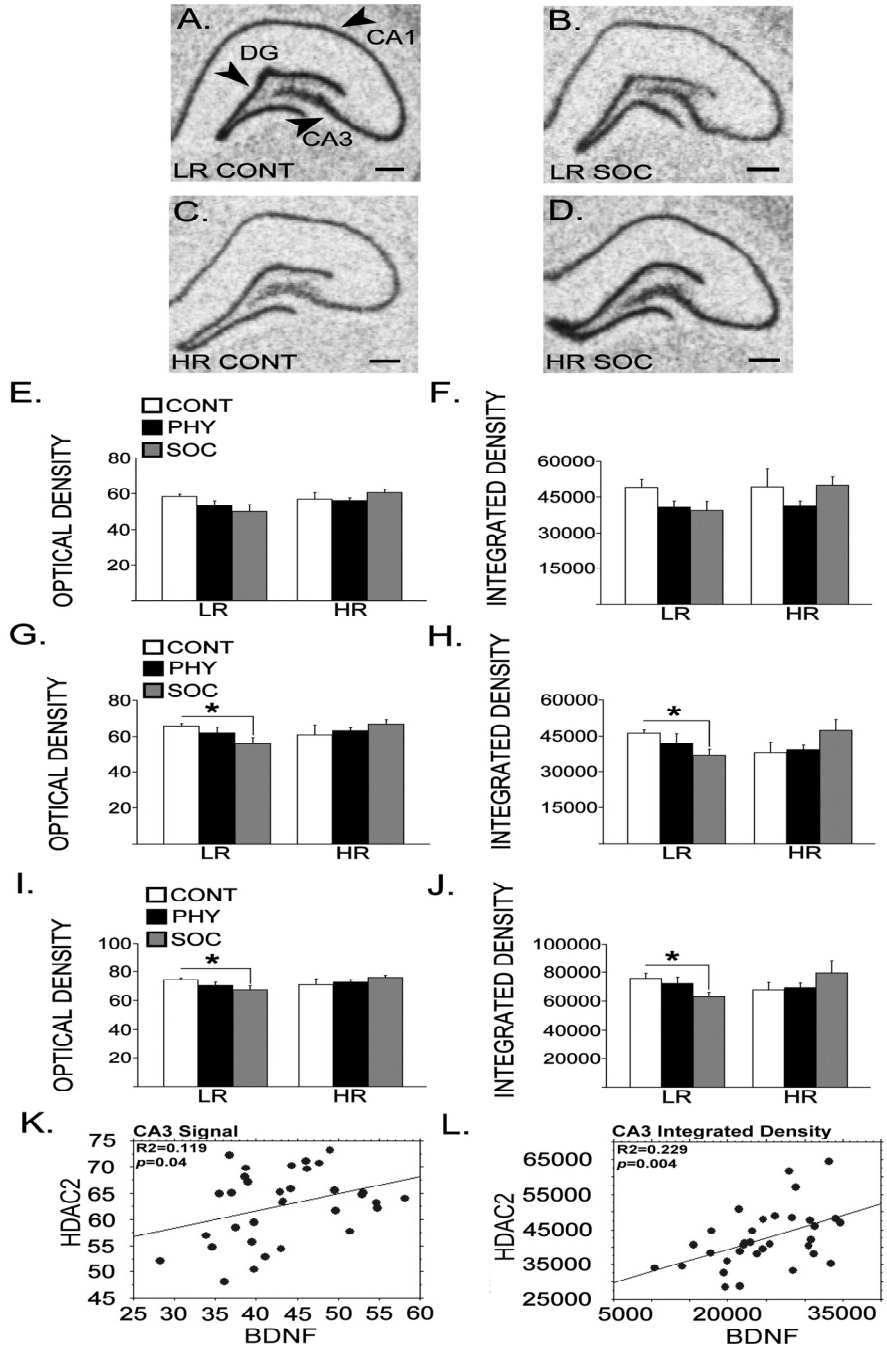

Figure 4: Panel A, B, C and D show x-ray film-exposed images of representative coronal hemisections of the dorsal hippocampus that were radioactively labeled with an antisense cRNA probe against HDAC2 mRNA in LR and HR animals

following control handling (A, C respectively), chronic variable physical stress (not shown) or chronic variable social stress (B, D respectively). Means for optical and integrated densities ± SEMs are plotted by bar graphs. (E), (G) and (I); CA1, CA3 and DG signals respectively*;* (F), (H) and (J); CA1, CA3 and DG integrated densities respectively. Regression plots between HDAC2 and BDNF mRNA signal (K) and integrated density (L) in the CA3 field of the hippocampus were depicted (*: $p \leq 0.05$). Scale bar = 250 μm.

DISCUSSION

Our results show that mRNA levels for neurotrophin genes, BDNF, NGF, and NT3 are differentially regulated in the hippocampus across chronic variable stress regimens and LRHR rats. Phenotype-independent downregulation is observed in NGF mRNA in the CA1 and CA3 pyramidal cell layers. Namely, both chronic stress regimens downregulate NGF mRNA in the CA3 in LRs compared to control levels (Figure 2, i.e., optical density effect with physical stress, and integrated density effect with social stress), and chronic variable social stress downregulates NGF mRNA in the CA3 in HRs compared to control levels. In both LRs and HRs chronic stress downregulates NGF mRNA in the CA1 field. Both types of stressors are implicated in this effect in LRs, whereas only social stress leads to downregulation of the NGF mRNA in HRs. Furthermore, phenotype-independent downregulations are also observed in NT3 mRNA in LRHR rats, in that NT3 integrated density is decreased following chronic variable social stress in the DG in LRs compared to control levels, and in the same region both signal and integrated density are decreased following chronic physical stress in HRs

compared to control levels (Figure 3). Conversely, phenotype-dependent effects are observed in the CA3 pyramidal cell layer for BDNF mRNA expression. Both chronic stress regimens downregulate BDNF mRNA in the CA3 in LRs compared to control levels; conversely, chronic variable social stress exposure causes upregulation in BDNF mRNA expression in HRs compared to control levels (Figure 1). In addition to phenotype-dependent regulation of BDNF in the CA3 field, both chronic social and physical stress downregulate BDNF mRNA in the CA1 in LR rats compared to control levels, whereas no significant changes are observed in the CA1 in HR rats. Interestingly, chronic variable social stress results in opposite regulation of HDAC2 mRNA expression in LRHR rats in the CA3 field of the hippocampus as also observed with BDNF mRNA results (Figure 4). Specifically, HDAC2 mRNA is dowregulated in LRs in the CA3 field of the hippocampus with chronic variable social stress compared to controls; whereas in HRs, HDAC2 mRNA shows a strong trend toward upregulation in the same region compared to controls. Furthermore, a significant positive correlation is found between HDAC2 and BDNF mRNA expressions in the CA3 region of the hippocampus when animals are pooled for a single regression analysis. This parallel regulation between HDAC2 and BDNF mRNAs in the CA3 field of the hippocampus suggests that these two mechanisms that are regulated by chronic stress may be causally related, although it is beyond the scope of this experiment to assess a direct link between HDAC2 and BDNF mRNA regulation. Epigenetic regulation of the BDNF gene is certainly a plausible hypothesis to be tested in future experiments. Nonetheless, these results implicate opposite regulation of

BDNF mRNA in the hippocampus in response to chronic variable social stress in the LRHR rats with possible implications for stress-induced neuronal plasticity in the novelty-seeking phenotype.

Many studies have demonstrated that chronic stress can result in atrophy and death of CA3 pyramidal neurons in the hippocampus (McEwen 1999; Sapolsky 1996). In addition, stress decreases the rate of neurogenesis of dentate gyrus granule neurons in the hippocampus of adult animals (Gould *et al* 1997, 1998). Decreased levels of BDNF in response to stress in the LRs could lead to a loss of cell survival, neurogenesis, neuronal differentiation and eventually damage and loss of neurons. Neurotrophic factors are critical for cell survival during the development, and in absence of neurotrophic support the neurons undergo a process of programmed cell death or apoptosis.

NGF mRNA levels are dowregulated in the CA3 in HRs following chronic variable stress (Figure 2), whereas BDNF mRNA levels are upregulated in the same region in the HR animals (Figure 1). It is plausible that upregulation of BDNF mRNA in the CA3 field in HRs may be a compensatory mechanism to downregulation of the NGF mRNA in the same region following chronic social stress exposure. Other molecular mechanisms may also be involved in the regulation of the NGF mRNA, such as apoptosis. ΔNp75 is N-terminally truncated p73 which is a member of the p53 family that is shown to recruit HDAC1 and HDAC2 to the TrkA promoter, thereby negatively regulating the NGF-mediated neuronal differentiation via transrepressing TrkA (Zhang *et al*., 2007). Upregulation of HDAC2 mRNA in HR CA3 field that I showed (Figure 4) may be

critical for effective transrepression of TrkA, and a subsequent inhibition of NGF signaling. This may have morphological implication on the CA3 neuron, specifically potential for atrophy and apoptotic cell death in the HR animals, all of which need to be investigated in future studies. Conversely, chronic social stress may lead to activation of other epigenetic mechanisms that may directly regulate NGF mRNA expression, such as HDAC1 in the LR CA3 field. Further studies examining the effect of chronic social stress on HDAC2 and HDAC1 function in the promoter of TrkA gene are needed in the LRHR animals to elucidate this mechanism of the NGF regulation. As mentioned in the general introduction, chronic stress decreases NT-3 in dorsal and ventral hippocampus in the adult rat (Uys *et al*., 2006). Since NT-3 is involved in neuronal differentiation, synaptic plasticity, and neuronal survival (Francis *et al*., 1999), downregulation of NT3 mRNA expressions in the hippocampi of LR and HR rats following chronic stress exposure could lead to granular neuron atrophy or even death. These data suggest that chronic stress may make cells in the DG vulnerable to structural damage in both phenotypes. In summary, results of this experiment show widespread downregulation of NGF and NT3 in the hippocampi of LRHR rats, coupled with an opposite regulation of BDNF and HDAC2 between LR and HR rats following chronic variable stress. Structural consequences of common neurotrophin downregulation, namely NGF and NT3, in LRHR rats will need to be investigated in future studies. However morphological implications of differential regulation of the BDNF in the CA3 field of the LRHR animals are examined in Experiment 3.

EXPERIMENT 2: Chronic variable stress and chromatin plasticity: a possible mechanism for BDNF gene transcription in the dorsal hippocampus

INTRODUCTION

Chromatin is the complex of DNA, histones and nonhistone proteins in the cell nucleus. Remodeling of chromatin is a dynamic process that modulates gene expression. The fundamental unit of chromatin is the nucleosome, which consists of ~147 base pairs of DNA wrapped around a core histone octamer (~1.65 turns). Each octamer contains two copies each of the histones H2A, H2B, H3 and H4. The nucleosomal structure of chromatin allows DNA to be tightly packaged into the nucleus by organized folding. Chromatin remodeling mechanisms ensure that DNA remains accessible to the transcriptional machinery. These epigenetic mechanisms alter gene activity by modulating DNA-protein interactions without changing the genetic code (Tsankova *et al*., 2007). The interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs) is critical to the dynamics of chromatin structure and function, thus, regulating gene expression in all eukaryotes (Peterson, 2002).

Recently HDAC activity is linked to gene transcription underlying drug taking behavior. Cocaine induces transient increases in histone acetylation at many immediate-early genes. Nestler and colleagues (2005) showed that transcription and promoter acetylation of BDNF and Cdk5 are specifically increased by chronic cocaine exposure. Furthermore, administration of HDAC inhibitors; trichostatin A (TSA) and sodium butyrate alter locomotor and rewarding responses to cocaine. Thus, they showed that chromatin remodeling is an important regulatory mechanism underlying cocaine-induced neural and behavioral plasticity. HDAC activity is also linked to gene transcription underlying chronic stress. Tsankova and colleagues (2006) have shown that social defeat stress causes decrease in BDNF gene transcription in the hippocampus of mice. Prolonged exposure to an aggressor induces lasting changes in mouse behaviour such as social avoidance, which are reversed by imipramine treatment which downregulates HDAC5 in the hippocampus. Specifically in the hippocampus, chronic social defeat stress results in downregulation of BDNF transcripts III and IV due to increased repressive histone methylation at the promoter regions, and antidepressant treatment reverses downregulation of these transcripts by increasing H3 but not H4 acetylation at the same promoters (Tsankova *et al.,* 2006). The effect of imipramine in this study is also attenuated by viral transduction of HDAC5 into the hippocampus. Thus, they have proposed that BDNF is important player for regulation of behavioral adaptation to stress.

The purpose of this experiment of is to assess if there are strong associations between BDNF and class I (HDAC2 and HDAC3) and a class 2

(HDAC5) HDACs following chronic variable stress exposures. To determine whether there is a strong association between epigenetic regulation and neurotrophic factor gene expression, specifically BDNF, two weeks after the chronic variable stress exposures, animals will be sacrificed via rapid decapitation and dorsal hippocampi will be collected to conduct real time-PCR. BDNF, HDAC2, HDAC3 and HDAC5 gene transcription levels will be assessed. We expect the vulnerable HR phenotype to show pronounced changes in chromatin plasticity following chronic variable stress exposure during adolescence.

METHODS

RNA extraction and clean up: Total RNA was extracted from the dorsal hippocampus by the Trizol method with an RNA extraction kit. Briefly, brain tissue samples (50mg) homogenized in 1 ml of Trizol with a Power Gen 125 tissue homogenizer (Fisher) for 30 sec at high speed. The homogenized samples were then incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After 50µl of bromoanisol was added to the tubes, the tubes were shaken vigorously by hand for 15 s and then incubated at room temperature for 10 min. The samples were centrifuged at 12,000 *g* for 15 min at 4°C. A 0.25 ml portion of the aqueous phase (RNA remainder) was collected and transferred into a fresh tube. Isopropanol (0.5 ml) was then added to the tube, followed by thorough mixing at room temperature for 10min and centrifugation at 12,000 *g* for 15 min at 4°C to precipitate the RNA. The supernatant was removed, and the RNA pellet was washed with 1 ml of 75%

ethanol. After the RNA pellet was air dried for 5 min, 100 µl of RNase-free water was added and the RNA pellet was dissolved completely and cleaned up with RNeasy MinElute Clean up Kit (Qiagen) according to manufacturer's instructions. RNA quantity was determined by NanoVue spectrophotometer (GE Healthcare) and the extracted RNA had a 280/260 OD ratio between 1.8 and 2.0. Following RNA extraction and quantification, RNA was pooled across n = 6 animals per experimental group.

cDNA synthesis and clean up: First-strand cDNA synthesis was carried out using the Gene Amp RNA PCR Core kit (Applied Biosystems.). Total RNA (1µg) was reverse transcribed using Oligo d(T) as primers and MuLV reverse transcriptase in a total volume of 8 µl was incubated for 10 min at room temperature for primer extension, 15 min at 42^0C for reverse transcription and 5 min at 99^0C for reverse transcriptase denaturation. The cDNA was cleaned up with QIAquick PCR Purification kit (Qiagen) according to manufacturer's instructions.

Quantitative real time-PCR (qPCR): Real-time PCR was performed on MxPro-Mx3005P QPCR System (Agilent Technologies). cDNA obtained from IRs was used as calibrator in all runs. According to the primer validation results two types of qPCR were conducted:

Singleplex real time-PCR: The singleplex real time-PCR reaction consisted of 12.5 µl of 2X Brilliant II qPCR Master Mix (Agilent Technologies), 1.2 µl of Taqman Gene Expression Assay (Hprt, control assay and BDNF, HDAC3

and HDAC5 experimental assays), 30nM reference dye (Agilent Technologies) and 250 ng template cDNA in a total volume of 25 µl.

Multiplex real time-PCR: Amplification reactions were performed in a volume of 12.5 µL of 2X Brilliant II qPCR Master Mix, 1.2 µl of Taqman Gene Expression Assay (HDAC2), 1.2 µl of Taqman Endogenous Control (GAPDH), 30nM reference dye and 250 ng template cDNA in a total volume of 25 µl. For both type of real time-PCR, cycling conditions comprised 10 min polymerase activation at 95°C, followed by 40 cycles at 95°C for 30 sec and 62°C for 1 min. Each assay was run in duplicate.

Quantitative evaluation: As indicated before, RNA samples were pooled across animals belonging to each experimental condition (Phenotype X Stress; n = 6 per group). Percent changes from control levels were reported as descriptive statistics.

RESULTS

Figure 5 depicts gene transcription levels of the BDNF (A), HDAC2 (B), HDAC2 / GAPDH amplification plot (C), HDAC3 (D) and HDAC5 (E) in the dorsal hippocampi of chronic stress exposed LRs and HRs. BDNF transcript levels were decreased with social stress in LRs by 33.3%, whereas increased with physical stress in HRs by 33.3% compared to control levels (Figure 5A). Chronic social stress led to decrease in HDAC2 transcript levels in LRs by 29.1% compared to control levels, whereas HDAC2 transcript levels were increased in HRs by 22.7% following chronic social stress compared to control levels (Figure 5B). HDAC3 transcript levels in LR controls were basally higher than HR controls by 14.2%.

Chronic social stress exposure caused a decrease in HDAC3 transcript levels in LRs by 28.5% compared to control levels; conversely chronic physical stress led to an increase in HDAC3 transcript levels in HRs by 41.6% compared to control levels (Figure 5D). HDAC5 transcript levels in LR controls were basally higher than HR controls by 59%. Chronic stress did not lead to any change in HDAC5 transcript levels in the LR dorsal hippocampus, whereas HDAC5 transcript levels were increased by physical stress in HRs by 50% compared to control levels (Figure 5E).

FIGURE 5.

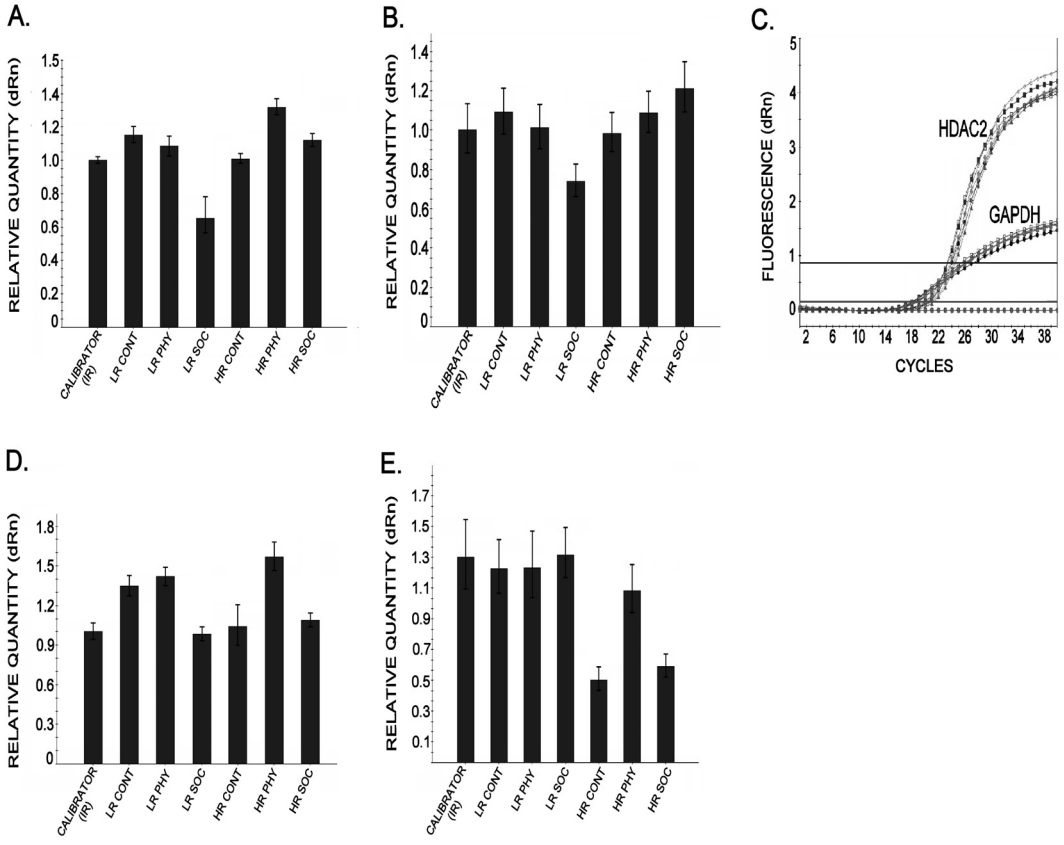

Figure 5: Relative gene transcription levels following quantitative real time-PCR. BDNF gene (A), HDAC2 gene (B), HDAC2/GAPDH sample amplification plot (multiplex, C), HDAC3 gene (D) and HDAC5 gene (E) are shown. Gene transcription levels were normalized with Taqman housekeeping (Hprt) gene expression assay with the exception of HDAC2. HDAC2 (A) gene transcription level was normalized with Taqman endogenous control (GAPDH) gene expression assay. RNA was pooled across n = 6 animals per experimental group. Gene transcription levels were reported as descriptive statistics in percent fluctuations from control levels.

DISCUSSION

These results show strong phenotype-dependent regulation of the BDNF and HDAC2 genes in the dorsal hippocampus following chronic variable stress. Namely, chronic variable stress exposures lead to a decrease in BDNF gene transcription in LRs compared to control levels, whereas chronic variable stress exposures lead to an increase in BDNF gene transcription in HRs compared to control levels. Interestingly, chronic variable social stress results in opposite regulation of HDAC2 gene transcription in LRHR rats. Specifically, HDAC2 gene transcription is decreased in LRs with chronic variable social stress compared to control levels, whereas in HRs, HDAC2 gene transcription is increased following chronic variable social stress compared to control levels. HDAC3 gene transcription along with HDAC2 seems to be going in the same direction with BDNF gene transcription in LRHR rats. Chronic variable stress exposure causes decrease in HDAC3 gene transcription in LRs compared to control levels,

whereas in HRs, HDAC3 gene transcription is increased with chronic variable stress compared to control levels. HDAC5 gene transcription is not altered with chronic variable stress in LRs compared to control levels, whereas in HRs, chronic variable physical stress causes an increase in HDAC5 gene transcription compared to control levels.

Class I HDACs that I tested, namely HDAC2 and HDAC3 appear to be regulated in similar pattern as the BDNF gene in the LRHR dorsal hippocampi following chronic stress (Figure 5). The RT-PCR data showed that, indeed different stressors are regulating HDACs in different directions in the LRHR animals and I found similar results in this experiment as in Experiment 1 showing opposite regulation of BDNF mRNA between LR and HR rats. As mentioned above, I observed a significant positive correlation between HDAC2 and BDNF mRNA expressions in the CA3 region of the hippocampus in Experiment 1, which suggests very strong association between these two gene transcripts. These effects are being regulated by chronic stress and may not necessarily suggest a direct link between HDAC2 modulation and BDNF gene transcription. In Experiment 2, I confirm this link and further show that HDAC3 is also regulated in the same direction as the HDAC2 and show correspondence to fluctuations in the transcript levels of the BDNF gene. Hence there appears to be a curious strong association between BDNF and class I HDACs that we tested, which I will further investigate in a causal study as a future direction.

Ajamian and colleagues (2004) have shown that inhibition of HDACs via histone deacetylase inhibitors; sodium butyrate and trichostatin A cause an

increase in class 1 and 2 HDACs. They reported auto-regulatory feedback loop to the expression levels of several HDACs. In our chronic stress paradigm we applied 14 stressors during the two weeks and it is plausible that early on in stress exposure HDAC activity is decreased, which can directly increase BDNF gene transcription by increasing acetylation of histones in the promoter region of the BDNF gene. However after repeated exposure to stress, HDAC inhibition could lead to increase in the transcription of the same HDAC genes, providing a robust autoregulatory mechanism. It is equally likely that increase in HDAC2 and 3 levels in the HR dorsal hippocampi following chronic stress may decrease transcription of genes that are inhibitory to BDNF activity; hence HDAC regulation can indirectly disinhibit BDNF gene transcription. Thirdly, it is also possible that epigenetic factors are not important for regulation of BDNF transcription; the observed increase in BDNF levels in the HR hippocampi could be a direct effect of traditional transcriptional regulators such as CREB. All of these are plausible hypotheses that are easily testable in future studies.

EXPERIMENT 3: Chronic variable stress and hippocampal mossy fibre morphology in LRHR rats

INTRODUCTION

This experiment will address neuromorphological adaptations chronic variable social stress may mediate in the hippocampus underlying the stress vulnerability phenotype (i.e., LRHR rats). In Experiment 1 an upregulation of the BDNF mRNA was observed in the CA3 field in HR animals exposed to chronic social stress compared to control levels and a downregulation of BDNF mRNA was observed in the same region in LR animals following same stress exposure. Since BDNF is predominantly stored in the axon terminals of the dentate granule neurons synapsing onto CA3 (i.e., mossy fibre terminal fields) it is interesting to see if there are any structural changes in the terminal fields of the mossy fibres following chronic variable social stress in the LRHR rats. Our lab has previously shown that a behaviorally-sensitizing regimen of nicotine induces a hypertrophy in the suprapyramidal mossy fibre (SP-MF) terminal fields in the HR but not LR rats (Bhatti *et al.,* 2007), and that there are phenotype differences in the terminal field size of the SP-MF in the adult LRHR rats (Isgor *et al.,* 2004b). The hippocampal mossy fibres comprise the axons of the dentate gyrus granule

neurons that innervate the CA3 field (for review see Gaarskjaer, 1986). Morpho-behavioral correlations in genetically-altered mice implicated hippocampal mossy fibres with exploratory behavior (Roullet and Lasalle, 1990; Ivanco and Greenough, 2002; Mineur *et al.,* 2002). The hippocampus, specifically the mossy fibre system, has been critically involved in detection of novelty (Vinogradova, 2001). The mossy fibre projection relays multimodal sensory information from the entorhinal cortex to the hippocampus proper, which upon receipt is compared with previously stored information (Amaral and Witter, 1989; Witter *et al.,* 1989; Vinogradova, 2001). Such a mechanism to detect novelty within the environment must be a necessary component of the novelty-seeking/risk-taking trait.

Central hypothesis of this experiment is that HR animals will show a hypertrophy in the terminal fields of the mossy fibres, particularly the SP-MF compartment following exposure to chronic variable social stress compared to control animals, where as LR animals will show an atrophy in the terminal fields of the mossy fibres, particularly the SP-MF compartment following the same stress exposure compared to their respective control group.

METHODS

Timm's method for silver sulfide staining: Same animals used for *in situ* hybridization study in Experiment 1 were used for mossy fibre analyses in this experiment (n=6 per experimental group). After the last chronic social stress exposure or control handling animals were sacrificed by rapid decapitation. Brains were harvested, frozen in isopentane (-30 to -40C), and stored at –80C. Frozen, 16 mm thick coronal sections were mounted on poly-L-lysine-subbed

slides. Sections were collected at 160-µm 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 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 stain and coverslipped. This protocol was successfully used on fresh frozen tissue before 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 mossy fiber system (shown in Figure 6; i.e., the intra- and infra-pyramidal mossy fibers, IIP-MF; and supra-pyramidal mossy fibers, SP-MF) 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 rostra-caudal extent of the hippocampus, yielding an average of 20 analyzed sections. The cross-sectional areas of the

IIP-MF and SP-MF 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 mossy fiber 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 total (SP + IIP) mossy fiber systems.

Statistical analysis: Two-way ANOVAs were used to analyze the SP-MF, IIP-MF, and total MF terminal field volumes between phenotypes (LR, HR) and stress condition (SOC, CONT). Significant main effects and interaction were followed by Fisher's Least Significant Difference tests, and significance was set at $p = .05$.

FIGURE 6.

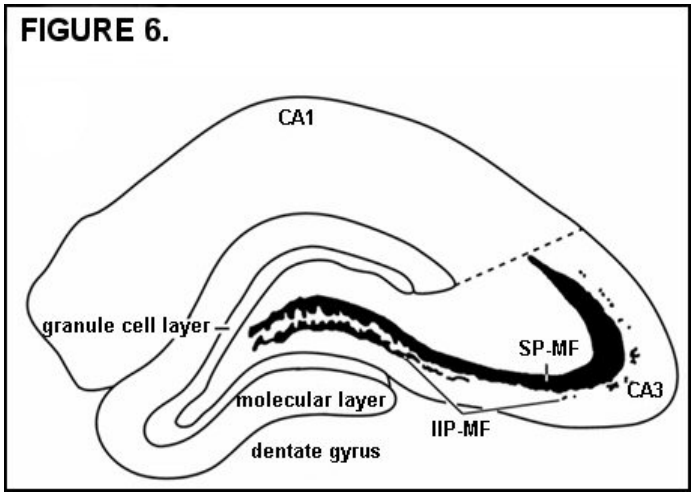

Figure 6: An illustration of a coronal hemisection of the dorsal hippocampus depicting the two compartments of the mossy fibre system (IIP-MF, intra- and

infra-pyramidal mossy fibres; SP-MF, supra-pyramidal mossy fibres) in relation to the major hippocampal subdivisions.

RESULTS

Figure 7 depicts dorsal hippocampal sections of Timm-stained hippocampal mossy fibres from a representative LR control (A), LR chronic variable social stress (B), HR control (C), HR chronic variable social stress (D) animals and estimated total mossy fibre terminal field volume (E) and estimated SP-MF volume (F). Two-way ANOVAs (Phenotype X Stress) showed a significant interaction and main effect of stress on total mossy fibre terminal field volume as well as SP-MF terminal field volumes [Fs $\geq$ 6.85, ps $\leq$ .021], whereas no significant effects were detected in IIP-MF terminal field volumes. Subsequent posthoc comparisons showed a significant increase in the total mossy fibre terminal field volume and SP-MF volume in HRs exposed to chronic variable social stress compared to control animals, and a significant decrease in the total mossy fibre terminal field volume and SP-MF volume in LRs exposed to chronic variable social stress compared to control animals [ps $\leq$ .014].

FIGURE 7.

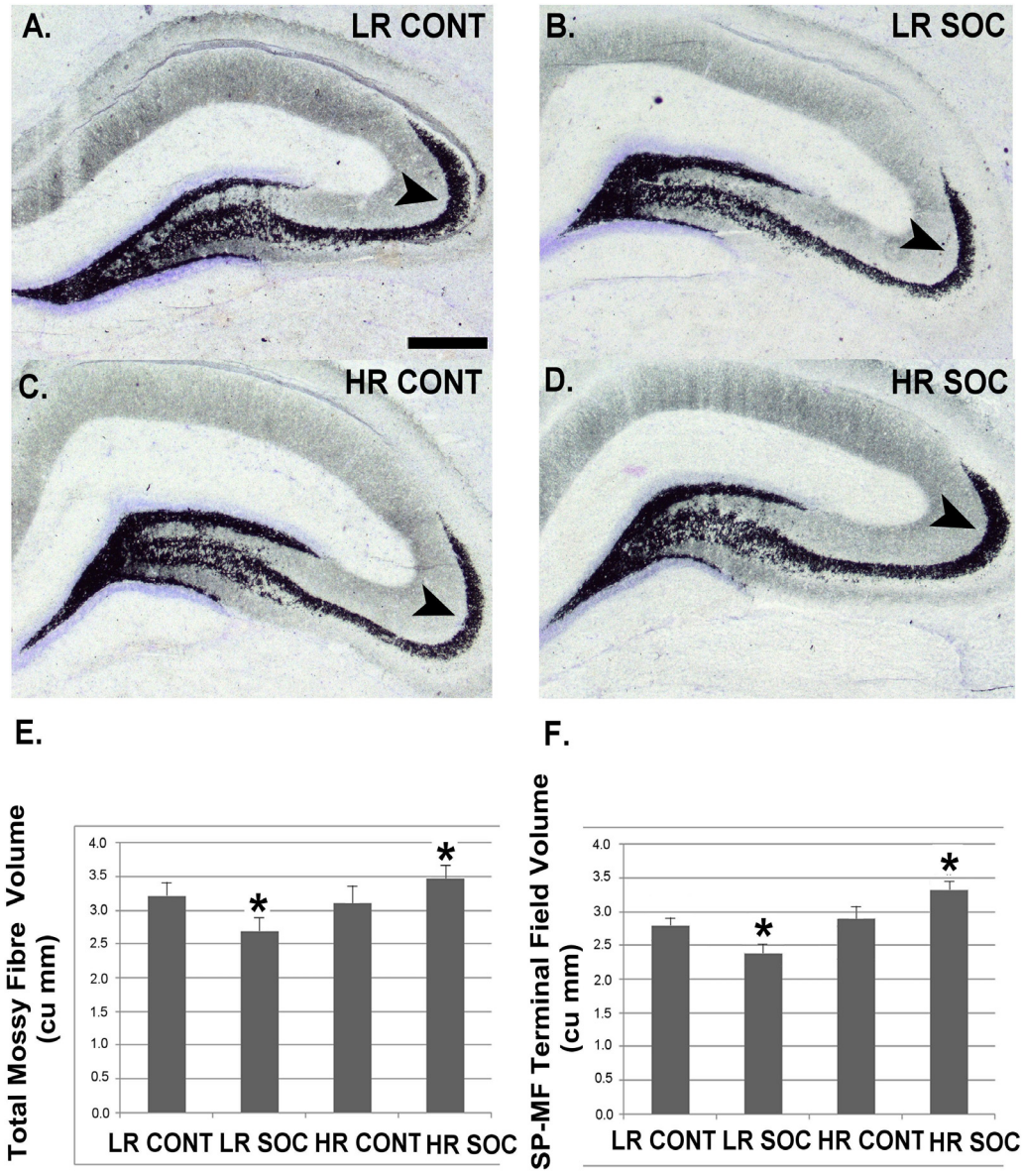

Figure 7: Coronal hemisections of Timm-stained mossy fibre pathway that is counterstained by cresyl violet representing LR control (A), LR chronic variable social stress (B), HR control (C), HR chronic variable social stress (D), estimated total mossy fibre terminal field volume (E), estimated SP-MF terminal field volume (F). Mean values for estimated volumes ± SEMs are plotted in bar

graphs. Black arrows are pointing at SP-MF compartment of the mossy fibre projections. $p \leq 0.05$; Scale bar = 250 μm

DISCUSSION

In line with the increase in BDNF mRNA reported in Experiment 1, these data show a hypertrophy in the SP-MF compartment of the mossy fibre terminal fields in the HR rats following chronic variable social stress compared to controls. Conversely, corresponding to the decrease in BDNF mRNA reported in Experiment 1, these data also show atrophy in the SP-MF terminal fields in the LR rats compared to controls. Hence chronic variable social stress induces morphological changes in the total mossy fibre terminal fields in the LRHR rats because of changes in the SP-MF compartment of this fibre pathway. These morphological changes coincide with the fluctuations in BDNF levels suggesting that neurotrophic support via BDNF may be important in the stress-induced axonal sprouting in the HR phenotype. These findings further support the notion that the hippocampus, specifically the mossy fibre system, in the HR animals is particularly vulnerable to the neuroadaptive alterations induced by chronic social stress exposure, which may be a substrate for the behavioral vulnerability observed in these animals.

It is important to understand how/if stress-induced hypertrophy of the mossy fibre terminal fields in the HR rats is reflected in the dendritic receptive fields of CA3 neurons, an aim that will be explored in future studies. It is also equally important to assess how long lasting these morphological changes are and if they reverse with recovery from stress. Mossy fibres have been

traditionally characterized as a prominent excitatory pathway in the adult organism. A decrease in mossy fibre synaptic facilitation is detected from 3 wks to 6 wks-old mice, which likely contributes to the stabilization of the late maturing hippocampal circuitry (Mori-Kawakami *et al.,* 2003). The amount of mossy fibre content is highly dependent on experiential factors which can induce sprouting and/or pruning (Gomez-Di Cesare *et al.,* 1997). In the face of the high level of late developmental neuroplasticity in the mossy fibre system, maintaining normal hippocampal function would require correct mossy fibre path finding and precise synaptic connections (Parent *et al.,* 1997), all of which may be affected by chronic stress exposure.

We can largely speculate on the functional significance of increased SP-MF innervation of the CA3 field in the HRs in response to chronic variable social stress. 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, more recent anatomical evidence shows 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 *et al.,* 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. To make things more complex, it is also 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. We speculate that the newly-formed SP-MF synapses in response to chronic stress in the HR animals are likely to have different physiological properties than those formed under basal, stress-free conditions, which remains to be elucidated.

GENERAL DISCUSSION

Our results show that chronic variable stress exposure differentially regulates BDNF gene expression in LRHR animals. In Experiment 1, *in situ* hybridization results showed that BDNF mRNA levels are upregulated in the HR CA3 field following chronic variable social stress, whereas the same stress regimen leads to a downregulation in BDNF mRNA levels in the LR CA1 and CA3 fields. This opposite regulation was also seen in Experiment 2 using RT-PCR method in that BDNF transcript levels are higher in the HR dorsal hippocampus compared to controls, whereas lower in the LR dorsal hippocampus compared to controls following chronic variable stress exposure. *In situ* hybridization histochemistry allows for assessment of mRNA levels with anatomical specificity as film images of section have higher resolution; however RT-PCR experiment is conducted on the dorsal hippocampus as a whole without anatomical specificity. Therefore our RT-PCR results may reflect diluted effects compared to *in situ* hybridization findings. Even though there are differences between these two assessment methods (i.e., *in situ* hybridization and RT-PCR) with regards to sensitivity, we found largely corresponding BDNF results where chronic variable stress, particularly social stress has a potential role for BDNF regulation in opposite direction in the LRHR rats using both methods. Interestingly, chronic variable stress resulted in opposite regulation of HDAC2

mRNA in LRHR rats as shown in Experiment 1. Namely, HDAC2 mRNA is downregulated in LRs with chronic variable social stress, whereas chronic variable social stress leads to upregulation in HDAC2 mRNA in HRs in the CA3 field of the hippocampus. HDAC2 findings are in perfect correlation with BDNF findings in Experiment 1, hinting at the potential for epigenetic regulation of the BDNF gene following chronic variable social stress in the LRHR rats. This possibility will be investigated in future studies.

We further investigated class 1 (HDAC2, 3) and class 2 (HDAC5) HDAC regulation following chronic stress exposure in Experiment 2. RT-PCR method was chosen to assess regulation of other HDACs because we could not clone riboprobes for HDAC3 and 5 for an *in situ* hybridization assessment due to lack of sequence information for the rat in the NCBI database until just recently. However, we were able to generate an *in situ* hybridization riboprobe for rat HDAC2 and ran it in Experiment 1 as well as using HDAC2 Taqman primers in Experiment 2 for confirmation between *in situ* and RT-PCR findings. Our RT-PCR results also showed an opposite regulation in HDAC2 gene expression between LR and HR rats following chronic variable social stress in the dorsal hippocampus (Experiment 2). Namely, HDAC2 transcript levels are decreased with chronic variable social stress in LRs, whereas in HRs, HDAC2 transcript levels are increased following chronic variable social stress as also observed with BDNF transcript results. HDAC3 gene transcriptional regulation is mostly in line with HDAC2 in that both HDACs show an opposite regulation following chronic variable stress in the LRHR rats which match in direction and are highly

associated with BDNF transcript levels. We found overwhelming correspondence between class 1 HDACs (HDAC2 and HDAC3) and BDNF gene expression following chronic variable stress, in particular chronic variable social stress in adolescent LRHRs; strongly suggesting a direct epigenetic control over the BDNF gene expression in this phenotype with chronic stress which needs to be investigated properly in future studies.

Despite an upregulation in BDNF mRNA in the HR CA3 field following chronic variable social stress, NGF mRNA expression is downregulated with chronic variable social stress in the HRs CA3 in the same region. Upregulation of BDNF mRNA expression in the CA3 field in HRs can be a compensatory mechanism to downregulation of the NGF mRNA in the same region following chronic social stress exposure. Enhanced expression of BDNF could replace the loss of NGF in response to chronic variable social stress and could thereby be an attempt to protect those neurons expressing inhibited NGF. Thus, BDNF may induce axonal sprouting to keep NGF expressing cells alive in the CA3 field of the hippocampus. This can be investigated by assessing cell death markers in the CA3 field following chronic variable social stress in the LRHR rats. In Experiment 3 we found an increase in supra-pyramidal mossy fibre terminal field size in HRs following chronic social stress in the CA3 field of hippocampus corresponding to increase in BDNF mRNA observed in Experiment 1, which may be construed as an attempt for BDNF-induced protection of CA3 cells via axonal sprouting. Conversely, in LRs, both BDNF and NGF mRNAs are downregulated in the CA3 field following chronic variable social stress as shown in Experiment

1. Consequently, we found a decrease in supra-pyramidal mossy fibre terminal field size in LRs following chronic variable social stress in Experiment 3. Downregulation of both BDNF and NGF mRNAs in the LR CA3 field may suggest lack of neuroprotective response in the DG-CA3 synapse. In agreement with the previous literature where chronic stress is shown to downregulate NT3 protein in the hippocampus (Uys et al., 2006), we also showed a phenotype-independent downregulation of NT3 in the DG of LRs and HRs following especially chronic variable social stress. Since NT3 is involved in neuronal differentiation, synaptic plasticity and neuronal survival (Francis *et al*., 1999), downregulation of NT3 mRNA expressions in DG in both LR and HR rats following chronic variable stress could lead to granular neuron atrophy or even death.

Present results show that BDNF and HDAC2 mRNA levels in the CA3 field is positively correlated and regulated in an opposite direction between LR and HR rats following chronic variable social stress exposure. It is commonly accepted that decrease in HDAC activity indicates increase in acetylated histones and accompanies increased gene transcription. However concerning BDNF gene transcription, we see opposite effect in the hippocampus; namely an increase in HDAC2 levels goes together with an increase in BDNF levels in HRs; and conversely a decrease in HDAC2 levels goes together with a decrease in BDNF levels in LRs. Although this seems like a contradiction at first glance, three possible explanations come to mind. First as mentioned previously, changes in HDAC activity can regulate its own gene transcription in a tightly managed autoregulatory loop. Explicitly, inhibition in HDAC levels could in turn

activate increase in the same HDAC transcription. Therefore, in my current chronic stress paradigm, it is possible that there is an early inhibition of class 1 HDAC activity in HRs, followed by observation of increased HDAC levels at the completion of chronic stress regimen at the time of assessment. Such an autoregulatory control is expected to maintain homeostasis in an epigenetic switch for widespread gene transcription. This hypothesis is testable in a kinetic study looking at HDAC levels at various time points along stress regimen in LRHR rats. Activity of class 1 HDACs, specifically HDAC2, can be assessed at different time points in response to acute and repeated stress exposures in LRHR rats. If indeed autoregulation is involved in HDAC2 activity, early on I would expect to see severely inhibited HDAC2 activity in HRs in response to chronic variable social stress, but later on HDAC2 activity will be enhanced by autoregulatory mechanism in HRs following chronic variable social stress. Conversely, in LRs, early on HDAC2 activity will be robustly enhanced in response to chronic variable social stress, which later on activates the autoregulation of its own transcription leading to a decrease in HDAC2 activity following chronic variable social stress. Expected initial inhibition of HDAC2 activity in response to chronic stress would also be expected to drive the upregulation in the BDNF gene observed in HRs. Once the kinetics of HDAC2 regulation is determined, the causal link can be tested using ChIP (chromatin immunoprecipitation) assay to determine whether there is a direct regulatory association between HDAC2 and BDNF gene transcripts. Specifically, I can assess acetylation of H3 and H4 on the promoter region of the BDNF gene using

the ChIP technique because these histones have been shown to have an important role for regulation of the BDNF gene transcription (Tsankova *et al*., 2004; Kumar *et al*., 2005; Tsankova *et al*., 2006). If there is a direct regulation of BDNF via class 1 HDACs, specifically HDAC2, I would expect that before completion of chronic stress regimen an increase in histone acetylation will be observed in the promoter region of the BDNF gene in HRs. Chromatin immunoprecipitation is a powerful method for analyzing epigenetic modifications and used to evaluate the association of proteins with specific DNA regions. The technique is based on crosslinking of proteins with DNA, fragmentation and immunoprecipitation of the fragmented DNA with an antibody recognizing the protein of interest. DNA is then purified and quantified using PCR method, mostly RT-PCR. Kumar and colleagues (2005) utilized ChIP assays to investigate mechanisms of chromatin remodeling associated with drug action. They chose gene promoters that represent distinct drug mechanisms and showed that chronic cocaine exposure causes an increase in transcription and acetylation on the promoter region of BDNF. Specifically they found H3 hyperacetylation on the both promoter regions of BDNF making this method readily available and particularly valid for future investigations. Second explanation why a positive correlation is observed between HDAC2 and BDNF contradictory to the traditional view of epigenetic regulation could be that increase in HDAC activity in the HRs following chronic stress may be inhibiting transcription of genes that also inhibit BDNF production in the HRs; net result being disinhibition of the BDNF gene transcription. Thirdly, it is possible that more traditional transcriptional

regulators such as CREB may indeed be driving the effect on BDNF gene rather than posttranslational mechanism. To test this latter hypothesis I can further assess CREB activation levels in LRHR rats following chronic variable social stress regimen. If this hypothesis is valid, I would expect to see an increase in CREB activity and thereby an increase in BDNF levels in HRs following chronic variable social stress, whereas in LRs, chronic variable social stress would be expected to inhibit CREB activity and consequently leading to a decrease in BDNF levels.

In the stress paradigm I used, I observed strong correlation between BDNF and class 1 HDACs (HDAC2 and HDAC3) following chronic social stress regimen but I should note this does not necessarily mean that class 2 HDACs (HDAC4, 5, 6, 7, 9, and 10) are not involved in regulation of the BDNF gene transcription. HDAC5 has also been shown to have an effect on the regulation of BDNF gene transcription. Tsankova and colleagues (2006) showed that, social defeat stress induces long-lasting downregulation in total BDNF mRNA levels in the hippocampus of mice which is mediated by means of the decreased expression of BDNF III and IV but not of any of the other variants (I, II and V). Prolonged exposure to an aggressor induces lasting changes in mouse behavior such as social avoidance, which are reversed by imipramine treatment which induces hyperacetylation of H3 at the BDNF promoter, an effect that seems to be mediated at least in part by downregulation of HDAC5 in the hippocampus. Thus, they have proposed that BDNF is important player for regulation of behavioral adaptation to stress. If HDAC5 is also involved in the regulation of BDNF gene in

my paradigm, I would expect to see same downregulation of BDNF III and IV variants in LRs following chronic variable social stress regimen, whereas, in HRs I would expect to see an upregulation of BDNF III and IV variants following chronic variable social stress exposure. Hence HDAC5 may have a regulatory function on the promoter region of the BDNF III and IV variants that should be tested.

As I mentioned before BDNF and CREB are negatively regulated by chronic stress (Duman *et al*., 1995) which is reversed by chronic antidepressant treatment (Duman *et al*., 1995; Duman *et al*., 2000). It has been also shown that electroconvulsive seizures (ECS) upregulates the levels of BDNF and CREB in the hippocampus (Nibuya *et al*., 1996). Tsankova and colleagues (2004) showed that chronic upregulation of the BDNF transcription in response to ECS may be sustained via control of H3 acetylation, selectively at the P3 and P4 promoters. This data may suggest that P3 and P4 promoters are important for posttranslational modifications of histones, specifically histone H3 which may be affected by chronic stress effects. In my stress paradigm, it is worthwhile to investigate possible differential epigenetic regulations on the P3 and P4 promoters of the BDNF in LRHR rats following chronic variable stress.

As mentioned before, mossy fibre projections are the primary excitatory input that has been traditionally accepted to be primary excitatory synaptic contact from dentate granular cells to CA3 primary neurons. I showed that there are changes in mossy fibre terminal field size, which is in line with findings on BDNF mRNA in Experiment 1. Chronic variable social stress resulted in an

increase in SP-MF terminal field size in HRs, whereas in LRs, SP-MF size is decreased with chronic variable social stress. BDNF mRNA levels are upregulated in the CA3 in HRs following chronic variable stress, whereas NGF mRNA levels are downregulated in the same region in the HR rats. Conversely, in LRs both BDNF and NGF mRNA levels are downregulated in the CA3 following chronic variable social stress. In our Timm's stain method I visualize the presynaptic axon terminals. An interesting hypothesis is to assess changes on the postsynaptic side, namely measuring changes in the CA3 dendritic processes. Based on our NGF findings I expect that LRs and HRs may have same response on CA3 neurons, namely, an atrophy of the dendritic processes of CA3 neurons that also express less NGF mRNA. However, if increase in BDNF in the mossy fibre axon terminals of HRs is an attempt to rescue these cells from atrophy by providing neurotrophic support on CA3 neurons, then atrophy may not be observed on the CA3 dendritic processes. It is plausible to expect that BDNF response could actually sustain the CA3 neurons. Conversely, an atrophy in dendritic processes may still be observed despite that there is an attempt to correct for it. I can test this possibility by using Golgi-Cox method to visualize the dendritic processes of the entire CA3 neurons. Thus, I can quantify how much dendritic material these cells have following exposure to chronic variable social stress in LRHR rats. Since I found an increase in SP-MF size and BDNF levels in CA3 field, I can hypothesize rescue of apical CA3 pyramidal dendritic field from atrophy in HRs following chronic variable social stress in line with a compensatory response. However, it is equally likely to still observe

atrophy of apical CA3 dendrites in both phenotypes. Axonal sprouting in HR raises a question about its functional significance. In other words the question needs to be answered if these newly formed synapses in the case of HRs are indeed making contact with the CA3 dendritic surfaces and they are integrated to circuitry. Electron microscopy images can enable us to have visual confirmation that these neurons are functional. If they are not making functional synapses, the CA3 neurons may still demonstrate dendritic atrophy in HRs animals that showed increased SP-MF size and increased levels of BDNF. Despite the traditional role, more recent anatomical evidence shows that inhibitory interneurons receive approximately 10 times more synapses from mossy fibers than do principal neurons (Acsady *et al*., 1998), generating a strong feed forward inhibition at physiological firing frequencies of dentate granule cells (Lawrence *et al*., 2003). The mossy fiber-CA3 network dynamics under pathophysiological circumstances may depend on the net innervation ratio between the excitatory primary neurons and inhibitory interneurons. To make things more complex, it is also shown that mossy fiber 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*., 2004) providing a state-dependent switch in the network, all of which need to be investigated in the current chronic variable social stress paradigm.

In situ hybridization histochemistry is anatomically specific, highly sensitive quantitative measure of mRNA expression but it does not allow us to determine the protein levels. To assess the protein levels I can run Western Blot

using whole dorsal hippocampus or to maintain the anatomical specificity I can run immunohistochemistry. Ultimately all of the mRNA findings I reported in Experiment 1 will need to be confirmed in a protein study. Moreover, increase in BDNF in HRs following chronic variable social stress may factor in an increase in proBDNF levels because our BDNF probe consisted of all BDNF variants and does not distinguish between different splice variants and immature form of BDNF. ProBDNF has been proposed to be only developmentally important but recently it has been shown that adult neurons produce and secrete proBDNF as well. 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 (Teng *et al*., 2005). In adult brain proBDNF can be regulated by certain parameters, such as stress. Even though as I mentioned above, enhanced BDNF levels following chronic variable social stress may be an attempt to rescue CA3 neurons from death in HRs, it is also possible that proBDNF can be activated by chronic variable social stress which then can induce cell death via binding to p75 receptor. Future experiments will be aimed to assess proBDNF levels in HRs hippocampus, specifically in the CA3

field, following chronic variable social stress and its effects along with BDNF on the neuronal function.

In summary, in this thesis I described the activational profile of various neurotrophins in the hippocampus following chronic variable stressors employed in adolescence on the LRHR rats. I found widespread downregulation of hippocampal NGF and NT3 in both phenotypes, however a phenotype-dependent regulation of BDNF following chronic variable social stress in the CA3 field. I also observed a positive association between class 1 HDACs, specifically HDAC2 and BDNF levels in the hippocampus in the LRHR animals. Both HDAC2 and BDNF levels were regulated in an opposite manner in LRHR rats following chronic variables social stress in the hippocampal CA3 field, namely increased in the case of HRs and decreased in the case of LRs. In conjunction with opposite regulation of BDNF in the CA3 field of LRHR rats, I also reported an opposite regulation of the SP-MF terminal field size; specifically an increase in the terminal field volume in HRs and conversely a decrease in the terminal field volume in LRs. These studies suggest that there may be an epigenetic control of the BDNF gene regulation in the mossy fibre axon terminals in response to chronic variable social stress in LRHR rats that also give way to individual differences in structural plasticity induced by stress in the terminal fields of mossy fibres.

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