Female and Adolescent Specific Roles for RasGRF1 in Regulating the Hypothalamic-Pituitary-Adrenal Axis

A thesis

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Abstract

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in the induction and prolongation of a variety of psychiatric disorders. As such, much effort has been made to understand the molecular mechanisms involved in its control. However, the vast majority of studies on the HPA axis have used adult animals, and among these the majority has used males. RasGRF1 (GRF1) and RasGRF2 (GRF2) form a family of calcium-activated guanine nucleotide exchange factors that activates Ras and Rac GTPases and regulates multiple forms of synaptic plasticity in the CA1 of hippocampus (HC). Since the HC is known to play a key regulatory role in controlling the HPA axis that regulates the stress response, we tested the involvement of GRF1 in this process by comparing the response of GRF1 knockout (GRF1(-/-)) mice and control (WT) mice after exposure to acute and chronic restraint stress.

Here we show that in knockout mice lacking GRF1, habituation to 30 minutes a day of restraint is markedly accelerated, such that these mice no longer display elevated corticosterone levels or enhanced locomotion after 7 days of stress exposure, while WT mice do not even begin to habituate after 14 days of stress. Strikingly, this phenotype is present in early-adolescent female Ras-GRF1 knockout mice, but not in their early-adolescent male, mid-adolescent female, or adult female counterparts. Moreover, not only is there a clear response to restraint stress in early-adolescent female Ras-GRF1 knockout mice after fewer stress exposures, their response is magnified ~3 fold compared to WT mice. Because animal wide knockout mice were used in these studies, these complex phenotypes are likely due to a composite of the effects of GRF1 loss in various brain regions known to regulate the HPA axis. In fact, we have shown that the loss of

GRF1 in the CA1 hippocampus is responsible for the super-activated HPA axis response observed after short-term stress, which is consistent with the role of this brain region in HPA axis negative feedback mechanism.

Mechanistic studies have suggested two levels of GRF1 control over the HPA axis response to short term stress in early-adolescent females; one as a master negative regulator of phosphorylation changes of proteins in the CA1, and the other as a regulator of gene expression changes via control over stress-induced alterations in histone H3K9 acetylation. A full understanding of how Ras-GRF1 controls the HPA axis response to stress may be required to design effective strategies to combat stress-induced psychiatric disorders initiated in stress-sensitive young females.

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List of Abbreviations

AAV, adeno-associated virus

ACTH, adrenocorticotropic hormone

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMY, amygdala

AV, adenovirus

AVP, vasopressin

BDNF, brain-derived neurotrophic factor

C/CTRL, control mice

cFos, immediate early gene

CHiP, chromatin immunoprecipitation

CORT, corticosterone

CRH, corticotrophin releasing hormone

DBKO, GRF1/GRF2 double knock-out mice

DST, dexamethasone suppression test

ECL, enhanced chemiluminescence

EPM, elevated plus maze

ERK, extracellular signal-regulated kinases

GC, glucocorticoid

GR, glucocorticoid receptor

GRF1(-/-), GRF1 knock-out

GRF1-AV, GRF1 adenovirus

HAT, histone acetyl transferase

HC, hippocampus

HDAC, histone deacetylase

HPA, hypothalamic-pituitary-adrenal axis

HYP, hypothalamus

LTD, long-term depression

LTP, long-term potentiation

MDD, major depressive disorder

mPFC, medial prefrontal cortex

MR, mineralocorticoid receptor

NAc, nucleus accumbens

NMDAR, N-methyl-D-aspartate receptor

OFT, open field test

OVX, ovariectomy

PBS, phosphate buffered-saline

pn, post-natal day

PTSD, post-traumatic stress disorder

PVN, paraventricular nucleus

S1, 1 day stress

S3, 3 days stress

S5, 5 days stress

S7, 7 days stress

SAL, saline

SCRM, scrambled sequence

SDS, sodium dodecyl sulfate

TSA, trichostatin A

WT, wild type mice

Chapter 1:

Introduction

1.1 RasGRF proteins

p140-Ras-GRF1 (GRF1) and p130-Ras-GRF2 (GRF2) constitute a family of calcium activated exchange factors for both Ras and Rac GTPases. They are expressed in neurons throughout the central nervous system but not in glia (Feig 2011). They have very similar domains including calmodulin (CaM)-binding IQ motifs, Rac-activating DH/PH, and Ras-activating CDC25 domains as seen in Figure 1.1. Upon an elevation in calcium concentration, calmodulin binds to the IQ motifs and stimulates the ability of GRF1 and GRF2 to activate Ras and Rac GTPases (Shou, Farnsworth et al. 1992, Farnsworth, Freshney et al. 1995).

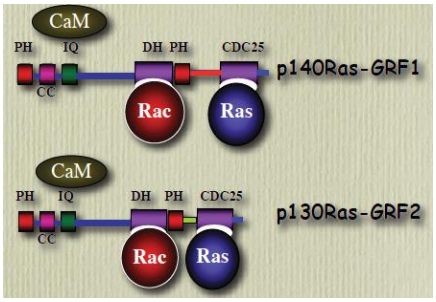

Figure 1.1 Structural domains of RasGRF1 and RasGRF2

Even though GRF1 and GRF2 are structurally very similar, they play very different roles in synaptic plasticity in the CA1 region of the hippocampus (HC) (Feig 2011). Previous work in our lab indicates that RasGRF1 promotes LTD by acting through NR2B containing NMDA receptors via p38 activation, whereas RasGRF2 protein promotes LTP by acting through NR2A containing NMDA receptors via ERK activation (Li, Tian et al. 2006). Our lab also showed that GRF1 can induce LTP when it is

mediated by calcium-permeable AMPA type glutamate receptors (CP-AMPARs) through p38 activation to promote contextual discrimination learning starting at 2 months of age (Jin, Arai et al. 2013) (Figure 1.2). GRF1 also induces LTP in the amygdala (Brambilla, Gnesutta et al. 1997) and perirhinal cortex (Silingardi, Angelucci et al. 2011) but its behavioral roles are not known. Our lab has also found that both GRF1 and GRF2 contribute to the survival of adult-born hippocampal neurons, whose survival rate is environmentally controlled through GRF2 (Darcy, Trouche et al. 2014, Darcy, Trouche et al. 2014).

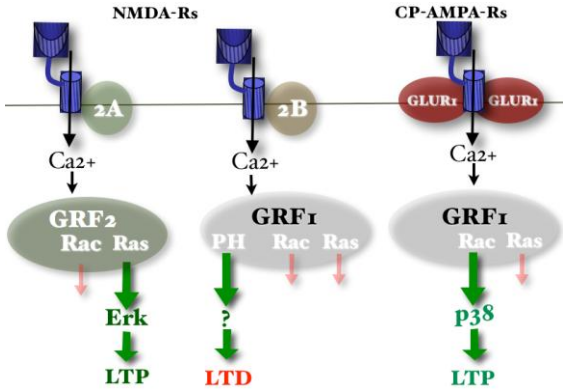

Figure 1.2 Roles of GRF1 and GRF2 in synaptic plasticity

GRF1 and GRF2 play distinct roles in synaptic plasticity besides in the hippocampus too. For example in the striatum, GRF1 mediates Erk regulation by dopamine receptors (Fasano, D'Antoni et al. 2009). Moreover GRF1(-/-) mice are resistant to the development of dyskinesia during chronic L-DOPA treatment, a treatment used for Parkinsons's disease. This suggests a role for GRF1 in Parkinson's disease through dopamine receptors (Fasano, Bezard et al. 2010). Furthermore, in a genome-wide

association study, the GRF1 gene is found to be in a locus that has been implicated in myopia. This suggests that alterations in GRF1 levels may play a role in this disease (Hysi, Young et al. 2010). Moreover GRF2 plays a role in alcohol induced dopamine release in ventral striatum through ERK activation. (Stacey, Bilbao et al. 2012)

Besides their roles in the central nervous system, GRF1 and GRF2 have some other functions. For example, GRF1 is a paternally imprinted gene such that it is expressed only from the paternal gene in young animals. The maternal allele of the gene is relaxed after 3 weeks of age, causing an increase in GRF1 expression (Shibata, Yoda et al. 1998, de la Puente, Hall et al. 2002, Tian, Gotoh et al. 2004). One common feature of paternally imprinted genes is their regulation of growth hormones. Consistent with this finding is that GRF1(-/-) mice are smaller in size compared to their WT counterparts. This is due to reduced levels of IGF-1 and its regulatory growth hormone (Itier, Tremp et al. 1998, Drake, Park et al. 2009).

GRF1 and GRF2 expression and function are both highly age-dependent. They play no detectable roles in hippocampal synaptic plasticity before early adolescence, when their expression levels are very low (Tian, Gotoh et al. 2004). These findings highlight that GRF proteins can play different roles in different brain regions, and their roles can be age-dependent. Due to the roles of GRF1 in hippocampus and the importance of hippocampus in HPA axis regulation, I investigated the role of GRF1 in stress induced HPA activation.

1.2 Stress and HPA Axis

Exposure to chronic stress plays a major role in psychological and physical problems by altering basic mechanisms of neuroendocrine and neurobiological systems (Franklin, Saab et al. 2012). Pathological conditions highly associated with stress include psychiatric disorders like major depressive disorder (MDD), post-traumatic stress disorder (PTSD), heart diseases and drug addiction (Marin, Cruz et al. 2007, O'Keane, Frodl et al. 2012).

The hypothalamic-pituitary-adrenal (HPA) axis is the major endocrine system that restores homeostasis following environmental challenge. Physical or psychological stress results in cortisol production in humans or corticosterone (CORT) production in rodents that alters a variety of adaptive animal responses (Marin, Cruz et al. 2007). As seen in figure 1.3, the HPA axis is activated upon stress exposure with the mechanisms well established. It is initiated by the release of corticotrophin-releasing hormone (CRH) and vasopressin (AVP) from the neurons in the paraventricular nucleus (PVN) of hypothalamus. This stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. Circulating ACTH initiates the synthesis of glucocorticoids (GC) (corticosterone in rodents and cortisol in humans) in adrenal glands in kidney and corticosterone (CORT) is released into the blood stream (Herman, Ostrander et al. 2005, O'Mahony, Sweeney et al. 2010).

Circulating GCs are needed to maintain the homeostasis to cope with stress mechanism by providing energy to the cells through glucose metabolism. They do so by binding to glucocorticoid receptors (GRs) or mineralocorticoid receptors (MRs) on

different organ systems to induce further signaling. A prolonged increase in GCs is known to have deleterious consequences, therefore the HPA axis needs to be very tightly controlled by positive and negative feedback mechanisms at the level of the brain and the pituitary. Therefore an increase in GC secretion inhibits further release of CRH and ACTH by interacting with receptors on specific CNS components (McCormick, Smith et al. 2008). Two types of feedback mechanisms exist; fast and delayed feedback. Both of these are mediated by binding of CORT to GRs. The fast feedback mechanism is mediated by CORT binding to GRs on the PVN to induce endocannabinoid mediated suppression in the hypothalamus. Delayed feedback is mediated by CORT binding to GRs in other brain regions. This binding leads to GRs shuttling from the cytosol to the nucleus to activate or repress different gene transcription patterns (Bangasser and Valentino 2014). Many brain regions influence the HPA axis such as hippocampus (HC), prefrontal cortex (mPFC) and amygdala (AMY). These regions are densely populated with GRs and MRs. The hippocampus and mPFC play an inhibitory role on the HPA axis whereas the amygdala activates the HPA axis through both region and stressor specific mechanisms (Herman, Ostrander et al. 2005, Joels 2008).

Altered activity of the HPA axis is known to be adaptive in short term, but prolonged alterations have detrimental health consequences. Moreover altered basal levels of cortisol in humans can be associated with certain psychiatric disorders. For example, HPA hyper-reactivity is linked to vulnerability to stress and heightened risk factor for depression and anxiety disorders (O'Keane, Frodl et al. 2012). In contrast, excessive suppression of the HPA axis is associated with the development of PTSD (Handwerger 2009).

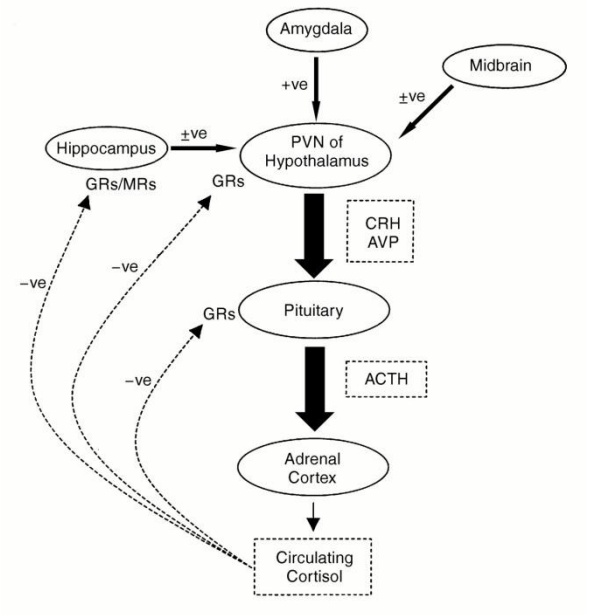

Figure 1.3 Schematic diagram of the HPA axis. Figure describes the regulation and negative and positive feedback mechanism (McQuade and Young 2000).

HPA axis negative feedback in humans can be measured by either the dexamethasone suppression test (DST) or a modified version, DST/CRF test. Dexamethasone is a synthetic glucocorticoid that can mimic CORT and bind to GRs in the brain and pituitary with high affinity. Therefore, injection of dexamethasone can suppress the cortisol release in healthy humans. However, 20-60% of people with depression fail to respond to DST (Bangasser and Valentino 2014).

Major sources of stress include psychological stress paradigms such as social defeat, social instability and restraint stress (used in this thesis) or physical stress paradigms such as foot shocking or cold water (Kearns and Spencer 2013). Several animal models have been developed to study mechanisms underlying the consequences of various stressors (Schmidt, Sterlemann et al. 2008). The intensity and duration of

stressors play a major role in the consequences of stress exposure. For example, acute or chronic stress have been shown to have similar or opposing behavioral or biochemical effects in rodents depending on the type of stressor. Chronic stress carries a risk of long-term detrimental consequences whereas acute stress may be involved in adaptation to changing natural and social environments (McEwen 2008, Buynitsky and Mostofsky 2009, Bali and Jaggi 2015).

Two important factors that change coping mechanism with stress are age and sex. Stress during prenatal period, adolescence or adulthood has different consequences on measures of fear, anxiety and depression related behaviors. Adolescence in humans and rodents is a critical time period for growth and maturation because of the changes taking place in the brain and the body (Laviola, Adriani et al. 2002, Lupien, McEwen et al. 2009). Also males and females have been shown to react differently to stress exposures however the mechanisms behind this difference are not well established (Hefner and Holmes 2007, Dedovic, Duchesne et al. 2009). Involvement of age and sex in stress mechanism is explained below in more detail.

Hippocampus and HPA axis negative feedback mechanism

The hippocampus (HC) is one of the key regulators of the HPA axis negative feedback mechanism. Of the three components of the main circuitry of the hippocampus, CA1, CA3 and dentate gyrus, the CA1 and DG regions are most enriched in MR and GRs that provide detection and binding in a wide range of circulating CORT (Joels 2008, O'Keane, Frodl et al. 2012). MRs have 5-10 fold higher affinity for CORT and therefore are extensively bound even at basal conditions. Upon stress exposure and increased

circulating CORT, CORT starts binding to the GRs on these brain regions (Jankord and Herman 2008). To provide interactions between HC and the PVN, indirect GABAergic projections are found through the bed nucleus of stria terminals (BST), medial preoptic area and dorsomedial hypothalamus (Jankord and Herman 2008, Ulrich-Lai and Herman 2009). Hippocampal loss-of-function experiments cause basal CORT elevation in rodents (Jankord and Herman 2008). Furthermore hippocampal lesions have been detected following certain stressors such as restraint stress or elevated plus maze exposures. Morphological changes in the CA1 region and dendritic retraction is known to occur after chronic stress. This leads to HC mediated spatial memory impairments (McLaughlin, Gomez et al. 2007, Joels 2008).

The MAPK pathway is one of the key pathways regulated upon acute and chronic stress in different brain regions including the HC, mPFC and the cingulate cortex depending on the type of stress and age or sex of the rodents. ERK1/2 is a member of the MAPK pathway that has been shown to be altered upon different stress exposures and is highly expressed in the central nervous system including the three regions of the HC (Ferland, Harris et al. 2014). Several studies have shown elevated p-ERK1/2 levels in HC as well as in mPFC upon different stress exposures (Meller, Shen et al. 2003, Yang, Huang et al. 2004, Chen, Tang et al. 2008, Ferland, Harris et al. 2014). Furthermore the study by Revest et al. showed that activation of GRs with glucocorticoid treatment is sufficient to enhance pERK1/2 levels in the HC (Revest, Di Blasi et al. 2005). However, the study by Shen et al did not detect an increase in pERK1/2 levels in the HC after acute swim stress (Shen, Tsimberg et al. 2004). These results suggest that the stress-induced levels of the components of the MAPK kinase family depend on the type and intensity of

the stressor. Moreover, all these findings were just correlative and detected in adult male mice and rats. Therefore, more studies need to be done to further understand the role of the MAPK pathway in HPA axis regulation in females and adolescents.

BDNF (Brain derived neurotrophic factor) is another major target of stress exposure in HC. Both human and animal studies have shown that levels of BDNF are decreased in depressed patients (Dwivedi, Rizavi et al. 2003) as well as rats submitted to acute and restraint stress (Nibuya, Morinobu et al. 1995, Barrientos, Sprunger et al. 2003). Moreover, BDNF levels can be increased by anti-depressant treatments and the antidepressant responses are lost in BDNF knockout mice in a sex dependent manner (Nibuya, Morinobu et al. 1995, Monteggia, Luikart et al. 2007, Sun, Kennedy et al. 2013).

1.3 Habituation to Chronic Stress

Multiple exposures to a homotypic stress response leads to a decreased responsiveness, a process called 'habituation' or 'adaptation'. It is a key mechanism to prevent damaging effects of prolonged GC levels and the transition to stress induced pathology (Herman 2013, Karatsoreos and McEwen 2013). Habituation can be measured by magnitude of the HPA axis activation such as CORT or ACTH levels, behavior of rodents such as escape or struggling response or minimal weight loss (Grissom and Bhatnagar 2011). Excessive habituation has been found in PTSD patients (Garcia, Marti et al. 2000). Habituation is known to be mainly regulated by positive and negative feedback mechanisms because blocking MR/GR activity systemically can prevent

habituation in rats (Cole, Kalman et al. 2000). However, adrenalectomized rodents are still able to habituate stating that negative feedback mechanism cannot fully explain the habituation process (Jaferi and Bhatnagar 2006). Habituation is observed after a wide intensity range of stimuli from a mild to a severe stressor. The habituation rate is dependent on the frequency and severity of the stressor. It has been shown that the habituation rate is inversely proportional to the severity of the stressor (Pitman, Ottenweller et al. 1988, Kearns and Spencer 2013). Manipulating stress parameters such as changing the context may also disrupt habituation (Grissom and Bhatnagar 2009, Kearns and Spencer 2013). Habituation is not considered as reversion to normal because it involves long-term changes to the CNS and is reversible over time. Also exposure to a novel stress paradigm after a repeated exposure to a homotypic stressor can reactivate the HPA axis, named as 'dishabituation' (Grissom and Bhatnagar 2009, Herman 2013). The neural mechanisms involved in habituation appear to involve the sensory cortex and the limbic system, including the medial prefrontal cortex (mPFC) (Weinberg, Johnson et al. 2010), basal amygdala (Grissom and Bhatnagar 2011) and paraventricular thalamus (Bhatnagar, Huber et al. 2002) with the molecular mechanism still not clear. Habituation is also known to cause morphological changes in these limbic brain regions (Herman 2013). Habituation to repeated stress is known to affect the levels of some immediate early genes in different brain regions. For example, c-Fos mRNA is one of the best characterized immediate early genes that are shown to be activated upon stress exposure. However, upon habituation, the levels of activated cFos are reduced mainly in paraventricular nucleus of hypothalamus (PVN), lateral septum (LS) and mPFC (Stamp and Herbert 1999, Kearns and Spencer 2013).

Habituation is a key mechanism for preventing the damaging effects of prolonged levels of glucocorticoids. The transition to stress induced pathology may involve a breakdown in the ability to habituate. In addition, failure to habituate to non threatening environments can promote damaging effects of excess glucocorticoids.

1.4 Stress and Adolescence

Adolescent development is known for a gain in many neurobiological and cognitive functions as well as being characterized as the 'period for developmental vulnerabilities (Eiland and Romeo 2013). It is defined as ages between 10-18 in humans and pn 21-59 in rodents. Adolescence is divided into 3 stages in rodents as early-adolescence (pn 21-34), mid-adolescence (pn 35-46) and late-adolescence (pn 46-59) (Burke and Miczek 2014). As the brain is developing throughout adolescence, early-adolescence is considered to be the most vulnerable stage since pubertal maturation is taking place during this phase (Romeo 2010). Excess stress during this developmental stage may influence maturation and contribute to anxiety and depression that is often observed during adolescence and associated with development of psychiatric disorders later in life (Kessler and Magee 1993). In addition, adolescents are at a greater risk of experiencing trauma than adults or children (Nooner, Linares et al. 2012).

Many critical neuroendocrine changes and structural and functional brain developments are taking place during adolescence especially in limbic regions such as the hippocampus, amygdala and medial prefrontal cortex. HC, Amy and mPFC are shown to increase in volume (Isgor, Kabbaj et al. 2004) and the dynamic grey- and white-matter

volume changes are also observed (Gogtay, Giedd et al. 2004) during adolescence. Due to the important roles of these limbic regions in the HPA axis, adolescence is thought to be a critical phase for stress exposures. Many studies have compared various stress paradigms in adolescents and adults and in general have found that the HPA axis in adolescents is more sensitive compared to adults (McCormick, Smith et al. 2008, Romeo 2010). For example, prepubertal animals display enhanced stress response to different stressors compared to adult animals owing it to incomplete maturation of the negative feedback mechanism (Romeo, Lee et al. 2004, Romeo, Karatsoreos et al. 2006); however the levels of the MRs and the GRs are found to be stable throughout adolescence and adulthood (Romeo 2013). Moreover, during adolescence it takes a longer time for the stress hormones to return to basal levels after the last stress exposure, displaying decreased habituation (Foilb, Lui et al. 2011). Also, adolescent mice can show a greater decreased weight gain compared to adult animals after a chronic stress exposure (Eiland, Ramroop et al. 2012). Interestingly stress appears to increase risk taking in adolescence but not adulthood (Peleg-Raibstein and Feldon 2011, Toledo-Rodriguez and Sandi 2011). Even though many biological and morphological changes between adolescents and adults have been observed upon stress exposure, the molecular mechanisms mediating these age-dependent shifts are not clearly understood. For example levels of BDNF and neurogenesis were reduced when adult animals were exposed to chronic mild stress whereas the levels were increased with adolescent animals (Toth, Gersner et al. 2008). Moreover studies have shown that corticosterone injection in mice increases hippocampal NR2A and NR2B receptor levels more in adolescents than adults (Lee, Brady et al. 2003).

Studies done with humans also show behavioral differences during adolescence and adulthood. It is thought that biochemical changes that take place during pubertal maturation are playing a role in shifting stress reactivity through adulthood (Romeo 2010). Human studies have also showed enhanced HPA response during adolescence. For example, adolescent boys and girls showed greater cortisol responses than children (Dahl and Gunnar 2009). Studies show that stress before puberty mainly depends on family and parent interactions whereas after puberty it begins to depend on social interactions and peer relations. For example, adolescents who grew up with mothers with postnatal depression have higher morning cortisol levels suggesting an intrafamilial risk for psychiatric disorders before puberty (Halligan, Herbert et al. 2007). Moreover, studies have shown that adolescents who grew up in poor economic conditions have higher basal cortisol levels then their middle-income counterparts (Evans and English 2002). Studies also show that timing of puberty plays a major role in developing psychological disorders among adolescent girls (Ge, Conger et al. 2001, Mendle, Turkheimer et al. 2007).

Even though adolescence is a very critical time period for stress disorders, many of the studies use adult animals. Therefore, more studies need to be done using adolescent animals to further understand the mechanism causing stress-induced behavioral differences compared to adults.

1.5 Stress and Females

Sex-dependent differences are other major factors in the development of stress responses. These differences emerge around adolescence with the start of puberty and are

known to persist throughout adulthood (Ordaz and Luna 2012). Some population-based studies have shown that there are sex differences in the prevalence of stress-related disorders. For example, women are roughly twice as likely to suffer from anxiety disorders, PTSD and depression (Sheikh, Leskin et al. 2002, Tolin and Foa 2006) and respond better to a certain class of antidepressants than men (Kornstein, Schatzberg et al. 2000). Moreover women show an earlier onset of depression than men (Marcus, Young et al. 2005). On the other hand men are more likely to develop drug and alcohol abuse upon stressful conditions (Bangasser and Valentino 2014). A consistent trend from animal studies shows that there is a higher and long-lasting CORT secretion after stress in adult female rats compared to male rats (Panagiotakopoulos and Neigh 2014). However, this trend is shown to be inconsistent among adult women and men depending on the stress type, age and basal hormone levels. Some studies find higher cortisol response in women whereas some studies find higher response in men (Bangasser and Valentino 2014, Panagiotakopoulos and Neigh 2014). Consistent findings of women being more likely to suffer from depression and the female rat studies support the idea that women have more dysregulated HPA axis. Sex differences at multiple sites in the HPA axis have been implicated including pituitary response to ACTH (Young and Altemus 2004), CRH and CRHR expression (Iwasaki-Sekino, Mano-Otagiri et al. 2009), GC feedback (Heinsbroek, Van Haaren et al. 1991) and GR metabolism (Bourke, Raees et al. 2013). Dexamethasone suppression test (DST) and DST/CRF tests are used to measure the defects in HPA negative feedback. Upon CRH treatment followed by pretreatment with dex, healthy and depressed women are shown to have a greater cortisol response than men suggesting a sex-dependent CRF sensitivity in women. This finding was consistent

among female rats exposed to DST and DST/CRF treatment as well (Bangasser and Valentino 2014). Studies have also showed a slower feedback mechanism in female rats compared to male rats since it takes longer for CORT to go back to basal levels in females (Weiser and Handa 2009). Besides the sex-dependent HPA response, sex differences have been detected in brain activity in response to negative emotions and region specific morphological changes as well (Ordaz and Luna 2012, Bangasser and Valentino 2014).

Pubertal transition is a very critical stage in stress exposure. Females are known to enter puberty 1-2 years earlier than males and sex differences are known to start with secretion of gonadal steroid hormones with puberty. For example increased rates of depression among women compared to men start with puberty and remain until menopause. However; not only the increase in ovarian hormones, decreased levels can also play roles in psychiatric disorders (Schmidt and Rubinow 2009, Solomon and Herman 2009). This suggests that sudden changes in ovarian hormones may play a role in psychiatric disorders. However, not all the females exposed to stress develop depression starting with puberty. Also females and males are known to react to different stressors differently after puberty. Moreover treating prepubertal male rats with adult-like physiological levels of testosterone is not sufficient to activate an adult-like stress response (Romeo, Lee et al. 2004). These findings suggest that besides the effects of the hormones, biochemical changes are also taking place during puberty (Ordaz and Luna 2012).

Although most studies factor in age and sex separately, a few studies have combined them. For example, female rats exposed to chronic restraint stress throughout

adolescence (Barha, Brummelte et al. 2011) or social instability stress during early- to mid-adolescence (McCormick, Nixon et al. 2010) exhibit decreases in adult neurogenesis compared to an increase in males (Toth, Gersner et al. 2008). Moreover adolescent male rats exposed to repeated daily restraint stress displayed reduced habituation relative to adult males while adolescent females were more similar (Doremus-Fitzwater, Varlinskaya et al. 2010). Also, 13-year old girls showed much greater cortisol reactivity to a social stress test than 13-year-old boys, suggesting sex differences impact stress reactivity prior to the completion of adolescent maturation (Gunnar, Frenn et al. 2009). One example studying early adolescence and females showed that upon chronic stress, adolescent female mice display a blunted stress response compared to their male counterparts due to differential transport of GRs to the nucleus. GRs are transported to nucleus by the help of co-chaperone proteins. This study showed that upon stress exposure, the levels of co-chaperones, Bag1 and Ppid that inhibit GR translocation was increased in females (Bourke, Raees et al. 2013).

Due to the lack of knowledge for the differences in stress response between early adolescent females and males and also to the significance of transition into puberty, more studies need to be performed to understand the molecular mechanisms behind.

1.6 Stress and Epigenetics

Every human being is exposed to some kind of stress throughout their lives; however, not all of them have the same outcomes. For example, not all individuals develop psychiatric disorders upon stress exposures. These findings lead to understanding

gene and environmental factor interactions and epigenetics (Sun, Kennedy et al. 2013, Zannas and West 2014). Epigenetics is defined as stable alterations in chromatin structures that lead to altered gene expressions without changing the sequence of the DNA. These epigenetic modifications are known to be heritable and can be modified by the environment and outside factors (Stankiewicz, Swiergiel et al. 2013, Sun, Kennedy et al. 2013). For example, it is shown that the identical twins have a number of epigenetic differences occurring throughout their lives causing their behavioral differences (Stankiewicz, Swiergiel et al. 2013). Epigenetic changes can start developing early in life, can induce persistent changes in gene expression and cause behavioral differences through adulthood (Sun, Kennedy et al. 2013).

Epigenetic changes include DNA methylation or histone modifications that alter gene expression without changing the genetic code. Some commonly studied histone modifications are acetylation, phosphorylation and methylation and less studied modifications include crotonylation, ADP ribosylation, ubiquitination and SUMOylation. These modifications are usually not isolated and more than one modification is coregulated (Tushir and Akbarian 2014).

Chromatin is a complex structure composed of DNA, RNA and proteins formed to package ~2m DNA in the ~10μm diameter nucleus and to control gene expression and DNA replication. 147bp of DNA is super-helically wrapped around the octamer of histones, H2A, H2B, H3 and H4 to form the nucleosome, the core structure of chromosome. Also another histone molecule, H1 is used as a linker protein to pack the nucleosomes together to form a higher order structure. Chromatin is normally in the tight state when there is no gene transcription and through different histone modifications, it

can switch to the relaxed state to turn on gene transcription. Histones can be post-transcriptionally modified on their N-terminal tails via the modifications described above. These modifications can alter the chromatin structures either through a change in their electrical charges or by serving as recognition sites for transcription factors and enzymes to turn the gene transcriptions on/off (Stankiewicz, Swiergiel et al. 2013, Sun, Kennedy et al. 2013).

DNA methylation, histone acetylation and histone methylation are the three most commonly studied epigenetic changes that the levels are shown to be altered upon different stress exposures (Tsankova, Renthal et al. 2007, Zannas and West 2014). Even though different histone modifications are usually coregulated to turn the gene expressions on/off, for the purpose of this thesis I will be focusing on the mechanism of histone acetylation. Histone acetylation is usually linked with activation of gene transcription. As seen in figure 1.4, when the lysine residues on the histone tails get acetylated, the chromatin switches to the relaxed state. This is mainly mediated by neutralizing positively charged histones with the negatively charged DNA therefore disturbing the binding, as well as mediating the cofactors and coenzymes easily accessible to DNA to turn on gene transcription (Sun, Kennedy et al. 2013). Histone acetylation is mainly localized within different gene promoters. Two key enzymes play a role in histone acetylation and deacetylation. Histone acetylation is mediated by histone acetyl transferase enzymes (HATs) and histone deacetylation is mediated by histone deacetylase enzymes (HDACs). HATs are classified into type A and type B based on their localization (Selvi and Kundu 2009). HDACs are classified into 4 classes. Class I includes HDAC 1-3 and 8, class II includes HDAC 4-7 and 9, class III are known as

sirtuins and class IV includes HDAC11 (Hildmann, Riester et al. 2007, Selvi and Kundu 2009). HATs and HDACs are not specific to a single histone acetylation and a single lysine residue can be acetylated/deacetylated by multiple HATs or HDACs (Sun, Kennedy et al. 2013).

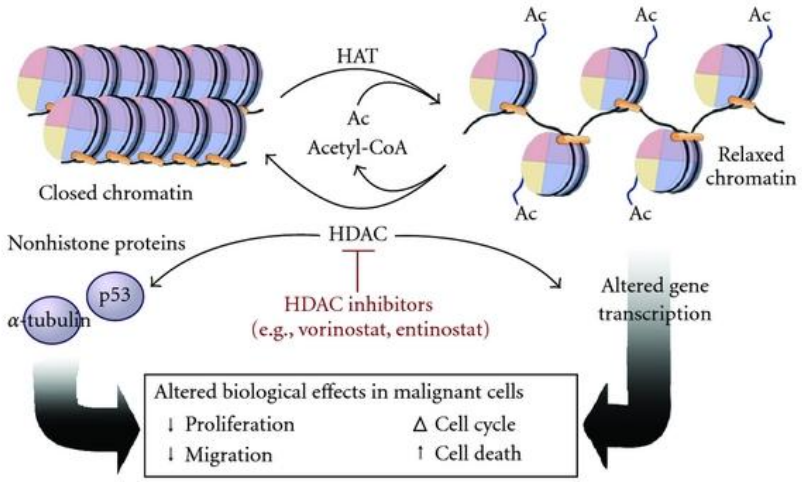

Figure 1.4: Histone acetylation and chromatin structure (Annabelle L. Rodd 2012)

Histone acetylations at different lysine residues have been shown to be altered upon acute and chronic stress exposures both in humans and in rodents in different brain regions. For example following a chronic social defeat stress H3K14ac was decreased in the HC and increased in the NAc of adult mice (Covington, Maze et al. 2009). Also another study showed an increase in acH3K18 and acH4K12 in the ventral HC of adult male rats (Kenworthy, Sengupta et al. 2014). In another study using adult male rats, decreased acH4K12 and phosphorylation H3K9S10 were observed in the DG (Ferland and Schrader 2011). Other studies have reported changes in levels of histone methylation and phosphorylation in different brain regions upon different stress exposures (Hunter,

McCarthy et al. 2009, Covington, Maze et al. 2011, Chiba, Numakawa et al. 2012, Rotllant, Pastor-Ciurana et al. 2013). Furthermore, some of the histone phosphorylation/acetylation changes upon different stress exposures were linked to ERK1/2 activation in the HC (Bilang-Bleuel, Ulbricht et al. 2005, Chandramohan, Droste et al. 2007, Chandramohan, Droste et al. 2008, Ferland, Hawley et al. 2013). Moreover some studies highlighted the specific gene promoters that the histone modifications are taking place on. Some of the most studied genes with the expression changes observed through epigenetic changes include GR (Nr3c1) (Weaver, Cervoni et al. 2004, Szyf, Weaver et al. 2005), CRH (Chen, Evans et al. 2012, Sterrenburg, Gaszner et al. 2012) and BDNF (Roth, Lubin et al. 2009, Keller, Sarchiapone et al. 2010).

Studies have shown that chronic administration of anti depressants can alter different histone acetylations at different brain regions (Lee, Wynder et al. 2006, Tsankova, Berton et al. 2006). Therefore using antidepressants with HDAC inhibitors is shown to have more effective anti-depressant responses (Weaver, Cervoni et al. 2004, Tsankova, Renthal et al. 2007, Covington, Maze et al. 2009). Some of the common HDAC inhibitors used for these studies include sodium butyrate (Yamawaki, Fuchikami et al. 2012), MS-275 (Weaver, Cervoni et al. 2004, Covington, Maze et al. 2009, Covington, Vialou et al. 2011) and Trichostatin A (TSA) (Fleiss, Nilsson et al. 2012, Dagnas, Guillou et al. 2013). However these inhibitors are not specific to a single HDAC and there are still treatment-resistant patients; therefore, more studies have to be done to find distinct molecular mechanisms. Also, majority of these studies focus on adult male mice and rats and there is a need for studies with adolescents and females to better understand the behavioral differences between different ages and sexes.

Chapter 2:

Materials and Methods

2.1 Animals

All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines of Tufts University. All mice were housed in a temperature and humidity-controlled facility on a 12 hour light-dark cycle with food and water ad libitum. All experimental manipulations were performed during light phase and mice were socially housed until a specified period of time prior to the start of behavioral experiments

Wildtype C57Bl/6mice

Male and female C57Bl/6 mice were supplied by our breeding pairs or The Jackson Laboratory. If purchased, they were socially housed until the stress exposures for at least 5 days.

GRF1(-/-) mice

Female and male *Ras-Grf1* homozygous knockout (GRF1(-/-)) mice generated as described previously (Giese, Friedman et al. 2001) and backcrossed onto a C57BL/6J background for more than 10 generations were used. Mice were maintained as described above.

GRF1/GRF2(-/-) (DBKO) mice

Female and male *Ras-GRF1/Ras-GRF2* homozygous knockout (DBKO) mice generated as described previously and backcrossed into a mixed C57BL/6jx129 background for more than 10 generations were used. Mice were maintained as described above.

2.2 Restraint Stress

Restraint stress was performed as described in previous studies (Rademacher, Meier et al. 2008, MacKenzie and Maguire 2015). Since the GRF1(-/-) mice were smaller in size, using 50 ml falcon tubes did not have the same effect on WT and GRF1(-/-) mice; therefore, I used plastic restrainer bags. Stressed mice were placed in plastic restrainer bags for 30 min/day for up to 7 consecutive days. Restrainer bags were cut on the small end to allow the noses to poke through for air and the back of the bag was tied with a twist tie around the tail to prevent escape and movement of mice. In contrast, unstressed control mice remained in their home cage and handled once a day during the other mice are being stressed. All mice are socially housed in their home cages (at most 4 mice in a cage). Single housing is strictly forbidden since that causes a stress on them as well. Immediately after the last stress exposure, mice were either used in behavioral tests or decapitated for blood corticosterone measurements. Stress procedures were performed between 1200 hr and 1500 hr.

2.3 Corticosterone Measurements

Stressed and control mice were decapitated and trunk blood was collected individually in 1.5ml EDTA-coated microcentrifuge tubes (Fisher Scientific). All blood samples were maintained on ice and centrifuged at 6000 rpm for 15 min at 4°C. Plasma was separated and stored at -80°C until analysis. Before measuring the corticosterone levels, blood samples are thawed on ice and diluted 1:20 in sterile saline solution (Teknova). Corticosterone levels in plasma were measured using a Corticosterone EIA Kit (Enzo

Life Sciences, Farmingdale, NY), according to the manufacturer's instructions. The intra-assay variability ranged from 6.6% to 8.0%, inter-assay variability ranged from 7.8% to 13.1% and mean assay sensitivity was 26.99 pg/ml. Stressed mice were decapitated immediately after the last stress exposure and none of the stressed or control mice were submitted to any behavioral tests before. All blood samples were collected during lights-on period between 1100 hr and 1500 hr.

2.4 CRH injections

150 µg/kg of CRH (Sigma) or Saline (Teknova) solutions were injected intraperitoneally between 1000hr and 1100hr. After injections mice were kept in their home cages for 1 hour and trunk blood was collected for CORT measurements.

2.5 Behavioral Paradigms

Elevated Plus Maze Test

The elevated plus maze test was performed as explained previously (Saavedra-Rodriguez and Feig 2013). The mice were transferred in their cages from the housing room to the behavior core with the elevated plus maze chambers. Before behavioral experiments, mice were kept in the behavior room for ~1 hour for habituation. Mice were placed in the center of a plus-shaped maze elevated 40 cm from the ground, composed of two open and two closed arms, each 35.5cm long and 5cm wide (Campden Instruments Ltd, Lafayette, IN). General mouse activity was analyzed for 5 min and the percent time spent in the

open arms was recorded using the Motor Monitor software (Campden Instruments Ltd, Lafayette, IN).

Open Field Test

The open field test was performed as explained before (Saavedra-Rodriguez and Feig 2013). The mice were transferred in their cages from the housing room to behavior core with the open field chambers. Before behavioral experiments, mice were kept in the behavior room for ~1 hour for habituation. Mice were placed in a 16 x 16 inch open arena connected to an activity frame (Campden Instruments Ltd, Lafayette, IN) and allowed to move under even illumination for 5 min. General locomotor activity was analyzed and total distance was recorded using the Motor Monitor software (Campden Instruments Ltd, Lafayette, IN).

2.6 Tissue Preparation and Western Blot

Immediately after the last stress exposure, mice were anesthetized with isoflurane in a separate cage and decapitated using a guillotine. CA1 and CA3/DG of HC were dissected. ~2 CA1 regions were transferred to 1.5 ml eppendorf tubes and immediately frozen on dry ice. Tissues were stored in -80 until further use. For total protein extraction, tissues were removed from -80 freezer and kept on dry ice to prevent from thawing. Tissues were immediately lysed with 200ul (for 2 CA1 regions) lysis buffer using a bio vortexer. Tissues are always kept on ice after this step to prevent protein degradation. Tissue lysates were spun down for 15 min at 14,000xrpm at 4°C in cold room. Protein concentrations were measured using BCA assay kit (Thermofisher). Protein lysates were

run on a gel immediately after lysis and the remaining lysates were stored in -80 freezer. Equal amounts of protein were mixed with 4X SDS loading buffer and were boiled for 5 min at 95°C. Western blotting was performed according to standard protocols. Briefly, protein lysates were run on 15% SDS-PAGE gels. SDS-PAGE gels were then transferred to PVDF membrane at 30V for 70min, blocked in 5% milk in PBS-T for 1 hour at room temperature. Membranes were then incubated with primary antibodies in 5%BSA/PBS-T overnight at 4°C. Next day, followed by a secondary antibody incubation for ~1 hour at room temperature. ECL Western blotting substrate (Thermofisher) was used to develop the signals on film (Denville Scientific). For histone blots, membranes were not stripped since histone proteins are very small in size and they may be lost from the membrane throughout stripping process.

2.7 Antibodies

The following antibodies were used: acH3K9 (1:2000) rabbit mAb, acH3K18 (1:1000) rabbit mAb and total H3 (1:6000) rabbit mAb for primary antibodies (Cell Signaling Technologies). Goat anti-rabbit and goat anti-mouse (Jackson Immunoresearch) for secondary antibodies.

2.8 Stereotaxic Surgery for GRF1 re-expression in CA1

Pn 24 day GRF1(-/-) mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). Once anesthetized, each mouse was placed in a stereotactic frame (myNeuroLab, St. Louis, MO). A surgical incision was made along the midline of the head to expose the skull. Two holes were made in the skull overlying the hippocampus. Coordinates for CA1 injection into the mice were: 2.5-mm posterior to Bregma, _2.5-mm lateral from the midline, and 1.75-mm below the surface of the skull. Injections were performed with a 10-ul Hamilton syringe fitted with a custom made blunt-ended 30-gauge needle (Hamilton). Each injection consisted of 1 µl of adenovirus expressing various GRF proteins infused at a rate of 0.06 µl/min. An infusion pump controlling the plunger on the Hamilton syringe precisely regulated the rate of injection. The needle was then left in place for 8 min prior to withdrawal from the brain. The stress paradigm began 7–11 days after stereotactic injection of the viral vectors, a date chosen to provide recovery time for mice and exogenous GRF proteins accumulate to levels normally found for endogenous proteins in WT animals around 7 days.

2.9 Statistical Analysis

All the statistical analyses were performed using Prism 5 software (GraphPad Prism Software). Data are presented as means +/- SEM. Two way ANOVA tests were used to analyze the differences between stressed and control groups for behavioral analyses, CORT measurements and histone western blots. Post hoc multiple comparisons using Bonferroni's correction were performed. For all comparisons, values of $P<0.05$ were considered as the level of statistical significance.

Chapter 3:

Early-Adolescence and Female Specificity of RasGRF1 Function in HPA Axis Regulation

3.1 Generation of a chronic restraint stress response in WT and GRF1(-/-) mice

GRF1 and GRF2 play critical roles in synaptic plasticity in CA1 region of the hippocampus (HC). Because the HC is also known to regulate the HPA axis, I investigated the role of GRF1 in the stress response. For this purpose I chose a simple and well-characterized stress paradigm, restraint stress for 30 min/day for up to 7 consecutive days (Rademacher, Meier et al. 2008, MacKenzie and Maguire 2015). I used an animal-wide GRF1 knockout (GRF1(-/-)) mouse in the C57Bl6/j background and compared the results to their WT C57BL6/j counterparts.

To begin to test whether GRF1 contributes to HPA axis response to chronic stress, early adolescent (pn 28) and adult (2 months), WT and GRF1(-/-) mice were submitted daily to 30 min of restraint stress for seven days. Age and sex differences have been implicated after different stress exposures; therefore, I separated different groups of mice before testing. One indication of a chronic stress response in mice is a decrease in weight gain throughout stress exposure. In order to be sure all mice were exposed to stress similarly, I measured their weight over time. Adolescent female (Figure 3.1A), adult female (Figure 3.1B) and adolescent male (Figure 3.1C) WT and GRF1(-/-) mice all exhibited a decrease in weight gain after 7 days of restraint stress compared to unstressed controls (Figure 3.1, A-C) indicating they all experienced stress. GRF1(-/-) males appeared to respond more strongly to this paradigm but as shown below they showed no HPA axis abnormalities.

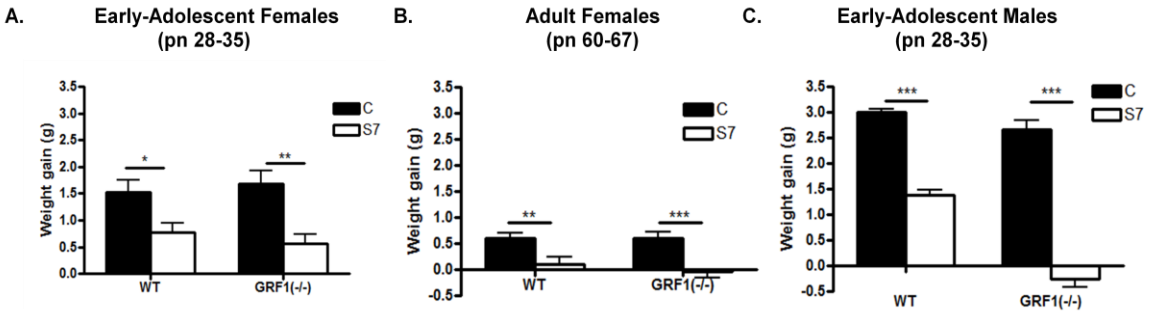

Figure 3.1: Similar suppression of weight gain in WT and GRF1(-/-) mice after 7 days of chronic restraint stress. Early-adolescent (pn 28) and adult (pn 60) WT and GRF1(-/-) mice were subjected to restraint stress (30min/day) for 7 days and total weight was then measured (on pn 35). (A) In early adolescent females stress had a significant effect on weight gain in both WT and GRF1(-/-) mice (WTC n=8, WTS n=9, GRF1(-/-)C n=8, WTS n=9); ($F_{(2,30)}$=20.21, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress, t=2.528, p<0.05; GRF1(-/-) control vs. stress, t=3.829, p<0.01). (B) In adult females stress had a significant effect in weight gain in both WT and GRF1(-/-) mice (n=9 for each group); ($F_{(2,32)}$=24.46, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress, t=3.093, p<0.01; GRF1(-/-) control vs. stress, t=3.900, p<0.001). (C) In early-adolescent males stress had a significant effect in weight gain in WT and GRF1(-/-) mice (n=5 for each group); ($F_{(2,16)}$=280.05, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress t=8.445, p<0.001; GRF1(-/-) control vs. stress t=15.22, p<0.001).

3.2 Early-adolescent female GRF1(-/-) mice have a blocked HPA response to chronic restraint stress

To test for the role of GRF1 in the HPA axis response to chronic stress, immediately after the last of seven daily 30 min/day restraint stress exposures, trunk blood CORT levels were compared in WT and GRF1(-/-) mice. I began with early adolescent animals because this is the time when GRF proteins begin to contribute to synaptic plasticity (Li, Tian et al. 2006). I also began my studies with females. As expected (Rademacher, Meier et al. 2008), CORT levels rose ~5-fold in early-adolescent female WT mice (Figure 3.2A, left panel). Strikingly, although baseline CORT levels in early-adolescent female GRF1(-/-) mice were similar to their WT counterparts, stress-induced increase in CORT observed in WT mice was completely blocked in GRF1(-/-) mice (Figure 3.2A, right panel)

To determine whether this altered HPA axis output in GRF1(-/-) mice was reflected in altered behavior, early-adolescent female WT and GRF1(-/-) mice were tested for several anxiety-related behavioral assays known to be affected by restraint stress immediately after the last stress exposure. In the Elevated Plus Maze (EPM), stressed early-adolescent female WT mice spent more time in the open arms than unstressed mice. This indicates decreased anxiety, which is counter to what might be expected. However, this has been observed by others studying adolescent mice and has been characterized as a stress induced increase in "risk-taking" behavior (Figure 3.2B, left panel) (Strekalova, Spanagel et al. 2005, Toledo-Rodriguez and Sandi 2011). Consistent with their blocked CORT response, this behavior was also completely blocked in GRF1(-/-) mice (Figure 3.2B, right panel).

In the Open Field Test (OFT), an assay for locomotion and anxiety, stressed WT mice displayed an increase in total distance travelled compared to the unstressed group indicative of hyperactive behavior (Marin, Cruz et al. 2007) (Figure 3.2C, left panel). This data can also explain the EPM behavior such that the hyperlocomotion may be masking the anxiety related behaviors and the observed increased risk taking behavior is also due to increased locomotion. Similar to the EPM data, this increase in locomotor behavior was also blocked in stressed adolescent female GRF1(-/-) mice (Figure 3.2C, right panel). These results indicate that, even though early-adolescent female GRF1(-/-) mice were clearly stressed as evidenced by a decrease in weight gain (Figure 3.1A), they failed to mount an HPA axis response to chronic restraint stress evidenced by elevated CORT levels and enhanced locomotion.

CNS Role for GRF1 in Regulating HPA Axis Response to Chronic Stress

Because we used an animal-wide knockout mouse, GRF1 could influence the HPA axis through its expression in any of the components of the HPA axis or brain regions that control it. However, GRF1 expression is undetectable in the pituitary or adrenal glands suggesting it plays a role in one or more regions of the CNS. To confirm this hypothesis, we tested for normal HPA axis function downstream of the hypothalamus by injecting CRH into GRF1(-/-) mice and measuring CORT output. Either saline (SAL) or 150µg/kg of CRH (CRH) solutions were injected intraperitoneally to early-adolescent WT and GRF1(-/-) females and 1 hour later trunk blood was collected for CORT measurements. CRH injections lead to an ~2.5 fold increase in CORT levels in both WT

mice (Figure 3.2D, left panel) and GRF1(-/-) mice (Figure 3.2D, right panel) suggesting that GRF1(-/-) mice can respond to CRH treatment with an intact HPA axis by an increased CORT secretion. Next, GRF1(-/-) mice were submitted to 6 days of restraint stress and on day 7, instead of subjecting mice to another restraint stress, the mice were injected with CRH. This increased the CORT response in GRF1(-/-) mice to levels comparable to WT mice exposed to 7 days of stress (Figure 3.2E). These findings confirm GRF1 in the CNS is responsible for regulating the HPA axis response to chronic restraint stress.

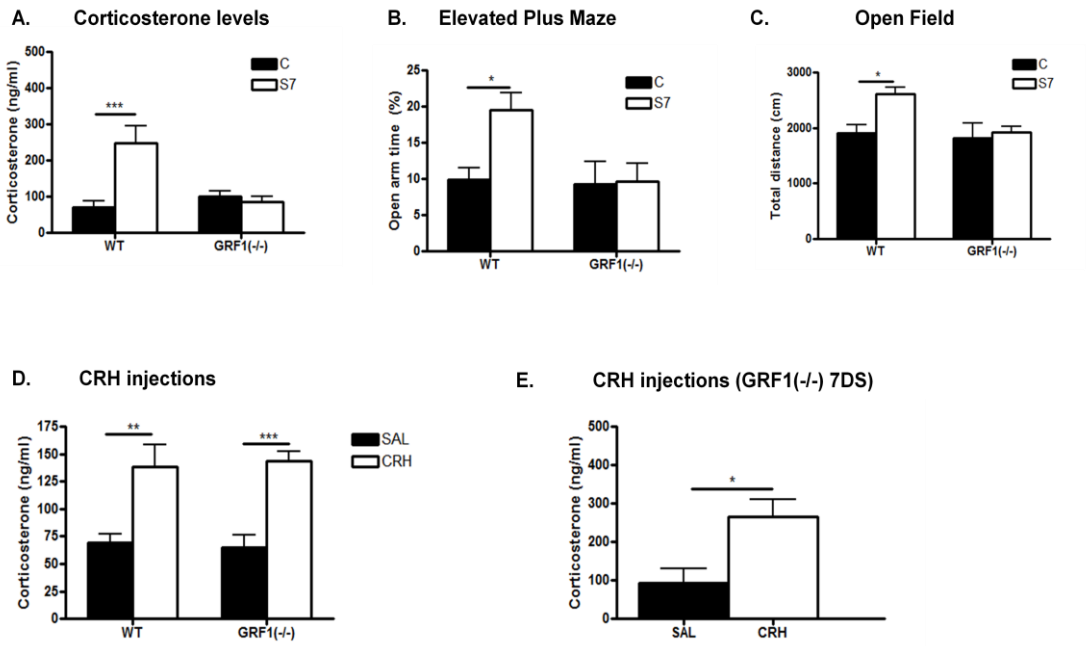

Figure 3.2: Failure of early-adolescent female GRF1(-/-) mice to generate a stress response after 7 days of chronic restraint stress. Early-adolescent female WT and GRF1(-/-) mice (pn 28) were subjected to 7 days of chronic restraint stress. Circulating CORT levels were measured or mice were submitted to several behavioral assays for evaluation of anxiety-like phenotype and locomotor behaviors on pn 35. (A) WT mice displayed an increase in CORT levels after stress exposure compared to unstressed counterparts whereas GRF1(-/-) mice displayed blocked CORT response (n=6 in each group); ($F_{(2,20)}$=7.94, p=0.0106; Bonferroni post-hoc tests for WT control vs. stress t=4.381, p<0.001); GRF1(-/-) control vs. stress t=0.395, p>0.05). (B) In the elevated plus maze, stressed WT mice spent more time in the open arms compared to unstressed counterparts and stressed GRF1(-/-) mice showed no change in EPM behavior (WTC n=8 and WTS7 n=6, GRF1C n=8 and GRF1S7 n=8) ($F_{(2,26)}$=3.832, p=0.0611; Bonferroni post-hoc tests WT control vs. stress t=2.560, p<0.05; GRF1(-/-) control vs. stress (t=0.116, p>0.05). (C) In the open-field test, stressed WT mice travel more distance in the open field and stressed GRF1(-/-) mice show no change in the total distance travelled (WTC n=8 and WTS7 n=6, GRF1C n=8 and GRF1S7 n=8) ($F_{(2,26)}$=5.138, p=0.0320; Bonferroni post-hoc tests for WT control vs. stress t=2.663, p<0.05; GRF1(-/-) control vs. stress (t=0.4602, p>0.05). (D) 150µg/kg of CRH was injected intraperitoneally to WT and GRF1(-/-) mice. Both of the groups showed a significant increase in CORT secretion upon CRH injection (WT-SAL n=5, WT-CRH n=5, GRF1-SAL n=5, GRF1-CRH n=6) ($F_{(2,17)}$=65.85, p<0.0001: Bonferroni post-hoc tests for WT SAL vs. CRH t=3.773, p<0.01; GRF1(-/-) SAL vs. CRH t=4.447, p<0.001). (E) Early-adolescent GRF1(-/-) females were submitted to 6 days of restraint stress and on day 7 one dose of CRH was injected to determine CORT levels. Secreted CORT was increased significantly in GRF1(-/-) mice (SAL n=3, CRH n=5) (t_6=2.603, p=0.0405).

3.3 Role of GRF1 in HPA axis response to chronic restraint stress is age-dependent

The role of GRF1 in synaptic plasticity in the hippocampus begins during early-adolescence and continues through adulthood. To determine whether GRF1 contribution to the HPA axis response to stress also continues through adulthood, adult (pn 60) female WT and GRF1(-/-) mice were submitted to seven days of chronic restraint stress, and then CORT levels and anxiety-related behaviors were measured as described above.

WT adult female mice displayed an increase in CORT levels (Figure 3.3A, left panel) that was similar to that observed in early-adolescent female mice (Figure 3.2A). Remarkably, unlike their early-adolescent counterparts, stressed adult female GRF1(-/-) mice showed a rise in CORT levels similar to that found in WT mice (Figure 3.3A, right panel). Similarly, stressed adult female WT and GRF1(-/-) mice showed comparable increases in the time they spent in the open arms of the EPM (Figure 3.3B). Also, stressed adult female WT and GRF1(-/-) mice showed similar increases in distance traveled in the OFT, compared to unstressed counterparts (Figure 3.3C).

To better define the developmental period when the HPA axis becomes independent of GRF1, I repeated the experiments described above with mice in mid-adolescence (pn 35) such that the CORT measurements were performed seven days later (pn 42). Remarkably, these mid-adolescent female GRF1(-/-) mice displayed a CORT response to seven days of restraint stress that was comparable to that of WT mice (Figure 3.3D). Thus, the HPA axis in female mice transitions from GRF1-dependent to GRF1-independent during a ~1-week period when mice transition from early to mid-adolescence.

The time period between early to mid-adolescence when HPA axis becomes independent of GRF1 is when C57Bl/6j mice enter puberty. To determine if the rise in estrogen by puberty plays an active role in our stress paradigm, early adolescent WT and GRF1(-/-) females were ovariectomized (OVX) (Beverly Rubin, Tufts University) and submitted to 7 days of restraint stress when they reach 2 months of age. Figure 3.3E shows that OVX-GRF1 mice display similar CORT increase compared to non-ovariectomized counterparts in response to stress. This result suggests that the rise in estrogen does not play a role in this stress paradigm.

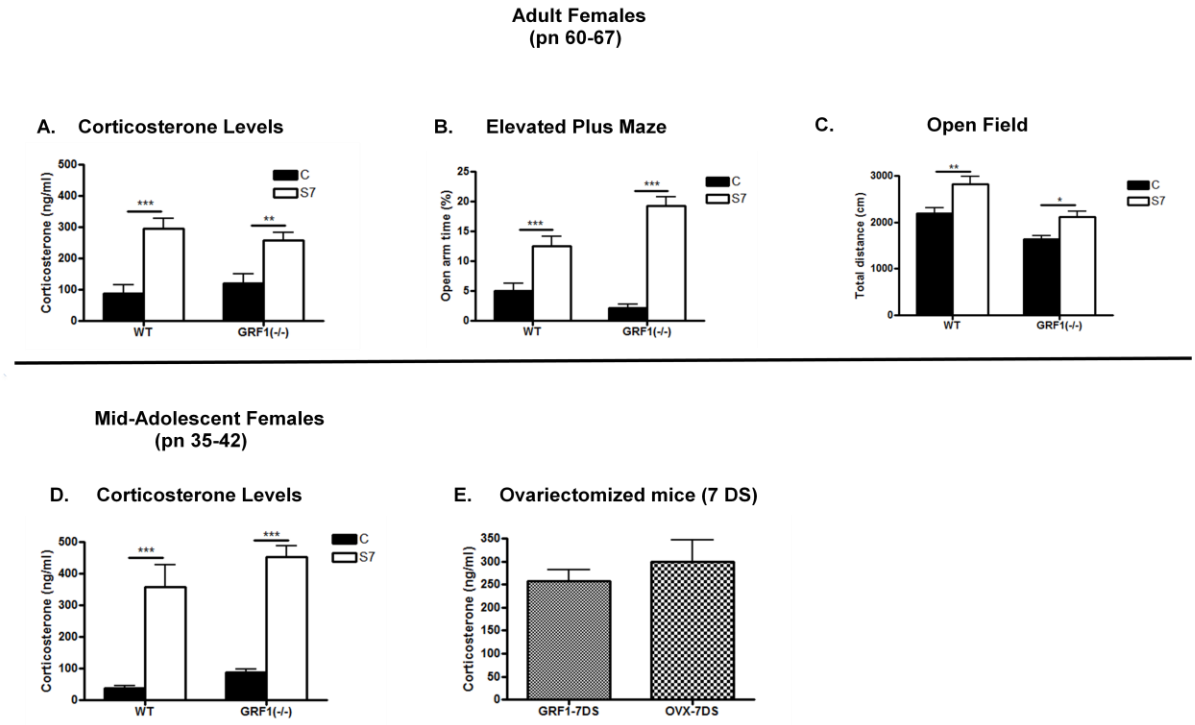

Figure 3.3: Normal chronic restraint stress response in mid-adolescent and adult female GRF1(-/-) mice. Mice were submitted to 7 days of chronic restraint stress. CORT levels were measured or anxiety-related behaviors were tested. (A) Stressed WT and GRF1(-/-) adult (pn 60) females displayed a similar increase in their circulating CORT levels compared to unstressed counterparts (n=5 for each group) ($F_{(2,16)}$=35.76, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress t=5.073, p<0.001; GRF1(-/-) control vs. stress t=3.384, p<0.01). (B) In EPM, adult female WT mice spent significantly more time in the open arms after stress exposure. A similar increase in the open arm time was observed for stressed GRF1(-/-) females compared to their unstressed counterparts (n=8 for each group) ($F_{(2,28)}$=88.57, p<0.0001); Bonferroni post-hoc tests for WT control vs. stress t=4.074, p<0.001; GRF1(-/-) control vs. stress t=9.235, p<0.001). (C) In OFT, stressed WT and stressed GRF1(-/-) adult females showed a significant increase in the total distance travelled compared to their unstressed counterparts (n=6 for each group) ($F_{(2,20)}$=19.92, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress t=3.617, p<0.01; GRF1(-/-) control vs. stress t=2.696, p<0.05). (D) Stressed mid-adolescent (pn 35) WT (control n=5, stress n=3) and GRF1(-/-) (control n=5, stress n=5) females displayed a significant increase in circulating CORT levels compared to their unstressed counterparts ($F_{(2,14)}$=117.8, p<0.0001); Bonferroni post-hoc tests for WT control vs. stress t=6.709, p<0.001; GRF1(-/-) control vs. stress t=8.831, p<0.001). (E) GRF1(-/-) mice and OVX-GRF1(-/-) show a similar CORT response upon 7 days of stress exposure. (GRF1 n=4, OVX n=3; t_5=0.5711, p=0.5927).

3.4 Role of GRF1 in HPA axis response to chronic restraint stress is sex-dependent

To determine whether GRF1 also contributes to HPA axis response to chronic restraint stress in males, early-adolescent and adult male WT and GRF1(-/-) mice were submitted to seven days of 30 min/day restraint stress. Like their female counterparts, WT male mice displayed a ~5-fold increase in CORT levels after the seventh exposure to stress (Figure 4.4A, left panel). In contrast to their female counterparts, early-adolescent male GRF1(-/-) mice displayed a CORT response similar to WT mice (Figure 3.4A, right side). Adult male GRF1(-/-) mice also showed a normal CORT response (Figure 3.4B). Thus, not only the role of GRF1 in regulating the HPA axis is age-dependent, it is also sex-dependent, such that it represents a distinct regulator only in early-adolescent females.

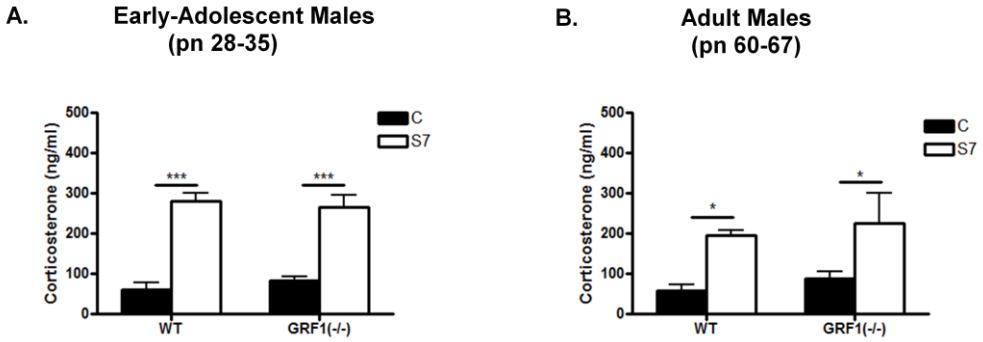

Figure 3.4: Normal chronic restraint stress response in early-adolescent male and adult male GRF1(-/-) mice. Mice were submitted to 7 days of chronic restraint stress and CORT levels were then measured. (A) Early-adolescent male WT mice showed significantly increased CORT levels upon stress exposure compared to unstressed counterparts (WT control n=5, stress n=2). A similar increase was observed with stressed early-adolescent male GRF1(-/-) males (GRF1(-/-) control n=5, stress n=3). ($F_{(2,11)}$=94.94, p<0.0001; Bonferroni post-hoc tests for WT control vs. stress t=7.057, p<0.001; GRF1(-/-) control vs. stress t=6.732, p<0.001). (B) Adult WT (control n=3, stress n=4) and GRF1(-/-) (control n=4, stress n=3) males displayed a similar increase in CORT levels after 7 days of stress exposure compared to unstressed counterparts ($F_{(2,10)}$=15.95, p=0.0025; Bonferroni post-hoc tests for WT control vs. stress t=2.831, p<0.05; GRF1(-/-) control vs. stress t=2.816, p<0.05).

3.5 Enhanced short-term HPA response to restraint stress specifically in early-adolescent female GRF1(-/-) mice

To determine whether the results observed after chronic restraint stress are due to the fact that early-adolescent female GRF1(-/-) mice have a completely defective HPA axis, these mice were tested for an acute stress response by measuring CORT levels immediately after a single 30 min exposure to restraint stress. Figure 3.5A (S1) shows this is not the case. While early-adolescent female WT mice displayed a ~2-fold increase in CORT levels after acute restraint stress, their GRF1(-/-) counterparts actually showed an enhanced CORT response reaching a ~4-fold rise. Thus, not only do GRF1(-/-) mice have a functional acute response to restraint stress, it is magnified.

To better understand the transition in the role of GRF1 from normally suppressing the HPA response after 1 stress exposure to promoting it after 7 stress exposures, the consequences of exposure numbers in between were determined. Figure 3.5A shows that WT mice maintained elevated CORT levels (~2-fold) after 3 (S3) and 5 days of daily exposures (S5) to 30 min/day restraint stress and CORT levels peaked (~3-fold) by 7 days of exposure. Moreover, elevated levels of CORT remained at peak levels even after 14 days of restraint stress (data not shown). In contrast, early-adolescent female GRF1(-/-) mice displayed an even more-magnified CORT response (~6-fold) compared to non-stressed mice after 3 days of restraint stress (S3). However, the mice became less responsive (~5-fold) after 5 exposures (S5) and no longer responded after 7 exposures (S7) as described in Figure 3.2 above. These results show that early-adolescent GRF1(-/-) females can, in fact, respond to restraint stress, but that they habituate to it more rapidly than WT mice.

I then repeated this set of experiments on adult female, early-adolescent male and adult male mice to reveal whether defective responses of GRF1(-/-) mice to fewer exposures to restraint stress are also female and early-adolescence specific. Fig. 3.5 B-D shows that this is the case because the response of both of these sets of GRF1(-/-) mice was similar to WT mice. Moreover, when mid-adolescent mice (pn 35) were tested after 3 and 7 days of restraint stress, no significant difference between WT and GRF1(-/-) mice was observed on day 7 (Fig. 3.5 E, S7). However, there was still an enhanced response at 3-days of stress in GRF1(-/-) mice, which might indicate that the transition from GRF1-dependent to GRF1 independent HPA axis in females occurs between 38 and 42 days of age.

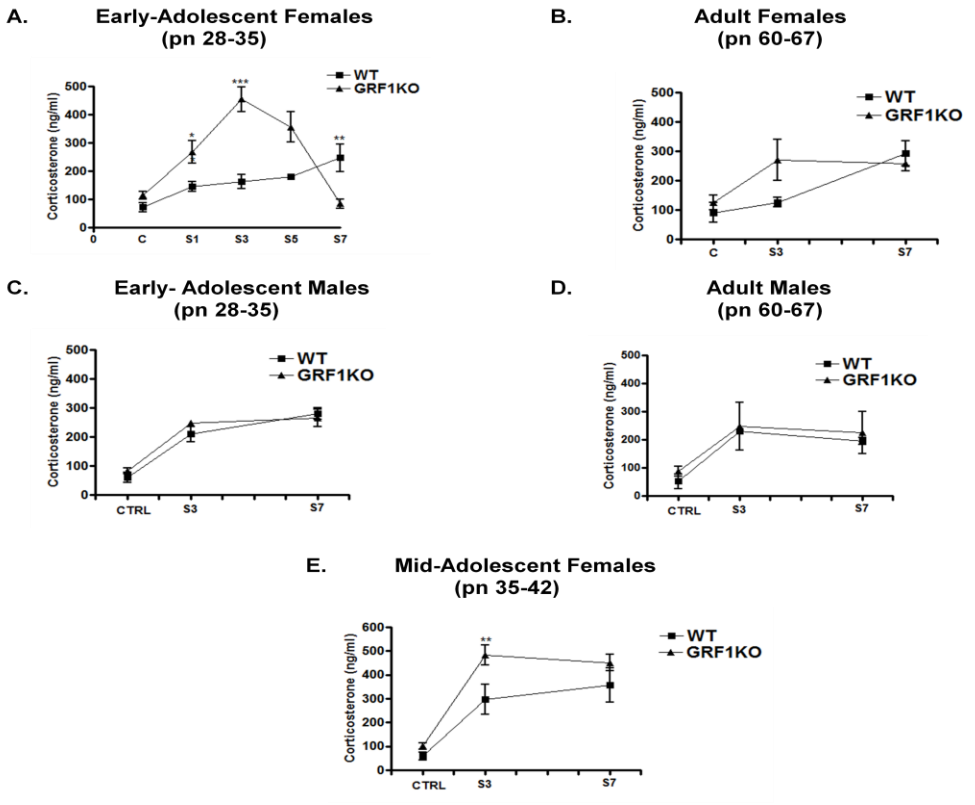

Figure 3.5: Enhanced short-term restraint stress response specifically in early-adolescent female GRF1(-/-) mice. (A) Early-adolescent female WT and GRF1(-/-) mice were submitted to 1, 3, 5 and 7 days of restraint stress (30-min/day) and then CORT levels were determined. GRF1(-/-) mice displayed enhanced short-term stress response compared to WT mice until day 3 followed by a blocked response by day 7. (WT C n=9, S1 n=8, S3 n=9, S5 n=3, S7 n=6; GRF1(-/-) C n=9, S1 n=7, S3 n=10, S5 n=3, S7 n=6). ($F_{(2,60)}$=17.80, p<0.001; Bonferroni post-hoc tests for WT vs. GRF1(-/-), S1 t=2.789, p<0.05; S3 t=7.445, p<0.001, S5 t=2.523, p>0.05, S7 t=3.319, p<0.01). (B) Adult GRF1(-/-) females displayed normal short-term restraint stress response on day 3 compared to WT counterparts (WT C n=6, S3=2, S7=4; GRF1(-/-) C n=6, S3 n=2, S7 n=5) ($F_{(2,19)}$=1.767, p=0.1995; Bonferroni post-hoc tests WT vs. GRF1(-/-), S3 t=1.951, p>0.05; S7 t=0.7105, p>0.05). (C) Early-Adolescent GRF1(-/-) males displayed normal short-term restraint stress response on day 3 of restraint stress exposure compared to their WT counterparts (WT C n=5, S3 n=3, S7 n=2; GRF1(-/-) C n=5, S3 n=2, S7 n=3) ($F_{(2,14)}$=0.7160, p=0.4117; Bonferroni post-hoc tests for WT vs. GRF1(-/-) S3 t=1.143, p>0.05; S7 t=0.4429, p>0.05). (D) Adult GRF1(-/-) males also displayed normal short-term restraint stress response on day 3 of restraint stress exposure compared to their WT counterparts (WT C n=3, S3 n=2, S7 n=4; GRF1(-/-) C n=4, S3 n=3, S7 n=3) ($F_{(2,13)}$=0.4732, p=0.5036; Bonferroni post-hoc tests for WT vs. GRF1(-/-) S3, t=0.2393 p>0.05, S7 t=0.480, p>0.05). (E) Late-adolescent (pn 35) WT and GRF1(-/-) females were exposed to 3 and 7 days of restraint stress. Both displayed normal stress behavior on day 7 (pn 42) CTRL. However, on day 3, late-adolescent GRF1(-/-) showed enhanced stress response (WT C n=7, S3 n=3, S7 n=3; GRF1(-/-) C n=7, S3 n=4, S7 n=5) ($F_{(2,23)}$=14.21 p=0.001; Bonferroni post-hoc tests for WT vs. GRF1(-/-) S3 t=3.376, p<0.01' S7 t=1.800, p>0.05).

3.6 Adolescent and male role for RasGRF2 in stress response

GRF1 and GRF2 play distinct roles in synaptic plasticity in CA1 of HC. After observing the early-adolescent and female specific role for GRF1 in restraint stress response, the next aim was to determine whether RasGRF2 played a role in HPA regulation as well. Until I could get enough GRF2(-/-) mice, I used GRF1/GRF2(-/-) (DBKO) mice with the same stress paradigm to compare the results to GRF1(-/-) mice. I started with adolescent male mice since GRF1 does not play a role in males. Figure 3.6 shows that adolescent DBKO male mice showed a normal CORT response to 3 days of (short-term) restraint stress response. However, by 7 days adolescent male DBKO mice, like adolescent female GRF1(-/-) mice no longer responded to stress. This data suggests that GRF2 can also suppress habituation to stress, but unlike GRF1, it does it in males. Whether is also functions in females, we will have to await testing GRF2(-/-) mice in the future.

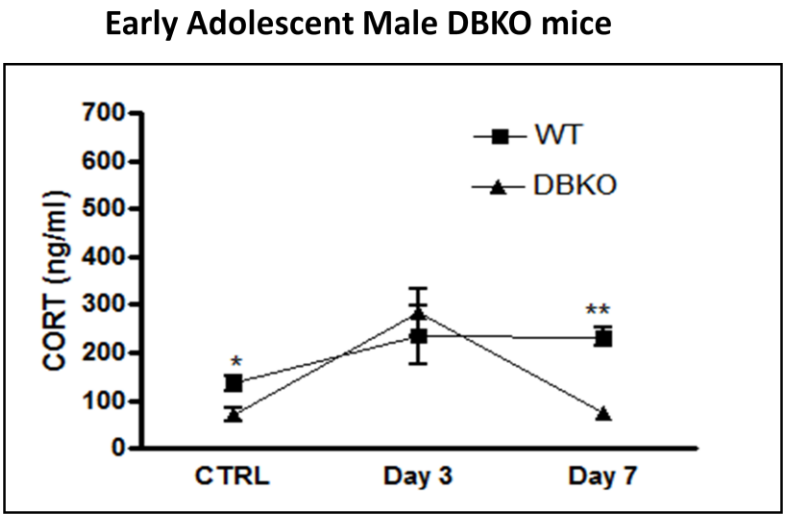

Figure 3.6: Early adolescent male DBKO mice show a normal CORT response to short-term stress but block CORT. Adolescent male DBKO mice are submitted to 3 days and 7 days of stress and CORT is measured immediately after the last stress exposures. DBKO mice show a similar rise in CORT levels after 3 days of stress compared to WT mice (t_5=0.5372, p=0.6141) and they show a blocked HPA response to 7 days of chronic restraint stress compared to WT mice (t_4=5.429, p=0.0056).

Chapter 4:

Role of Hippocampus RasGRF1 in Regulating the HPA Axis Response to Short-term Stress

4.1 Restoration of GRF1 expression in the CA1 of GRF1KO mice restores normal HPA axis response to short term restraint stress

The fact that GRF1(-/-) mice display a complex phenotype with an enhanced HPA axis response after short-term stress and yet blocked HPA axis response after chronic restraint stress suggests that the phenotype reflects the combination of defects due to the loss of GRF1 in multiple brain regions.

We propose that the enhanced short-term response is due to a defect in one of the negative feedback mechanisms known to regulate the HPA axis. We also hypothesize that this is over-ridden after 7 repeated stress experiences by a function of GRF1 in a different region of the brain involved in habituation. Since the hippocampus is one of the key regulators of negative feedback on the HPA axis and our lab has showed previously that GRF1 regulates synaptic plasticity there, I started to focus on the hippocampus. Our lab has previously published that (Jin, 2014) we can reconstitute all of the known synaptic plasticity functions of GRF1 in GRF1(-/-) mice by stereotactic injection of adenoviruses expressing GRF1 into the CA1 region of the hippocampus. We also described a GRF1 mutant, (PCQ_2)GRF1, that expresses at the same level as WT GRF1 but has none of the synaptic plasticity functions of GRF1 (see Fig 1 and Table 1 from (Jin, 2014)). Thus, Shan-Xue Jin in the lab injected the CA1 of GRF1(-/-) mice with virus expressing either WT (GRF1) or inactive GRF1 ((PCQ_2)GRF1) and we waited for 7 days, a period we showed previously allowed both forms of GRF1 to restore GRF1 expression to exogenous levels. Then I exposed the injected mice to 3 days of restraint stress. Immediately after the last exposure, I measured the CORT levels. Fig 4.1 shows that CORT levels in GRF1(-/-) mice reconstituted with a catalytically dead GRF1 mutant

(second bar) were comparable to those uninjected stressed GRF1(-/-) (first bar) mice and still elevated compared to stressed WT mice (fourth bar). However, stressed GRF1(-/-) mice reconstituted with functional GRF1 (third bar) displayed a CORT response that was back to that found in stressed WT mice. These findings show that the CA1 hippocampus is the main site where GRF1 regulates the HPA axis response to short-term stress.

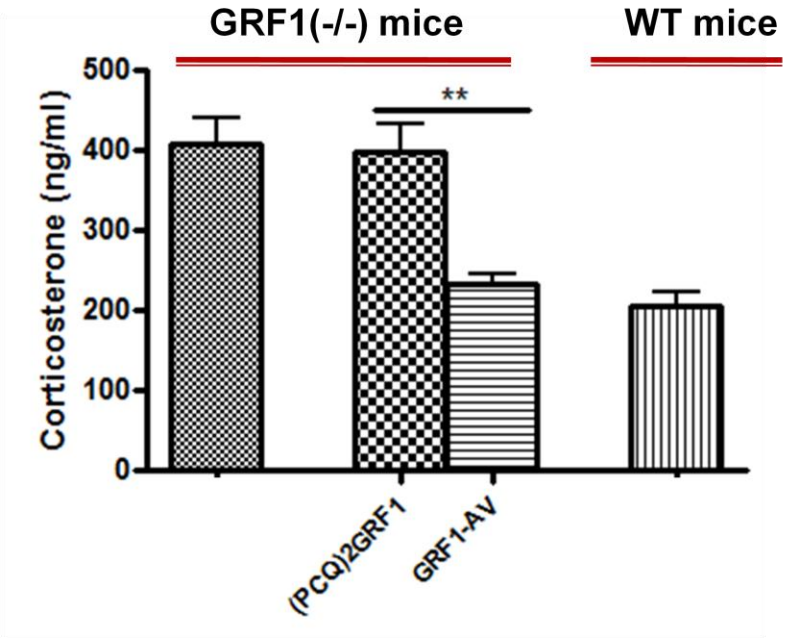

Figure 4.1: Restoration of CA1 GRF1 can rescue the short-term HPA defects. Stereotaxic GRF1-AV surgery was performed on GRF1(-/-) females ~pn 24 to re-express GRF1 in CA1. After 7-10 days of recovery time mice were submitted to 3 days of restraint stress and trunk blood was collected for CORT measurements. (A) Re-expression of GRF1 in CA1 of hippocampus (right panel) compared to SCRM controls (left panel). (B) CORT levels of GRF1 restored mice was at least partially reduced to the levels of WT mice after the same stress paradigm, n=5 for each group (t_8= 4.395, p=0.0023).

4.2 Phosphoproteomic screen to detect GRF1 dependent changes in the activity of components of signaling networks that control the HPA in response to short-term stress.

We hypothesize that GRF1 controls the HPA axis by altering the activity and/or expression of key proteins that regulate neuron function in the hippocampus to influence negative feedback on the HPA axis. Because in many cases, changes in activities of proteins can be regulated by their level of phosphorylation, we decided to screen for stress-induced changes in phospho-proteins that are regulated by GRF1. One advantage of this approach is that we may identify not only novel proteins involved in HPA axis regulation but also novel regulatory sites on them that control the HPA axis specifically in early adolescent females. Thus, we set up a screen to identify stress-induced phosphopeptides that are dependent on the presence of GRF1. In particular, WT and GRF1(-/-) early-adolescent female mice were exposed to 3 days of restraint stress (30 min/day), which yields a ~2-fold increase CORT response in WT mice, but a ~8-fold increase in GRF1(-/-) mice (see Fig 3.5). The CA1 regions of the 12 hippocampi from control or stressed, WT or GRF1(-/-) mice were dissected. Lysates were enriched for phosphopeptides by our collaborator Charles Farnsworth at Cell Signaling Technologies using IMAC (Fe^{3+}) columns. Phosphopeptides were quantified by LC-MS/MS Analysis using Orbitrap-Elite, ESI-CID and proteins were identified by Sorcerer. Label-free quantitation from phosphorylated peptide intensities was performed twice for each sample. MS/MS spectra were evaluated using SEQUEST. Comparisons were made between stressed vs control for each peptide from WT and GRF1(-/-) mice.

The vast majority of the peptides (>98.4%) did not change significantly (<2-fold) in intensity in either direction in stressed vs. unstressed WT mice, demonstrating the high accuracy of this technique. A striking difference between WT and GRF1(-/-) adolescent female mice was that while only ~1% of peptides changed significantly (either up or down) in WT mice after 3 days of restraint stress, ~10% did so in GRF1(-/-) mice. The majority of changes were in the positive direction, but many were in the negative direction. These findings are displayed in a scatter plot where each circle represents the relative abundance of a phospho-peptide between the two conditions with colored ones having >2-fold changes. Importantly, overall baseline phosphorylations of peptides in WT and GRF1(-/-) mice were not significantly different in most altered peptides, so the increases and decreases in p-peptide intensities reflects enhanced stress sensitivity of proteins in GRF1(-/-) mice. Moreover, the phosphopeptide changes occurred across a broad spectrum of protein types including, actin-binding proteins, scaffolds, kinases, cyclases, trancription factors, GAPs, GEFs, integral membrane proteins and proteases.

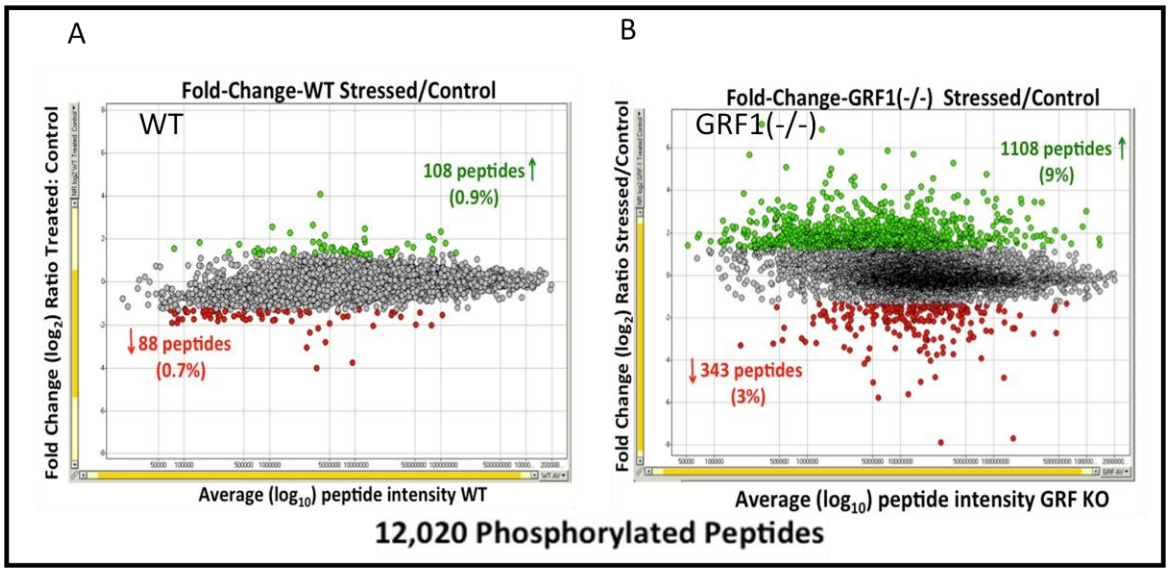

Figure 4.2: Scatter plot for the phosphorylated peptides after 3 days of restraint stress. Early-adolescent and female WT and GRF1(-/-) mice were submitted to 3 days of restraint stress. Phosphorylated peptides with >2 fold change in response are plotted in a scatter plot in A. WT mice and B. GRF1(-/-) mice.

One reasonable interpretation of this data is that a key function of GRF1 in the HPA axis response to short-term (3-day) restraint stress is to suppress global phosphorylation changes (up and down) that might otherwise arise in the CA1 hippocampus in response to the heightened neuronal network activity associated with stress. When excessive phosphorylation changes take place in the absence of GRF1, the ability of the CA1 to signal to the hypothalamus to suppress the HPA axis is defective. Thus, GRF1 may regulate the expression of a key phosphatase(s), whose activity is normally enhanced upon short-term stress to carry GRF1 function. Among the phosphatase targets could be kinases that are activated by dephosphorylation, which could explain how the loss of GRF1 could also decrease phosphorylation of some proteins.

To begin to understand how GRF1 regulation of stress-induced phosphorylation controls negative feedback on the HPA axis we used STRING: protein-protein interaction networks analysis coupled with KEGG pathway analysis to identify signaling pathways that might be most affected by the excess changes in phosphopeptides (green and red dots in Fig. 4.2) observed in the CA1 hippocampus of stressed mice lacking GRF1. Despite the fact that ~1000 phosphopeptides changed preferentially in GRF1(-/-) mice over control mice after stress, a clear leading candidate is the oxytocin signaling pathway, whose statistical significance was ~10X higher than the next three signaling pathways.

The 23 phosphoproteins containing these phosphopeptides implicated in oxytocin signaling by this technique are highly connected and include many related proteins. None of these proteins is specific for the oxytocin pathway, but the set has been reported to be regulated most by oxytocin.

The next most significant pathways identified regulate tight junctions, the actin cytoskeleton and glutamatergic synapses. Tight junctions may regulate electrical synapses between neurons but little is known how that may function in stress. The actin cytoskeleton pathways may be of particular interest since they are known to regulate synaptic function and because two of them, Fak kinase and myosin 9 are differentially phosphorylated in GRF1(-/-) mice after stress. Finally, we already know GRF1 contributes to glutamatergic signaling but we did not know that it may regulate the phosphoryation of components of this pathway.

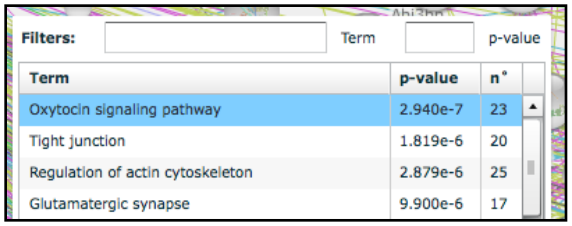

Figure 4.3: Results of the STRING network analysis. Top four signaling pathways to be altered in phosphorylation states in early adolescent GRF1(-/-) mice upon 3 days of stress.

GRF1 regulating oxytocin signaling is an exciting possibility because oxytocin is implicated in stress response and known to influence hippocampus function (Cohen, 2010, Amico, 2004 , Kumsta, 2013). The hypothalamus sends axons to the hippocampus where local release of oxytocin occurs after stress. Oxytocin in concert with estrogen is known to be an anxiolytic agent in adult females that decreases the stress response in the long-term. However, infusion of oxytocin into dorsal hippocampus of males enhances

baseline and magnifies stress-induced CORT elevation soon after stress implying that excessive oxytocin signaling magnifies the short-term response to stress. Based on these observations and our new findings, we have generated a working model for how GRF1 may contribute to CA1 function to promote negative feedback on the hypothalamus specifically in early-adolescent females (Fig 4.4).

Stress increases the activity of the oxytocin-regulated signaling pathways in the hippocampus that enhances CORT output by suppressing negative feedback on the hypothalamus. We propose that GRF1 normally suppresses this signaling effect indirectly in response to stress by increasing the expression of a phosphatase. Thus, in GRF1(-/-) mice, super activation of the oxytocin pathway occurs, negative feedback is suppressed, and CORT levels are super activated.

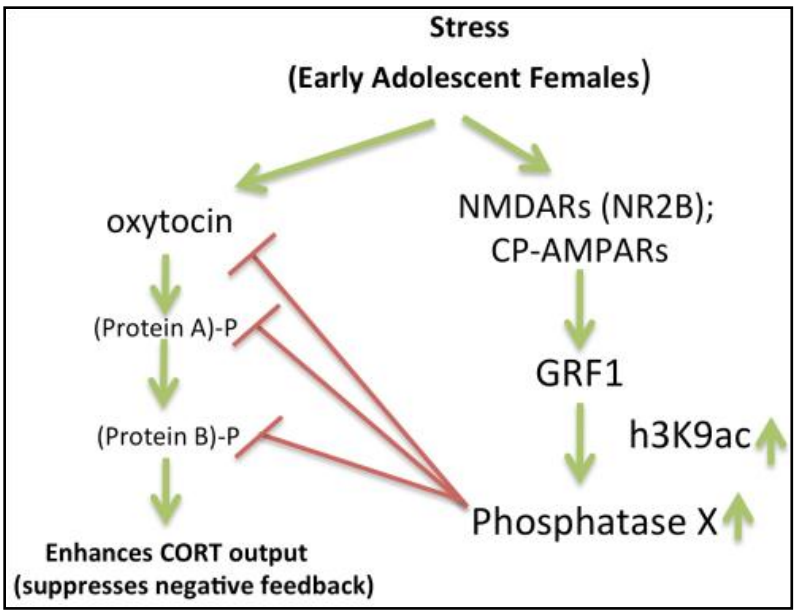

Figure 4.4: Model for role for GRF1 in oxytocin signaling upon stress exposure. Our working model on the role for GRF1 in regulating a phosphatase involved in oxytocin signaling. We hypothesize that GRF1 normally regulates a phosphatase in oxytocin signaling; therefore lack of GRF1 causes super activation of oxytocin pathway.

4.3 Defect in H3K9 acetylation in early-adolescent GRF1(-/-) females after short-term stress response

A body of evidence suggests that stress-induced histone post-translational modifications contribute to the adaptation of the HPA axis to a changing environment (Stankiewicz, Swiergiel et al. 2013, Sun, Kennedy et al. 2013, Zannas and West 2014). Moreover, phosphoproteomic studies (chapter 4.2) also suggest a role for GRF1 in regulating gene expression, possibly a phosphatase. To determine if GRF1 might control the HPA axis response by regulating gene expression through stress-induced histone modifications, I measured the levels of different histone modifications after 3 days of stress exposure by immunoblotting extracts of isolated CA1 and combined CA3/DG regions of the hippocampus.

Early-adolescent and female WT and GRF1(-/-) mice were submitted to either acute or 3 days of 30 min/day restraint stress. CA1 regions were dissected immediately after the last stress exposure. Total protein extracts from these tissues were used to measure different histone modifications. Figure 4.5A shows that in WT mice, levels of H3K9 and H3K18 acetylation are increased after acute stress and increase even more after 3 days of stress in CA1 of HC. However, GRF1(-/-) females showed a blocked increase in H3K9 acetylation after acute stress and day 3 of restraint stress whereas H3K18 acetylation increase was normal compared to WT mice. Figure 4.5A shows a representative image from a western blot and quantifications of 3 independent experiments are shown in figure 4.5B. This data suggests that the block in CA1 H3K9 acetylation in GRF1(-/-) mice upon stress exposure may be the cause of the defect in HPA negative feedback mechanism.

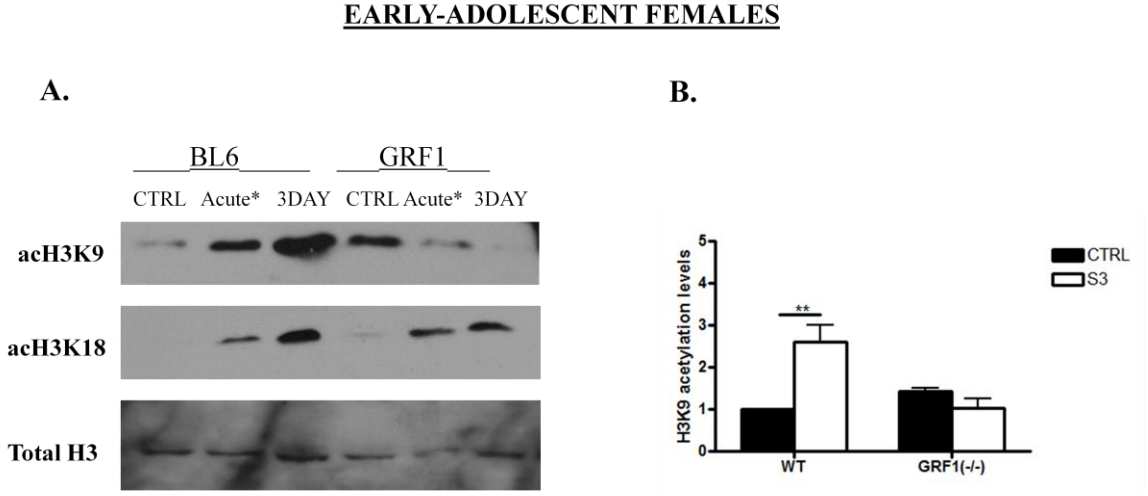

Figure 4.5: Early-adolescent and female GRF1(-/-) specific defect in H3K9 acetylation after acute and short-term stress. Early-adolescent and female WT and GRF1(-/-) mice are submitted to either acute or 3 days of restraint stress. CA1 of HC is dissected and further analyzed to measure H3K9 and H3K18 acetylation protein levels. **(A)** H3K9 and H3K18 acetylation levels are increased after acute and 3 days restraint stress in CA1 of WT mice. Block in H3K9 acetylation increase is observed in CA1 of GRF1(-/-) females whereas H3K18 acetylation increase is normal compared to WT females. **(B)** H3K9 acetylation increase $F_{(2,8)}$=6.903, p=0.0303; Bonferroni post-hoc test for WT control vs. S3 t=4.900, p<0.01; GRF1 control vs. S3 t=1.184, P>0.05.

4.4 Defect in H3K9 acetylation GRF1(-/-) females after short-term stress response is sex dependent

To determine if the H3K9 acetylation defect in GRF1(-/-) mice is female dependent, early-adolescent WT and GRF1(-/-) males were submitted to 3 days of restraint stress, CA1 of HC is dissected and analyzed for protein expression of H3K9 acetylation. Figure 4.6 shows that H3K9 acetylation is increased after 3 days of restraint stress in CA1 of early-adolescent WT males. In contrast to their female counterparts, early-adolescent male GRF1(-/-) mice showed a similar increase in H3K9 acetylation in CA1 of HC. Figure 4.6A shows the representative image of the western blots and figure 4.6B shows the quantification of 3 independent experiments. This result suggests that adolescent male GRF1(-/-) mice show normal H3K9 acetylation increase after short-term stress, correlate with their normal CORT increase. Therefore, this finding supports the idea that female specific exaggerated CORT release may be due to female specific H3K9 acetylation defect.

ADOLESCENT MALES

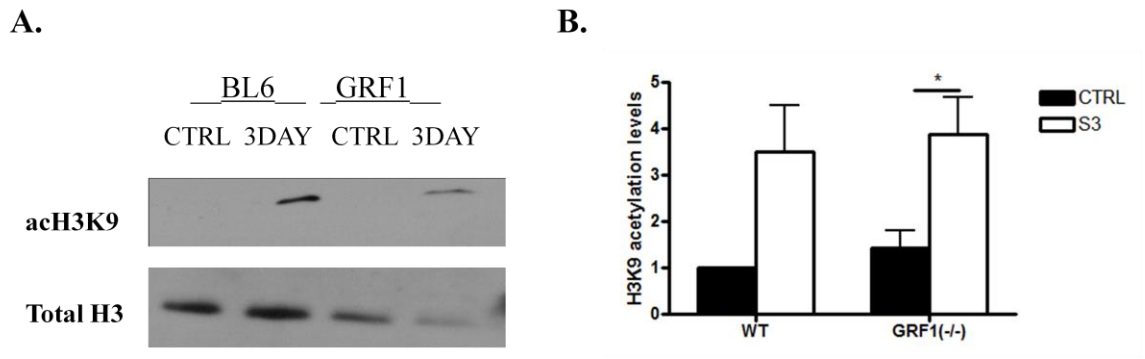

Figure 4.6: Normal H3K9 acetylation increase in early-adolescent and male WT and GRF1(-/-) mice. WT and GRF1(-/-) mice were submitted to 3 days restraint stress and CA1 of HC is dissected for protein analysis. (A) Early-adolescent and male WT mice showed an increase in H3K9 acetylation in response to 3 days restraint stress. A similar increase was observed in GRF1(-/-) males after 3 days restraint stress. (B) H3K9 acetylation increase $F_{(2,7)}$=16.65, p=0.0047; Bonferroni post-hoc test for WT control vs. S3 t=2.772, p>0.05; GRF1 control vs. S3 t=3.021, P<0.05.

4.5 H3K9 acetylation increase is not observed in adult female WT or GRF1(-/-) mice

To determine whether GRF1 contribution to stress-induced H3K9 acetylation continues through adulthood, adult WT and GRF1(-/-) females were submitted to 3 days of restraint stress and H3K9 acetylation protein levels were measured in CA1 of HC. Surprisingly, the increase that was observed in adolescent WT females was not observed even in adult WT females (Figure 4.7A,B). This suggests that HC mediated HPA axis negative feedback is activated through a different mechanism upon 3 days of restraint stress exposure that does not require enhanced H3K9 acetylation. As expected, adult GRF1(-/-) females also do not show an increase in H3K9 acetylation after 3 days stress exposure.

ADULT FEMALES

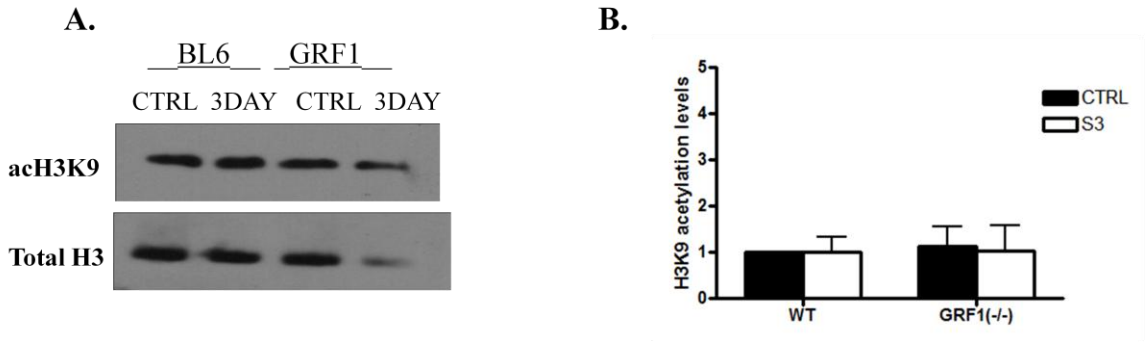

Figure 4.7: H3K9 acetylation in adult WT and GRF1 (-/-) females. Adult female WT and GRF1(-/-) females were submitted to 3 days restraint stress and CA1 of HC is collected for protein analysis. (A) Increase in H3K9 acetylation is not observed in adult female WT and GRF1(-/-)mice. (B) H3K9 acetylation increase $F_{(2,8)}$=0.23, p=0.8953; Bonferroni post-hoc test for WT control vs. S3 t=0.0062, p>0.05; GRF1 control vs. S3 t=0.1859, P>0.05.

4.6 H3K9 acetylation increase in WT and defect in GRF1(-/-) females is specific to CA1 of HC

Hippocampus is composed of 3 sub regions including CA1, CA3 and dentate gyrus (DG). The flow of information is unidirectional where the signals propagate through DG, to CA3 then CA1 and the projections go out of the HC to different regions in the cortex. Each sub region may have distinct functions in stress mechanism, synaptic plasticity and learning and memory (Farovik, Dupont et al. 2010, Brickman, Stern et al. 2011, Hawley, Morch et al. 2012). Also our lab and others have shown that GRF1 plays CA1 specific roles in synaptic plasticity (Feig 2011). To determine whether the early-adolescent and female specific defect of H3K9 acetylation increase was specific to the CA1 of the HC, I tested a pool of CA3 and DG regions. Early-adolescent female WT and GRF1(-/-) females were submitted to 3 days restraint stress and CA3/DG pool of HC was collected immediately after last stress exposure and H3K9 acetylation levels were determined.

Figure 4.8 shows that, the increase in H3K9 acetylation observed in CA1 of WT mice was not present in CA3/DG regions and as expected, the CA3/DG pool from adolescent GRF1(-/-) females also did not show an increase in H3K9 acetylation. However, it is possible that a small enhancement of H3K9 does occur in one region of adolescent females but is masked by no change in the other. Nevertheless, the findings highlight the CA1 region of HC as a key region where GRF1 plays a role in HPA axis response to short-term stress.

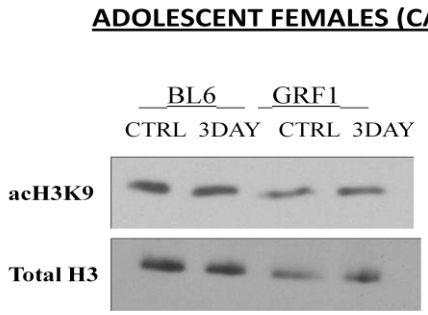

Figure 4.8: H3K9 acetylation in CA3/DG pool of HC in WT and GRF1(-/-) females after 3 days of restraint stress. WT and GRF1(-/-) females were submitted to 3 days restraint stress and CA3/DG pool is collected immediately after last stress exposure for protein analysis. The increase in H3K9 acetylation is not observed in WT and GRF1(-/-) female CA3/DG regions.

4.7 Model for early-adolescent and female specific role for RasGRF1 in CA1 mediated HPA axis negative feedback after short-term restraint stress

Figure 3.5 shows that early-adolescent female GRF1(-/-) mice display an exaggerated HPA axis response to 3 days of restraint stress, after which their response becomes blunted and by 7 days they do not respond at all. We propose that the enhanced HPA axis response after 3 days of stress exposure is due at least in part to a defect in HPA axis negative feedback mechanism of the HC. This idea is supported by our finding in Fig. 4.1 that restoring GRF1 to WT levels in the CA1 hippocampus of early-adolescent female GRF1(-/-) mice returns the CORT response to normal.

Moreover phosphoproteomic studies suggest that GRF1 plays a role in regulating a gene expression, possibly a phosphatase in early-adolescent females after 3 days of restraint stress. Furthermore, figures 4.5-4.8, imply that the defective stress-induced H3K9 acetylation in the CA1 of early-adolescent female GRF1(-/-) mice is involved in the enhanced HPA axis in GRF1(-/-) mice after 3 days of restraint stress because this defect correlates precisely with the age and sex-dependency of the GRF1 contribution to HPA axis regulation. Thus, we hypothesize that without GRF1 enhancement of H3K9ac, a phosphatase is not induced by stress. This leads to excessive phosphorylation changes that block the ability of the hippocampus to feedback on the hypothalamus. Moreover, in early adolescent GRF1(-/-) males, that have a normal HPA axis function, restraint stress also leads to enhanced H3K9 acetylation in the CA1, implying that this modification is also important in males. However, some other signaling pathway, possibly through GRF2, mediates its action possibly through the same phosphatase. Moreover, because in adult female mice, restraint stress does not lead to enhanced H3K9 acetylation, they

couple this form of stress to HPA regulation in a novel way. Figure 4.9 shows a model explaining these ideas.

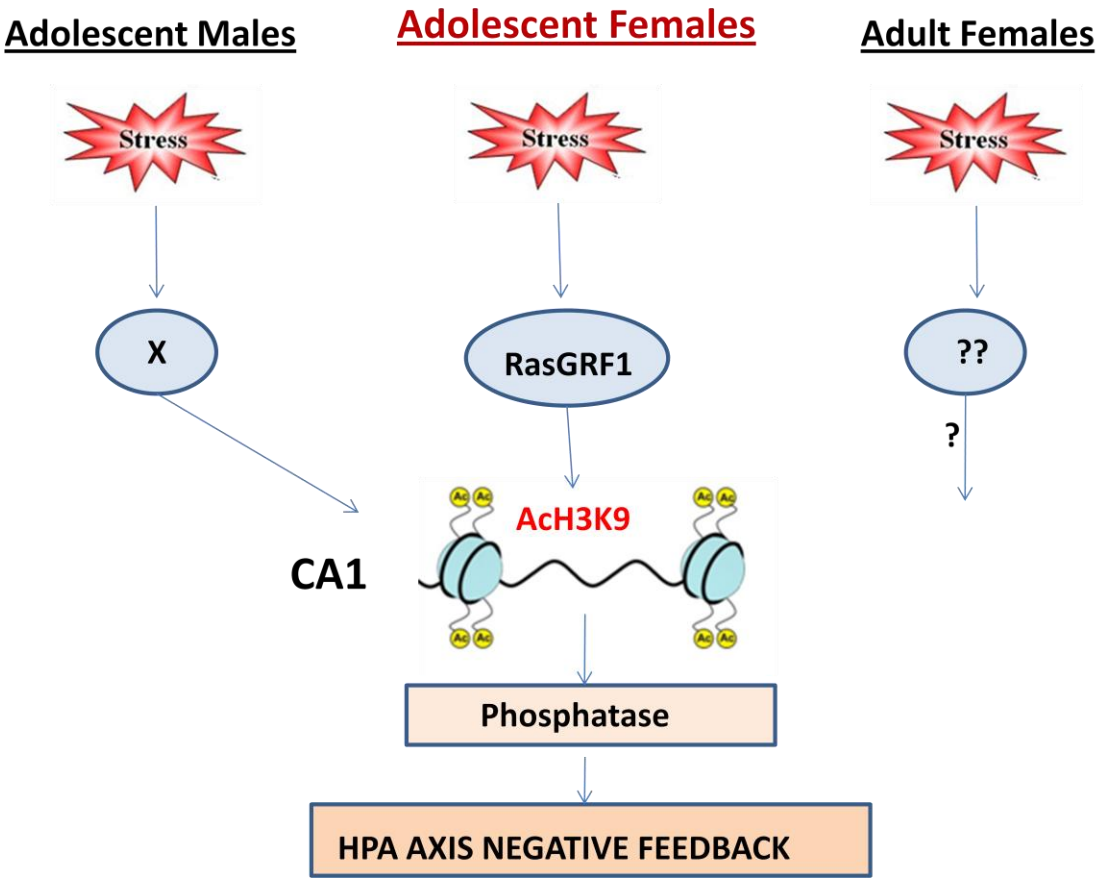

Figure 4.9: Model for early-adolescent female role for GRF1 in CA1 mediated HPA negative feedback mechanism through H3K9 acetylation. Accelerated CORT response in early-adolescent GRF1(-/-) females is due to a defect in CA1 mediated HPA negative feedback. Upon short-term restraint stress exposure in early-adolescent females, CA1 of HC is needed to activate the negative feedback mechanism that is mediated by GRF1 dependent H3K9 acetylation and a phosphatase activation. In early-adolescent males, H3K9 acetylation is still needed; however; this pathway is independent of GRF1. In adult females, H3K9 acetylation in CA1 is not needed suggesting a different mechanism that is independent of GRF1.

I then began to test the significance of the observed block in the stress-induced increase in H3K9 acetylation in GRF1(-/-) mice. To this end I tried to rescue this defect by increasing H3K9 acetylation levels in GRF1(-/-) mice and see if this restores the CORT response back to normal. In particular, I tested the effect of infusing different inhibitors of HDAC1, a deacetylase that targets H3K9 and of infecting AAV viruses expressing shRNA against HDAC1. The hope was that this would restore the stress induced H3K9 acetylation in GRF1(-/-) mice and suppress the excess CORT production in these animals. 3 different HDAC1 inhibitors were tested; CI-994, TSA and MS-275 by both systemic and intrahippocampal injections. HDAC1 shRNA AAV was injected into the CA1 of HC. Even though I was able to detect an increase in H3K9 acetylation levels in GRF1(-/-) mice, the enhanced CORT response was not lowered to normal levels of WT mice (data not shown). Possible explanations include: 1. Since HDAC1 is not specific to H3K9 acetylation; these methods may increase other histone acetylations that may have other side effects. 2. Histone modifications are usually coregulated. So H3K9 acetylation may not be the only histone modification change that is GRF1 dependent therefore changing only itself was not sufficient to rescue the CORT defects. 3. Although the defective H3K9 acetylation correlates precisely with the age and sex dependent effects of GRF1, it may not play a role in HPA response.

Chapter 5:

Discussion

5.1 Overview

The studies performed in this thesis provide new insights into how the HPA axis is regulated specifically in early-adolescent females. By expanding our studies on RasGRF1 from learning and memory to the stress response, we have revealed novel functions for the protein in regulating the HPA axis response to stress. Remarkably this function for GRF1 is limited to early adolescent females.

RasGRF1 and RasGRF2 form a family of calcium activated guanine nucleotide exchange factors that activate Ras and Rac GTPases and regulate multiple forms of synaptic plasticity in the CA1 of hippocampus (HC). Since the HC is known to play a key regulatory role in controlling the HPA axis, we tested the involvement of GRF1 in this process by comparing the response of GRF1 knockout (GRF1(-/-)) mice to their WT counterparts after acute (30 min) and chronic restraint stress (30 min/day) exposure.

Chapter 3 of this thesis reveals behavioral and hormonal responses of GRF1(-/-) and WT mice to both acute and chronic restraint stress. We found that the hyper locomotor behavior in the elevated plus maze and the open field tests, as well as elevated CORT levels that normally occur after chronic restraint stress in mice are blocked in GRF1(-/-) mice in an age- and sex-dependent manner. In particular, these responses are blocked in early-adolescent and female GRF1(-/-) mice but are normal in their adult female, adolescent male and adult male GRF1(-/-) counterparts. This phenomenon appears to be due to accelerated habituation to restraint stress since early-adolescent female GRF1(-/-) mice do respond to fewer exposures of restraint stress. In fact, these responses are enhanced compared to WT mice when GRF1(-/-) mice were exposed to 1,

3 and 5 days of stress. These findings imply that different mechanisms exist for how GRF1 regulates the HPA axis to short term stress and habituation to chronic stress. Presumably, the enhanced HPA axis response observed after short-term stress (1-3 days) in GRF1(-/-) mice, which we show is due to the loss of negative feedback by the HC, is over-ridden by the appearance of another effect of the loss of GRF1, enhanced habituation to stress. This begins after 5 days and is fully active by 7 days of stress. This is likely due to the loss of GRF1 in different brain regions known to regulate habituation.

In Chapter 4 we focus on the role for GRF1 in the HPA axis response to short-term restraint stress, where its natural function appears to be to suppress it because GRF1(-/-) mice display an exaggerated CORT response. We provide strong evidence that GRF1 function in the CA1 region of the hippocampus is responsible for this activity because we could restore a normal HPA axis response to short-term stress in GRF1(-/-) mice by re-expressing GRF1 through adenovirus infection solely in the CA1. Moreover, epigenetic changes in the brain have been associated with both acute and chronic stress mechanisms. We found that early adolescent female GRF1(-/-) mice have a defect in stress induced enhanced acetylation of H3K9 in CA1 of HC after 3 days of stress. In contrast, early adolescent male or adult females that show normal HPA axis response show no defects. These results imply that GRF1 influences the HPA axis response to short-term stress through regulation of H3K9 mediated gene expression changes in CA1.

Even though HPA axis regulation has been studied extensively, the majority of the studies use adult and male animals. Yet, it has been shown that adolescents and females are more sensitive to stress due to the mechanisms that are poorly understood. In addition, these populations are known to respond differently to treatments for diseases

associated with altered HPA axis. Thus, further studies based on our observations may yield new insights into the unique mechanisms underlying stress-induced activation of the HPA axis that occurs specifically in early adolescent females.

5.2 Early adolescence and female specificity of GRF1 in stress response

Our lab has shown previously that the age-dependent roles for GRF1 play a role in both synaptic plasticity and adult neurogenesis (Jin, Arai et al. 2013, Darcy, Trouche et al. 2014). Interestingly, the age-dependence of the role of GRF1 in HPA axis that I uncovered disappears after ~40 days of age, despite the fact that GRF1 continues to play a role in learning and memory throughout adulthood. The time period, between 28 days when the HPA axis is dependent upon GRF1 and 40 days when it is not, is the transition from early to mid-adolescence when Bl6 mice enter puberty. This raised the possibility that the rise in estrogen that occurs in mid-adolescence plays an active role in making the HPA axis independent of GRF1. This does not seem to be the case because ovariectomy of GRF1(-/-) mice at 28 days of age did not result in rapid habituation to 7 days of restraint stress in 2 month old mice. Rather, hormone-independent development of specific components of the HPA axis at the onset of puberty is likely involved.

Many studies have shown that adolescent animals respond differently to stress than adults (Panagiotakopoulos and Neigh 2014) but distinctions between early, mid and late adolescence have not been studied in detail. Early adolescence in mice (pn 21-34) is thought to be similar to human ages of 10-14 (Burke and Miczek 2014). It is a period of rapid brain development and high vulnerability to excessive stress (Andersen and Teicher

2008). Animal and human studies have observed differences between these two developmental stages in response to specific environmental perturbations. For example, long-term effects on future behavior can occur after exposure of early but not late adolescent rats (Raver and Keller 2014). Risk of depression upon early experience is shown to be maximal around early adolescence and decrease as humans age through mid and late adolescence (Andersen and Teicher 2008).

In addition, many studies have focused on sex differences in HPA axis response to stress and to responses to treatments for psychiatric disorders. Studies have shown sex differences in the development of depressive symptoms during early-, mid- and late-adolescence among boys and girls (Dekker, Ferdinand et al. 2007). Moreover, women are more likely than men to suffer from depression (Marcus, Young et al. 2005, Bangasser and Valentino 2014) and respond better to certain class of antidepressants than others (Kornstein, Schatzberg et al. 2000). Also, studies have consistently found PTSD to be more common in women than in men, and some studies have found that women with PTSD respond more robustly to SSRI antidepressants than men (Norris F 2002). However few studies have focused specifically on adolescent females, and fewer on early-adolescent females. One example did show that upon chronic stress, adolescent female mice display blunted stress response compared to their male counterparts due in part to differential transport of GRs to the nucleus (Bourke, Raees et al. 2013).

How and why GRF1 controls signaling pathways in cells that control the HPA axis specifically in early-adolescent females remains to be determined. GRF1 may regulate the HPA axis through its known ability to couple NMDA and calcium-permeable glutamate receptors to both the Erk, p38 Map kinases, since various stress paradigms

have been observed to alter the activities of these kinases (Cole, Kalman et al. 2000, Revest, Di Blasi et al. 2005, Ferland, Harris et al. 2014). However, to date these findings are just correlative and they were detected in adult males. Thus, GRF1 may contribute specificity to HPA axis regulation in early-adolescent females by coupling these kinases or other downstream effectors to distinct upstream signals that allow the HPA axis of early-adolescent females to respond to unique environmental stimuli, but use a downstream mechanism common to older females and males. Alternatively, GRF1 may be engaged by the same environmental signals, but connect to the HPA axis differently in this population of animals.

The fact that GRF1(-/-) mice display a complex phenotype, with an enhanced HPA axis response after short-term restraint stress, and yet blocked HPA axis response after chronic restraint stress may reflect defects in different brain regions where GRF1 is expressed that have distinct functions in regulating the HPA axis. For example, we already showed that the magnified CORT response observed in these animals is caused by a defect in the CA1 hippocampus from the loss of GRF1, which is consistent with the negative role this region plays in regulating the HPA axis (Jankord and Herman 2008). Moreover, the block in CORT response to chronic restraint stress may involve the loss of GRF1 in brain regions that have been reported to play a role in habituation (Herman 2013). However, all of the studies identifying these brain regions used adult males, so novel regions may be involved in early-adolescent females.

5.3 Role of the hippocampus GRF1 in regulating the HPA axis response to short-term stress

Excessive CORT secretion in GRF1(-/-) mice after short-term (1-5 days of 30min/day) restraint stress raised the possibility that GRF1 normally contributes to either positive HPA axis stimulation or negative feedback on it. To begin to test which one is correct we began by studying the hippocampus because we already knew from our lab's work that GRF1 contributes to synaptic plasticity in the CA1 HC and the HC is one of the key regulators of HPA axis negative feedback mechanism (Jankord and Herman 2008, Feig 2011). In Figure 4.1B, we showed that by re-expressing GRF1 in CA1 of early-adolescent female GRF1(-/-) mice, we were able to reverse the excessive CORT secretion after 3 days of restraint stress to the levels found in stressed WT mice. This finding demonstrates that the CA1 is the major site where GRF1 contributes to the HPA axis response to short-term stress and thus contributes to the negative feedback role of HC in early-adolescent females. It also demonstrates for the first time that a novel biochemical mechanism regulates the negative feedback role of the HC in early-adolescent females.

Our mechanistic studies on how GRF1 controls the HPA axis response to short-term restraint stress suggests GRF1 functions through control of both protein phosphorylation and gene expression.

A) *Regulation of phosphorylation*. A phospho-proteomic study was performed in collaboration with Cell Signaling Technology to detect GRF1 dependent changes in the activities of components of signaling networks controlling the HPA axis in response to short-term stress in CA1 of early adolescent female mice.

Results of the MS/MS data revealed the surprising finding that GRF1 is a major regulator of global phosphorylation changes in response to stress. In particular, while only ~1% of all CA1 phospho-peptides from WT mice changed more than 2-fold after 3 days of restraint stress, ~10% did in GRF1(-/-) mice. Most of the changes were in the positive direction, but many were suppression of peptide phosphorylation. They also involved a wide variety of protein classes. For the most part, baseline levels of phosphorylations were similar between WT and GRF1(-/-) mice indicating that GRF1 is specifically involved in preventing excess phosphorylation changes, both up and down, that would otherwise occur after restraint stress that likely super-activates brain circuits. The level of phosphorylation change in GRF1(-/-) mice after stress is similar to that which occurs after drug treatment of tissues (findings from Cell Signaling Technology) indicating the large magnitude of the effect the loss of GRF1 has in this system.

One possible explanation for this result is that GRF1 mediates the enhanced expression of a stress-induced phosphatase(s) with broad specificity. This could explain the increase in the phosphorylation of many proteins. Since kinases can be activated by dephosphorylation, the loss of a phosphatase could also lead to the decrease in some phosphopeptides observed. One analogous example is, CagA, a cytotoxin that regulates the function of host phosphatases and kinases (Stein 2014). It is also possible that the changes in phosphopeptides observed is due to GRF1 regulating the expression levels of proteins containing the phosphopeptides, but this is not very likely since we usually saw just a small subset of potential phosphorylation sites on target proteins changing in GRF1(-/-) mice exposed to stress.

When the proteins with altered phosphorylation states in stressed GRF1(-/-) mice were analyzed with STRING pathway analysis, the top four pathways containing proteins altered were found to be oxytocin, tight junctions, actin cytosekeleton and glutamatergic signaling pathways. Oxytocin is a neurohypophysial hormone that is secreted from the brain's hypothalamic paraventricular and supraoptic nuclei. Oxytocin signaling is mainly involved with reproduction and maternal behaviour. It is released from posterior pituitary in response to external stimuli (Insel, 2010; Viero, 2010). Nevertheless, oxytocin signaling is an exciting possibility because it has been implicated in the HPA axis regulation in response to stress and is also known to regulate hippocampal function. Oxytocin in concert with estrogen is known to be an anxiolytic agent in adult females, such that it decreases the stress response in the long-term. Also, oxytocin deficient female mice show increased CORT levels (Amico, Mantella et al. 2004). However, it appears to have the opposite acute effect in the hippocampus, where acute infusion of oxytocin into the hippocampus increases CORT release, presumably by inhibiting negative feedback on the hypothalamus from the hippocampus. Moreover, stress is known to induce oxytocin secretion from axons emanating from the hypothalamus (Cohen, Kaplan et al. 2010). These findings support the hypothesis that in early-adolescent females GRF1 may negatively regulate the oxytocin pathway via its control of the expression of a phosphatase(s) to keep CORT output in balance. In this way in early-adolescent female GRF1(-/-) mice the oxytocin signaling is super activated and mice display an exaggerated stress response.

B) *Regulation of Gene Expression:* Results from the phosphoproteomic studies suggest that GRF1 plays a role in promoting the expression of a phosphatase(s) in response to short-term stress. Moreover, epigenetic studies also suggest a role of GRF1 in regulating gene expression through H3K9 acetylation in response to restraint stress. These independent findings support our hypothesis that GRF1 normally suppresses oxytocin signaling indirectly in response to stress by increasing the expression of a phosphatase through enhanced H3K9ac. Moreover, CA1 specificity of H3K9 acetylation increase in adolescent females after restraint stress can be supported by the idea that this defect can be rescued by re-expressing GRF1 only in CA1 of HC.

Epigenetic changes have been implicated after both acute and chronic stress mechanisms (Tsankova, Renthal et al. 2007, Zannas and West 2014). For example, 30 minute of restraint stress increases H3S10p in various brain regions (Rotllant, Pastor-Ciurana et al. 2013) and H3K9 methylation in the CA1 (Hunter, McCarthy et al. 2009) although how this is connected to the stress response has not been revealed. We observed an increase in H3K9 acetylation, which has the opposite effect of H3K9 methylation, in CA1 after short-term stress in adolescent female and in adolescent male WT mice. Importantly we saw no change in adult WT mice. This finding strongly suggests an adolescent specific mechanism for HPA axis negative feedback regulation in CA1 through H3K9 acetylation.

HDAC inhibitors have been shown to augment antidepressants effects (Weaver, Cervoni et al. 2004, Tsankova, Renthal et al. 2007, Covington, Maze et al. 2009). They have also been used to reverse the behavioral effects of stress associated with histone changes (Weaver, Cervoni et al. 2004, Covington, Maze et al. 2009, Fleiss, Nilsson et al.

2012, Dagnas, Guillou et al. 2013). However, injecting HDAC inhibitors Ci-994, TSA or MS-275 did not rescue the hyper CORT secretion in GRF1(-/-) mice after 3 days of restraint stress. This may have possible explanations. First, none of these HDAC inhibitors are specific to H3K9 acetylation; therefore, they elevate other histone acetylations. Second, histone modifications are usually coregulated thus H3K9 acetylation may not be the only histone modification change needed to rescue the CORT defects. Third, although the defective H3K9 acetylation we detected in GRF1 (-/-) mice correlates precisely with the age and sex dependent effects of GRF1, it may not play a role in HPA response.

Defects in HPA axis negative feedback have been implicated in several disorders such as depression, PTSD and anxiety. Even though molecular mechanisms have been studied in detail, many of the studies use adult male animals whereas, adolescents and females are shown to be more vulnerable in developing these diseases. Our findings on early-adolescent and female roles for GRF1 may highlight new insights in developing anti-depressants and anti-anxiolytic drugs specifically for early-adolescent females.

5.4 Role of GRF1 in regulating the HPA axis response to chronic stress

Habituation to stress is defined as a decrease in response to repeated homotypic stress exposures. The fact that early-adolescent female GRF1(-/-) mice show an enhanced response to short-term restraint stress followed by a blocked response after 7 days of exposure suggests that GRF1 plays a role in regulating the habituation in early-adolescent and female mice. With our stress paradigm, WT mice do not even begin to habituate after

14 days of stress exposures (data not shown). This suggests that GRF1 normally functions to suppress habituation process. The biochemical mechanisms involved in how the rate of habituation is regulated have not been revealed but the neural mechanisms involved in habituation appear to include mPFC, amygdala and paraventricular thalamus (Grissom and Bhatnagar 2009, Weinberg, Johnson et al. 2010). However, these results were obtained with adult male animals. Therefore GRF1 in one of these brain regions or novel ones may be involved in regulating the habituation.

Habituation can be defined by the following criteria (Grissom and Bhatnagar 2009). First, habituation is reversible, such that after a few weeks an HPA response returns. Adolescent female GRF1(-/-) mice regain responsiveness to restraint stress as early as 7 days after cessation of stress exposure (data not shown). However, this also coincides with the developmental transition to mid-adolescence when female GRF1(-/-) mice show a normal stress response, so it is not possible for us to assign this reemergence of responsiveness to the reversal of habituation. Second, habituation can be enhanced by modifying certain parameters. For example, the rate of habituation can be enhanced by factors such as by elevating frequency of stress exposures or by reducing stress magnitude. The fact that the acute HPA response is exaggerated in GRF1(-/-) mice raises the possibility that this drives an accelerated habituation. However, this is unlikely because it has been demonstrated that the stronger the stress response, the slower the habituation (Grissom and Bhatnagar 2009). Third, exposure to any heterotypic stress after habituation to a homotypic stressor like restraint stress does not elicit cross-stressor habituation but instead facilitation of HPA activity (Armario, Hidalgo, & Giralt, 1988). We have not tested this criterion yet.

Habituation is needed to cope with different stress mechanisms because it helps prevent damage due to constitutive elevation of stress hormones after repeated exposures. Lack of habituation may lead to major depressive disorder or PTSD (Grissom and Bhatnagar 2009) and mechanisms underlying the habituation process are not well understood particularly in early-adolescent females. Therefore, our findings on the role for GRF1 in regulating the habituation is the first to show a distinct mechanism in early-adolescent females and thus reinforces the idea that age and sex-specific methods for treating stress associated psychiatric disorders deserve a study.

5.5 Conclusion and Future Perspectives

Distortion of the HPA axis caused by excessive stress has been linked to various psychological disorders such as depression, post traumatic stress disorder, anxiety and addiction (Marin, Cruz et al. 2007). Even though the mechanisms of the HPA axis regulation have been studied extensively, most of the studies use adult and male animals. Moreover, adolescents and females are known to be more sensitive to stress exposures and are more likely to develop stress-induced disorders (Kornstein, Schatzberg et al. 2000, Tolin and Foa 2006). Yet, molecular mechanisms underlying the differential susceptibility are poorly understood and there are many treatment resistant patients. This thesis highlights specific roles for RasGRF1 in regulating the HPA axis response to short term (1-3 days) and chronic restraint (7 days) specifically in early-adolescent females. Future studies on the role for GRF1 and GRF2 in the HPA axis regulation can highlight these differences and offer an opportunity to reveal insights that may be used in future

strategies to target the unique features of the stress response in early-adolescents and females.

Role of GRF1 in short-term stress response:

A) *GRF1 function through regulation of phosphorylation.* We will repeat the phosphoproteomic scan using adolescent males and adult females to focus on phosphorylation changes specific to early-adolescent female GRF1(-/-) mice and then repeat the STRING Analysis to determine if phosphorylation changes in components of oxytocin signaling are specific to early adolescent females.

If we find that the changes are specific, we will then confirm the role of GRF1 in regulating oxytocin signaling. Our findings suggest that oxytocin signaling is super activated in GRF1(-/-) mice and to test this hypothesis we will use an inhibitor of oxytocin receptor in CA1 of GRF1(-/-) to reverse the excess CORT secretion after 3 days. Another approach will be to test whether the activities of individual components that came up with the phospho-proteomic scan in GRF1(-/-) mice change after stress only in GRF1(-/-) mice. For example we will measure the activities of PKA, PKC, CamK, VGCC (voltage gated calcium channel). A limitation of this approach however is that none of these components are specific for the oxytocin pathway. An alternative approach could be to confirm that the oxytocin pathway is enhanced in GRF1(-/-) mice after stress by defining an oxytocin gene expression signature after infusing oxytocin into the CA1 hippocampus followed by RNAseq. We then could see if this pattern appears in the CA1 of GRF1(-/-) mice after stress.

B) *GRF1 function through regulation of gene expression*. The phosphoproteome work above suggests that a key function of GRF1 is to mediate stress induced increase in the expression of a phosphatase. We also generated independent data implicating GRF1 in stress-induced gene regulation through its ability to promote stress-induced increase in H3K9ac specifically in CA1 of hippocampus after short term stress. Additional histone modifications will be tested in future studies since epigenetic regulations usually involve concerted effects of different histone modifications. With this research we will be able to highlight new insights in the GRF1 dependent and independent epigenetic regulations in response to stress exposure .

To determine gene targets regulated by GRF1, like phosphatases, that might be regulated by GRF1 dependent histone modifications, a gene expression screen will also be performed. Stress induced gene expression changes in the CA1 of adolescent females, but not in adolescent males or adult males or females will be determined. This approach may also suggest other mechanisms GRF1 uses to regulate the HPA axis besides overall phosphorylation regulation. After determining stress-induced gene changes that are specific to early-adolescent GRF1(-/-) mice, we will test whether H3K9ac (or other histone modifications we detect) is involved in their regulation by performing ChiP analysis on these gene promoters.

If we can confirm the role of a specific histone modification in GRF1 dependent gene regulation that controls the HPA axis, upstream regulators and downstream effectors of GRF1 that regulate histones will also be tested. Based on previous studies, GRF1 can mediate its effects in the CA1 through NMDA receptors, CP-AMPA receptors and D1 dopamine receptors (Feig 2011). Thus, inhibitors of these receptors will be injected in

CA1 to determine if they block stress induced changes in histone modifications. Meanwhile, downstream GRF1 effectors that may mediate histone modifications will also be determined. For example, GRF1 can activate p38 and ERK in the hippocampus and other brain regions (Feig 2011). Involvement of these GRF1 effectors in HPA regulation will be tested by infusing the inhibitors of p38 and pERK to CA1 to determine if they also block stress induced changes in specific histone modifications. We will then test whether these inhibitors mimic the super-activation of the HPA axis to short-term stress observed in GRF1(-/-) mice.

Role of GRF1 in chronic stress response:

First, the brain region that GRF1 regulates habituation needs to be determined. Previous studies have implicated the amygdala, lateral septum, paraventricular thalamus and mPFC, not the hippocampus, in regulating habituation. But these studies were done on adult males. So studies on GRF1 could reveal the novel finding that different brain regions regulate habituation in early adolescent females. We already showed that GRF1 likely functions in the brain not the pituitary or adrenal glands, (see Fig. 3.2) to suppress habituation. Thus, we will begin by repeating the experiment where re-expressed GRF1 in the CA1 of early-adolescent female GRF1(-/-) mice and showed it rescued the HPA defect after 3 days stress to see if it also rescues the effect after 7 days stress. If the CORT levels rise as in WT mice it will be quite a novel result because this region does not appear to be involved in habituation in adult male mice. Next we will do a similar analysis in the 4 regions shown to be involved in habituation in adult males. If restoring

GRF1 in a specific brain region is not sufficient to restore normal HPA axis response to chronic stress in GRF1(-/-) mice, we will determine whether the loss of GRF1 in a specific brain region is sufficient to mimic the effect seen in GRF(-/-) mice by knocking out GRF1 in a tissue specific manner with our newly derived conditional GRF1 knockout mice. In particular we will inject adeno-CRE virus into specific brain regions as we did with GRF1 expressing virus.

After the brain region involved is determined, similar epigenetic, phosphoproteomic and gene expression studies will be performed in adolescent female WT and GRF1(-/-) mice as explained in the above section.

Role of GRF2 in HPA regulation to stress exposure:

Figure 3.6 shows that adolescent **male** double GRF1/2(-/-) knockout (DBKO) mice have defects in the stress response, in contrast to adolescent male GRF1(-/-) mice that are normal. This finding suggests that GRF2 may play a role in HPA axis regulation but in adolescent males. Interestingly, like early-adolescent GRF1(-/-) mice, early-adolescent male DBKO mice also display enhanced habituation to chronic restraint stress, but unlike GRF1(-/-) counterparts they display normal response to short-term stress. This supports the idea that the mechanism after short-term stress and chronic stress can be independent from each other. Also, DBKO mice have lower baseline CORT levels compared to their WT counterparts. These findings suggest distinct roles for GRF1 and GRF2 in HPA axis regulation. First, single GRF2(-/-) male mice will be used to confirm this hypothesis. Then, we will test whether GRF2 is male specific or the generic HPA

axis mediator by testing adolescent and adult female as well as adult GRF2(-/-) mice After determining the age and sex specificity of GRF2(-/-) to HPA regulation, similar behavior and gene analyses will be performed. Thus, we will likely learn more novel aspects of HPA axis regulation upon stress exposure from studying RasGRF2.

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