REGULATION OF THE HYPOTHALAMIC-PITUTARY-ADRENAL AXIS BY THE GLUCOCORTICOID RECEPTOR AND CORTICOTROPIN-RELEASING HORMONE

By

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First and foremost this is dedicated to my Lord Jesus Christ through whom I can accomplish all things

and

To my parents, sister, brothers, and family for their love and support

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11β-HSD	11β-hydroxysteroid dehydrogenase
A Pit	Anterior pituitary
ACTH	Adrenocorticotropic hormone
ASR	Acoustic startle response
AVP	Arginine vasopressin hormone
BAC	Bacterial artificial chromosome
BAR	Barrington's nucleus
BDI	Beck Depression Inventory
BLA	Basolateral amygdala
Bmal1	Aryl hydrocarbon receptor nuclear translocator-like gene
BnST	Bed nucleus of the stria terminalis
CA1, CA2, CA3	Hippocampal areas
CaMKIIα	Calcium calmodulin kinase 2 alpha
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid binding globulin
CCRF	Cincinnati Children's Research Foundation
CeA	Central nucleus of the amygdala
Cereb	Cerebellum
Cing Ctx	Cingulate cortex
CLOCK	Circadian locomotor output cycles Kaput gene
CNS	Central nervous system
CORT	Corticosterone
CREB	cAMP response element binding
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CRHBP	CRH binding protein
CRHR1	CRH Receptor 1
CRHR2	CRH Receptor 2

Cry1,2	Cryptochrome genes 1 and 2
CSF	Cerebrospinal fluid
DA	Dopamine
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DD	Dark dark
DEPC	Diethyl pyrocarbonate
DG	Dentate gyrus
Doxy	Doxycycline
DST	Dexamethasone suppression test
DTT	Dithiolthreitol
EE	Environmental enrichment
EPM	Elevated plus maze
EPSC	Excitatory postsynaptic currents
EZM	Elevated zero maze
fMRI	Functional magnetic resonance imaging
FPS	Fear potentiated-startle
Fr Ctx	Frontal cortex
FST	Forced swim test
GABA	Gamma-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
GPe	Globus Pallidus (external)
GR	Glucocorticoid receptors
H & E	Hemotoxylin & eosin
HPA	Hypothalamic-pituitary-adrenal axis
HPC	Hippocampus
HSP90	Heat shock protein 9
ICV	Intracerebroventricular
InfC	Inferior colliculus
IO	Inferior Olive

IRES	Internal ribosome entry site
kDa	kilo Dalton
KO	Knockout
L Sep	Lateral septum
LC	Locus coeruleus
LD	Light dark
LDT	Lateral dorsal tegmental nucleus,
LS	Lateral Septum
LTP	Long term potentiation
LTR	Long terminal repeats
LV	Lentiviral vectors
M Sep	Medial septum
MC2R	Melanocortin 2 receptor
MDD	Major depressive disorder
MeA	Medial nucleus of the amygdala
MR	Mineralocorticoid receptors
MWM	Morris Water Maze
NeuN	Neuron-specific nuclear protein
NMDA	N-methyl-D-aspartate receptor
NRC31	Glucocorticoid receptor gene
NSG	Normal goat serum
NTS	Nucleus tractus solitarii
OB	Olfactory bulb
Occ Ctx	Occipital cortex
OE	Overexpression
OF	Open field test
ORF	Open reading frame
P	Postnatal day
P Pit	Posterior pituitary
PAG	Periaqueductal gray
Par Ctx	Parietal cortex

Per1,2,3	Period genes 1, 2, and 4
PET	Positron emission tomography
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKA	Protein kinase A
POMC	Proopiomelanocortin
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus of the hypothalamus
REM	Rapid eye movement
RIA	Radioimmunoassay
RLU	Relative light units
RN	Raphe nucleus
rPFA	rostral Perifornical Area
rtTA	Reverse tetracycline transactivator
SCN	Suprachiasmatic nucleus of the hypothalamus
SEM	Standard error of the mean
SHRP	Stress hyporesponsive period
siRNA	Small interference RNA
SNPs	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
StAR	Steroidogenic acute regulatory protein
SupC	Superior colliculus
Tetop	Tetracycline-responsive promoter
Thal	Thalamus
TRE	Tetracycline Response Element
TST	Tail suspension test
Ucn	Urocortin
US	Unconditioned stimulus
UTR	Untranslated region
VMH	Ventromedial hypothalamus

PROLOGUE

BACKGROUND AND RESEARCH GOALS

INTRODUCTION

Stress and the HPA axis

Stress is the disruption in an organism's homeostasis caused by physiological or psychological deviations (1). To maintain well-being, all organisms require the ability to re-establish homeostasis in the presence of stress. The brain responds to stress by activating the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is a system of interactions between the hypothalamus, pituitary gland and adrenal gland that regulates hormonal responses to internal or external stimuli. Stress activates the HPA axis, causing the secretion of corticotropin-releasing hormone (CRH) and vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus to activate corticotroph cells in the anterior pituitary (1). There, both hormones induce synthesis of adrenocorticotropic hormone (ACTH), which causes glucocorticoid (GC)) secretion from the adrenal cortex into the bloodstream (Figure 1). Cortisol is the GC in humans, while corticosterone (CORT) is the GC in rodents. Secreted CORT binds to glucocorticoid receptors (GR), expressed in peripheral organs and limbic brain regions such as the amygdala, thereby facilitating responses aimed at adapting to the stressor (1). As a mechanism of control in the stress response, after GR is occupied by CORT, the sustained increase in levels of plasma CORT cause it to negatively feedback at the level of the PVN and anterior pituitary to inhibit its own secretion, and thus return the system to homeostatic levels (1). This negative feedback

prevents unnecessary prolongation of the stress response and thus averts maladaptive responses that may increase susceptibility to affective disorders.

Generally, the biology of the stress response is characterized by an initial rapid response, followed by a secondary slow, sustained response. The rapid response occurs seconds after stress exposure and consists of SNS activation of catecholamines, epinephrine and norepinephrine, and PVN release of CRH into the hypophyseal portal system (2). These hormones are fast-acting, driving second messenger signaling pathways to initiate changes necessary for stress mediation. HPA axis function can increase the release of epinephrine in order to mediate autonomic functions to increase arousal, vigilance and a decision to 'fight or flight' (3, 4). The secondary slow response is driven by secretion of glucocorticoids that mediate genomic functions that are protracted, such GC-induced increase in CRH mRNA in the amygdala (5) (Figure 1).

GC secretion occurs in a diurnal manner, with levels rising during the onset of an organism's active phase and decreasing during the inactive phase. The GCs present before a stressor, act in a permissive manner ready to initiate defense mechanisms in the event of stress. Within the first few minutes of stress exposure, there is an increase in energy mobilization, cardiovascular tone, immune activation, cerebral blood flow and memory consolidation (necessary for stress modulation), and a decrease in appetite and reproductive behavior (unnecessary for stress modulation) (Review (2)). Stress-induced increases in GCs levels further enhance this initial rapid stress response. The excess glucocorticoids in the first few hours are usually beneficial to survival of the organism. Stress-induced increase in GCs also causes delayed functions that serve to restrain the stress response and prevent exaggerated deviations from homeostasis This is through

negative feedback on GCs on GR in the PVN and anterior pituitary (Figure 1) as well as hippocampus and prefrontal cortex (6, 7). After the immediate stress response, GC signaling prepares the system to better respond to subsequent stressors. However in the event of chronic or repeated stress, the hormonal response of the HPA axis is hyperactivated causing an aberrant rise in CORT that is resistant to negative feedback, further disrupting homeostasis (8, 9). GR is pivotal in governing the actions of GCs in an organism, and represents an essential target to study in the understanding stress adaptation.

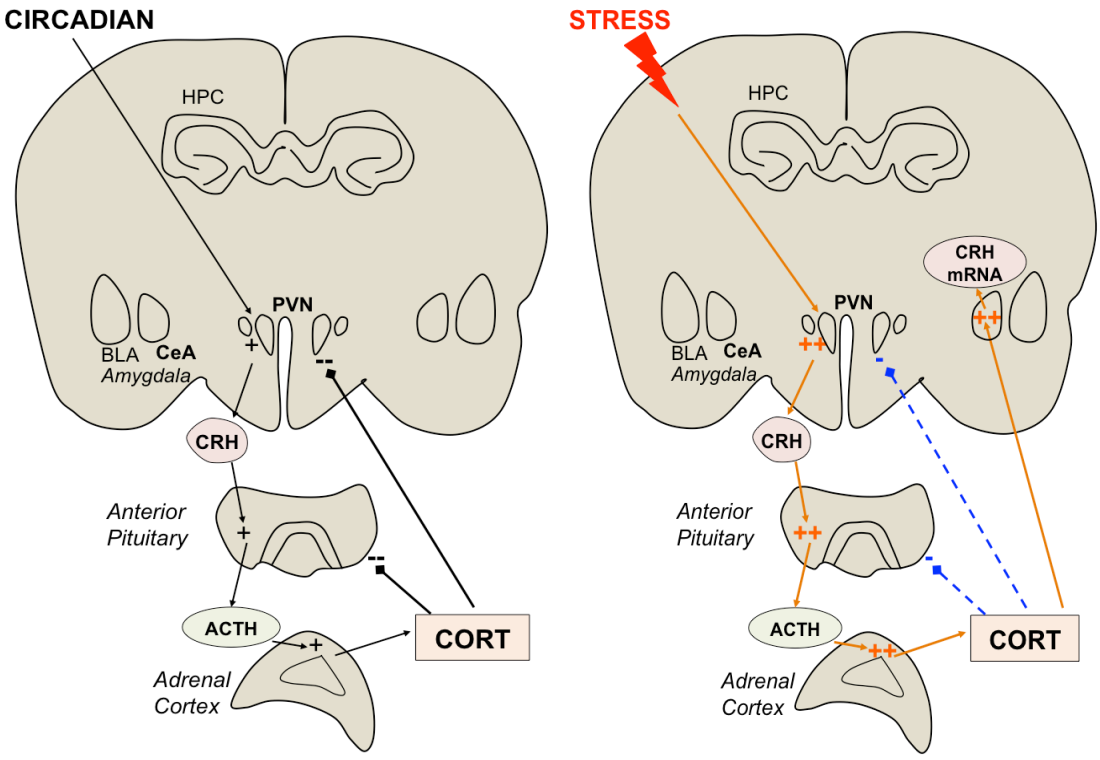

Figure 1. Hypothalamic-Pituitary-Adrenal (HPA) Axis Circuitry: Left panel: Normal circadian regulation of HPA axis. Right panel: Chronic stress can cause HPA axis hyperactivation and impaired negative feedback. GR, glucocorticoid receptor; HPC, hippocampus; CeA, central nucleus of the amygdala; BLA, basolateral amygdala; CRH, corticotropin releasing hormone; ACTH, adrenocorticotropic hormone, CORT, corticosterone. + activation, - inhibition

Glucocorticoid receptor

The GR gene, *NRC31*, after alternative splicing, alternate translation start sites, and post-translational modifications has many protein isoforms. The processing events result in the formation of at least five alternatively spliced isoforms and eight translational isoforms of GR. Post-translation modifications such as phosphorylation and acetylation also produce a variety of tissue-specific localizations and differential GC sensitivities that allow for the plethora of GR functions (Review (10)). For instance, while CORT acts on GR in the PVN to inhibit CRH and thus the cascade that results in its secretion, it acts in the amygdala to increase CRH mRNA and activate the HPA axis (11). This makes it important to evaluate the function of GR in specific regions of the brain to understand it's site-specific role in modulating stress.

The effects of genetic GR disruption throughout the brain was first studied by Tronche et. al using Nestin-Cre mice, expressing Cre-recombinase in neural progenitor cells, to excise the exon 3 region of GR in GR floxed mice (GR^{NesCre}) (12). These mice display GR deletion during embryonic development in the entire central nervous system including the PVN (Figure. 2B). As a result, the GR^{NesCre} mice display extreme hyperactivity of the HPA axis that causes Cushing-like symptoms and decreased anxiety and despair-like behaviors (12).

Limiting GR disruption specifically in forebrain neurons during adulthood (FBGRKO) (Figure 2C) in male mice, on the other hand, demonstrated increased basal and stress-stimulated CORT and despair behavior in male mice, although there are differences between studies (Table 1) (9, 13, 14). For instance, whereas Boyle et al revealed increases in plasma CORT at nadir and peak in FBGRKO mice, Furay and

colleagues do not observe changes in peak CORT between control and FBGRKO mice. Moreover, whereas stress increases CORT levels of FBGRKO in both studies, the time line of this increase differs between studies. Given ultradian fluctuations in glucocorticoid secretion, the differences in these studies may be due to the time when blood samples were collected. However, both studies implicate a role for forebrain GR in negative feedback regulation in adult male mice (9, 13, 14). The fact that male FBGRKO mice displayed elevated levels of CORT despite intact GR in the PVN and pituitary confirmed the existence of extrahypothalamic negative feedback sites. Indeed, lesion studies as well as GR inhibition studies in the hippocampus and prefrontal cortex both disrupt HPA axis activity, identifying these regions as sites of GC feedback regulation in the CNS (6, 15–21). The despair behavior observed in male FBGRKO mice was reversed after chronic antidepressant treatment, with consequent increases in hippocampal MR expression (9). There are overlapping regions of MR and GR expression in limbic forebrain regions such as the hippocampus, prefrontal cortex, and amygdala, where MR has a role in appraisal processes and response selection (1, 22). The data in FBGRKO mice supports a role for MR in compensating for GR loss in the hippocampus to mediate therapeutic responses to antidepressants.

In contrast to male mice, female FBGRKO mice do not show any changes in HPA axis activity compared to controls despite the fact that they had the same level of GR deletion observed in males (23). Moreover, female FBGRKO mice do not exhibit the despair-like behaviors in the forced swim or sucrose preference tests that were observed in the males. These gender differences may be due to the influence of gonadal hormones on the HPA axis and stress sensitivity, or the possibility that the GR sites targeted in the

forebrain are differentially regulated in males and females. Differences in gender-specific glucocorticoid actions have been exemplified in the evaluation of GC effects on gene expression in the rat liver (24). The authors found unique sets of genes regulated by GR that were male-specific or female-specific genes that were induced to a higher degree in males than females or vice versa, and genes that were repressed to a higher degree in males than females or vice versa. When the pathways regulating some of these genes were evaluated, it was determined that GC-mediated anti-inflammatory actions were more effective in males than females, and this was caused by ovarian hormones suppressing GC-mediated anti-inflammatory faction. The data from this paper presents evidence for differential GC signaling in males and females that results in distinct gene expression patterns (24). Although this data explored gender differences in liver GR-mediated gene regulation, it is conceivable that the underlying concept can be extrapolated to other tissues as well.

The effects of global GR loss, as well as disruption in the CNS and forebrain, have been reviewed in detail in many articles (25–29). This dissertation will serve to evaluate studies of GR targeting along major regulatory sites of the HPA axis and comparisons of phenotypes between the different transgenic mice.

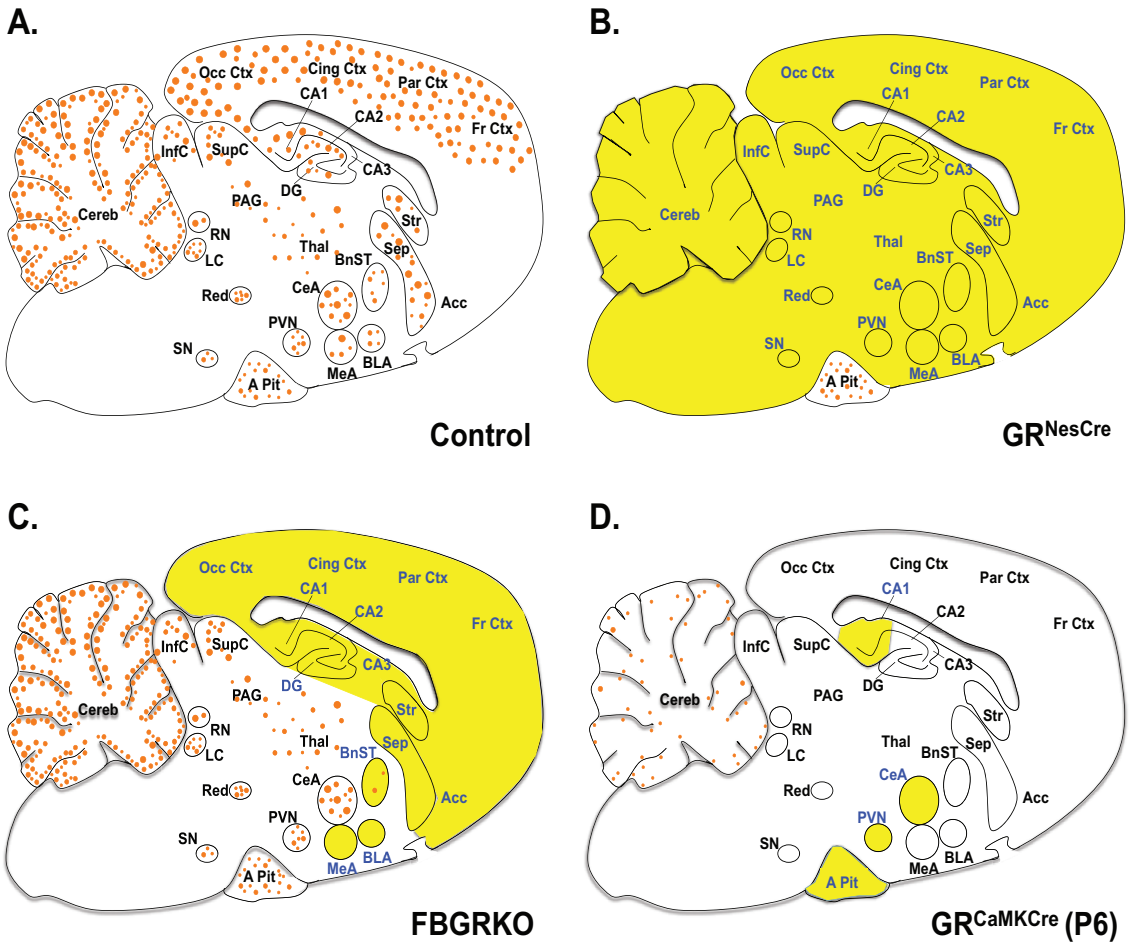

Figure 2. Glucocorticoid Receptor Expression in Genetic Mouse Models. Sagittal brain sections depicting areas of GR mRNA expression and deletion in mice with targeted GR deletion. Orange circles (●) represent GR targeted regions are in yellow highlight and blue font). (A) Adult control mice ubiquitously express GR mRNA in the entire brain and pituitary, with higher expression in limbic regions. (B) Adult GR^{NesCre} mice have GR loss in the entire brain. (C) FBGRKO mice have GR loss in forebrain nuclei during adulthood. (D) At postnatal day 6 GR expression is low and limited to few brain region (blue font). $GR^{CaMKCre}$ mice have GR loss in most of the brain and the pituitary at P6. ***Abbreviations***: Anterior pituitary, A Pit; Basolateral nucleus of the amygdala, BLA; Bed nucleus of the stria terminalis, BnST; Central nucleus of the amygdala, CeA; Hippocampal areas, CA1, CA2, CA3; Cerebellum, Cereb; Cingulate cortex, Cing Ctx; Dentate gyrus, DG; Frontal cortex, Fr Ctx; Inferior colliculus, InfC; Locus coeruleus, LC; Medial nucleus of the amygdala, MeA; Occipital cortex, Occ Ctx; Periaqueductal gray, PAG; Parietal cortex, Par Ctx; Paraventricular hypothalamic nucleus, PVN; Raphe nucleus, RN; Septum, Sep; Supraoptic nucleus, SN; Superior colliculus, SupC; Thalamus, Thal;

Corticotropin-releasing hormone

As a central stress hormone in the HPA axis pathway, CRH has been implicated in stress-induced psychiatric disorders, reproductive and cardiac function, as well as energy metabolism. In the context of psychiatric disorders, pathological increases in extrahypothalamic CRH expression is associated with the occurrence of post-traumatic stress disorder, major depression, anorexia nervosa, and anxiety disorders. One extrahypothalamic source of CRH is the central nucleus of the amygdala (CeA). Studies have provided evidence that CORT binding to GR in the CeA increases CRH mRNA synthesis there (30). Effects of CORT on CeA CRH expression have been further demonstrated in studies where CORT pellet implantation (5, 31) or repeated CORT injections (32) increases CRH mRNA in the CeA of rodent (3, 5, 31–33). Consequently, when GR is deleted in the CeA, it results in a subsequent decrease in the levels of CRH mRNA (34). The increased CRH mRNA in the amygdala leads to changes that heighten maladaptive emotional behavior (35).

Numerous brain functions are sensitive to stress and can be evaluated in behavioral paradigms that have been validated over the years. Two such brain functions are learning and memory, some aspects of which are mediated by the CeA(34). CeA lesions in rodents impair memory retention in the inhibitory avoidance task(36), the defensive burying task(37), and conditioned fear test(38). Although there are other peptides found in the CeA, such as neuropeptide Y, neurotensin, and enkephalin(39), the role of CRH as the major HPA axis activator in the stress response, as well as activator of the SNS (1) makes it a prime target of interest.

Early life stress increases the risk of psychiatric disorders later in life. Therefore, the developmental time period that stress occurs also plays a role in the neuroendocrine and behavioral outcomes(40). Adults who experienced some form of childhood stress show elevated basal cortisol, increases in ACTH responsiveness, and heightened emotional responses to stressful stimuli than control patients(40). In the CSF of these patients, increased CRH was predicted by perceived stress during pre-school years but not during preteen years(40). These models of early life stress have been recapitulated by studies of maternal deprivation in both primate and rodent models. The maternal deprivation model consists of taking the young away from the mothers a few hours a day for 2 weeks(41) and studying the behavior of the young when they reach adulthood. These studies show increased anxiety and depression behavior in rodents and primates that were maternally deprived(40, 42). In adulthood, maternally deprived primates show an increase in CSF CRH and rodents show increases in anxiety behavior(40). Furthermore, CRH mRNA in the amygdala and hypothalamus, and CRH immunoreactivity in the median eminence are increased due to maternal deprivation (41). This indicates that the time period during which one is exposed to a stress can affect the CRH system's response later in life. There is a need for animal models to explore temporal effects of CRH over-expression in the CeA.

The aforementioned evidence provides a solid basis for investigating the role of amygdalar CRH early in life. Isolating the effects of CeA CRH represents an avenue to discover its effects on neuroendocrine and behavioral correlates of stress to enhance understanding of CeA CRH in stress and psychiatric disorders.

RESEARCH OBJECTIVES

GR and CRH are important molecular regulators of an individual's ability to respond to stressful stimuli in an adaptive manner. Impaired signaling of both GR and CRH often leads to dysfunction of the hypothalamic-pituitary-adrenal axis, which underlies the etiology of many affective disorders such as anxiety and depression. Studies focusing on how GR and CRH influence the stress response are generalized to broad brain regions, thus hindering identification of how specific CNS nuclei contribute to maladaptive stress responses. Our research goals are to distinguish the site-specific involvement of GR and CRH in the stress response.

Part 1 of this dissertation focuses on the regulation of GR by the HPA axis activity during the stress response. The paraventricular nucleus (PVN) is a major site of negative feedback to coordinate the degree of the HPA axis activity with the magnitude of the exposed stressor. To define the function of endogenous PVN GR, we used Cre-*loxP* technology to disrupt different GR exons in *Sim1*-expressing neurons of the hypothalamus. Part 2 focuses on the use lentiviral (LV) vectors in combination with tetracycline transactivation system in transgenic mouse lines, to enable overexpression of CRH with spatial and temporal specificity.

The complete work in this dissertation represents an exploration of the neuroendocrine, molecular and behavioral consequences of GR and CRH signaling in mediating adaptation to stress.

REFERENCES

1. De Kloet ER, Joëls M, Holsboer F. 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6:463–75.

2. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

3. Ulrich-Lai YM, Herman JP. 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. 10:397–409.

4. Rodrigues SM, LeDoux JE, Sapolsky RM. 2009. The influence of stress hormones on fear circuitry. Annu. Rev. Neurosci. 32:289–313.

5. Makino S, Gold PW, Schulkin J. 1994. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Exeter Software.

6. Diorio D, Viau V, Meaney MJ. 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J. Neurosci. 13:3839–47.

7. Herman JP, Spencer R. 1998. Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. J. Neurosci. 18:7462–73.

8. Jankord R, Herman JP. 2008. Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Ann. N. Y. Acad. Sci. 1148:64–73.

9. Boyle MP, Brewer J a, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ. 2005. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc. Natl. Acad. Sci. U. S. A. 102:473–8.

10. Oakley RH, Cidlowski J a. 2013. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J. Allergy Clin. Immunol. 132:1033–44.

11. Lupien SJ, McEwen BS, Gunnar MR, Heim C. 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10:434–45.

12. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schütz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23:99–103.

13. Boyle MP, Kolber BJ, Vogt SK, Wozniak DF, Muglia LJ. 2006. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. J. Neurosci. 26:1971–8.

14. Furay AR, Bruestle AE, Herman JP. 2008. The role of the forebrain glucocorticoid receptor in acute and chronic stress. Endocrinology 149:5482–90.

15. Herman JP, Cullinan WE, Young E a, Akil H, Watson SJ. 1992. Selective forebrain fiber tract lesions implicate ventral hippocampal structures in tonic regulation of paraventricular nucleus corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression. Brain Res. 592:228–38.

16. Herman JP, Dolgas CM, Carlson SL. 1998. Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. Neuroscience 86:449–59.

17. Van Haarst AD, Oitzl MS, de Kloet ER. 1997. Facilitation of feedback inhibition through blockade of glucocorticoid receptors in the hippocampus. Neurochem. Res. 22:1323–8.

18. McKlveen JM, Myers B, Flak JN, Bundzikova J, Solomon MB, Seroogy KB, Herman JP. 2013. Role of prefrontal cortex glucocorticoid receptors in stress and emotion. Biol. Psychiatry 74:672–9.

19. Feldman S, Conforti N. 1985. Modifications of adrenocortical responses following frontal cortex simulation in rats with hypothalamic deafferentations and medial forebrain bundle lesions. Neuroscience 15:1045–7.

20. Jacobson L, Sapolsky R. 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr. Rev. 12:118–34.

21. Radley JJ, Kabbaj M, Jacobson L, Heydendael W, Yehuda R, Herman JP. 2011. Stress risk factors and stress-related pathology: neuroplasticity, epigenetics and endophenotypes. Stress 14:481–97.

22. Joëls M, Sarabdjitsingh RA, Karst H. 2012. Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes. Pharmacol. Rev. 64:901–38.

23. Solomon MB, Furay a R, Jones K, Packard a EB, Packard B a, Wulsin a C, Herman JP. 2012. Deletion of forebrain glucocorticoid receptors impairs neuroendocrine stress responses and induces depression-like behavior in males but not females. Neuroscience 203:135–43.

24. Duma D, Collins JB, Chou JW, Cidlowski JA. 2010. Sexually dimorphic actions of glucocorticoids provide a link to inflammatory diseases with gender differences in prevalence. Sci. Signal. 3:ra74.

25. Arnett MG, Kolber BJ, Boyle MP, Muglia LJ. 2011. Behavioral insights from mouse models of forebrain--and amygdala-specific glucocorticoid receptor genetic disruption. Mol. Cell. Endocrinol. 336:2–5.

26. Kolber BJ, Muglia LJ. 2009. Defining brain region-specific glucocorticoid action during stress by conditional gene disruption in mice. Brain Res. 1293:85–90.

27. Kolber BJ, Wieczorek L, Muglia LJ. 2008. Hypothalamic-pituitary-adrenal axis dysregulation and behavioral analysis of mouse mutants with altered glucocorticoid or mineralocorticoid receptor function. Stress 11:321–38.

28. Kellendonk C, Gass P, Kretz O, Schütz G, Tronche F. 2002. Corticosteroid receptors in the brain: gene targeting studies. Brain Res. Bull. 57:73–83.

29. Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Brühl B, Beier K, Metz J, Garcia-Arraras JE, Roig-Lopez JL, Monaghan P, Schmid W, Cole TJ, Kellendonk C, Tronche F, Schütz G, Unsicker K. 1999. Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. Development 126:2935–44.

30. Makino S, Hashimoto K, Gold PW. 2002. Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol. Biochem. Behav. 73:147–58.

31. Shepard J. 2000. Corticosterone delivery to the amygdala increases corticotropin-releasing factor mRNA in the central amygdaloid nucleus and anxiety-like behavior. Brain Res. 861:288–295.

32. Thompson B. 2004. Corticosterone facilitates retention of contextually conditioned fear and increases CRH mRNA expression in the amygdala. Behav. Brain Res. 149:209–215.

33. Van Den Eede F, Van Broeckhoven C, Claes SJ. 2005. Corticotropin-releasing factor-binding protein, stress and major depression. Ageing Res. Rev. 4:213–39.

34. Kolber BJ, Roberts MS, Howell MP, Wozniak DF, Sands MS, Muglia LJ. 2008. Central amygdala glucocorticoid receptor action promotes fear-associated CRH activation and conditioning. Proc. Natl. Acad. Sci. U. S. A. 105:12004–9.

35. Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME. 2009. Continuous expression of

corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol. Psychiatry 14:37–50.

36. Roozendaal B, McGaugh JL. 1996. Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. Neurobiol. Learn. Mem. 65:1–8.

37. ROOZENDAAL B, KOOLHAAS J, BOHUS B. 1991. Central amygdala lesions affect behavioral and autonomic balance during stress in rats. Physiol. Behav. 50:777–781.

38. Pitts MW, Todorovic C, Blank T, Takahashi LK. 2009. The central nucleus of the amygdala and corticotropin-releasing factor: insights into contextual fear memory. J. Neurosci. 29:7379–88.

39. Sah P, Faber ESL, Lopez De Armentia M, Power J. 2003. The amygdaloid complex: anatomy and physiology. Physiol. Rev. 83:803–34.

40. Carpenter LL, Tyrka AR, McDougle CJ, Malison RT, Owens MJ, Nemeroff CB, Price LH. 2004. Cerebrospinal fluid corticotropin-releasing factor and perceived early-life stress in depressed patients and healthy control subjects. Neuropsychopharmacology 29:777–784.

41. Ladd CO, Owens MJ, Nemeroff CB. 1996. Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. Endocrinology 137:1212–1218.

42. Aisa B, Tordera R, Lasheras B, Del Río J, Ramírez MJ. 2008. Effects of maternal separation on hypothalamic-pituitary-adrenal responses, cognition and vulnerability to stress in adult female rats. Neuroscience 154:1218–26.

PART 1

Glucocorticoid Receptor Regulation of Stress Pathways

CHAPTER I

INTRODUCTION TO GLUCOCORTICOID RECEPTOR MEDIATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

INTRODUCTION

Glucocorticoids (GCs) are endogenous steroid hormones that were first identified circa 1929 when studies in adrenalectomized animals demonstrated that adrenal cortex extracts ameliorated the effects of adrenal insufficiency (1–3). Two decades later, GCs isolated from the adrenal cortex were shown to treat symptoms of Addison's disease and rheumatoid arthritis leading to the Nobel Prize in Physiology for Edward Kendall, Tadeus Reichstein, and Philip Hench in 1950 (4–6). Since then, GC function has been demonstrated as important in the stress response for promoting coping and adaptation to stress as well as recovery from the stressor (7–12). In addition, GC function has been demonstrated in metabolism, reproduction, and inflammatory and immune responses (9, 13). As a steroid hormone, GC synthesis begins in the adrenal gland from cholesterol. Specifically, in the adrenal cortex, cholesterol is transported into the mitochondria by the steroidogenic acute regulatory (StAR) transport protein (14). In the mitochondria, a series of enzymatic reactions ultimately results in the conversion of 11-deoxycortisol by 11β-hydroxylase (CYP11B1) into cortisol (humans) or corticosterone (rodents) (15). Embryonic GC synthesis begins as early as E14.5 in mice (16) and at approximately 50 days post-conception in humans (17). Secretion of GCs is regulated in a circadian rhythm, through the suprachiasmatic nucleus of the hypothalamus (SCN) function, and by stress. Secretion of GCs shows ultradian and circadian regulation as well as

modulation by stress. Circadian regulation of GC results in a diurnal rhythm of GC secretion that has a 24-hour period and is governed by function of the suprachiasmatic nucleus of the hypothalamus (SCN) (18, 19)

The SCN is a located in the anterior hypothalamus just dorsal to the optic chiasm. It receives light input from the optic region through the retinohypothalamic tract. This light input synchronizes the SCN to cause the generation of the 24-hour circadian cycle of biological processes that regulates mammalian physiological homeostasis. One of the most robust endocrine processes governed in a circadian fashion is the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the major output of which is GC production. Neuronal tracing has provided evidence showing that arginine vasopressin (AVP) neurons from the SCN project into the paraventricular nucleus of the hypothalamus (PVN) through the dorsal hypothalamus and regulates the circadian rhythm of corticotropin-releasing hormone (CRH) (20, 21). The circadian release of CRH then regulates adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary, which subsequently triggers the secretion of corticosterone in rodents and cortisol in humans (CORT) from the adrenal cortex. This circadian rhythmicity is confirmed in SCN lesion studies that result in abolished CORT rhythms (22–24) as well as disrupting secretion of CRH and ACTH (25, 26). CORT secretion however does not occur only through the HPA axis drive. Studies suggest that the adrenal cortex contains its own endogenous clock system that regulates circadian GC secretion that is independent of HPA axis activity but is dependent upon the SCN (27–29). This adrenal modulation may be mediated by multisynaptic projections from the SCN through the autonomic nervous system to the adrenal cortex (30). The result is a diurnal rhythm of CORT secretion over

a 24-hour period that provides a basis for an organism's daily internal homeostasis. Underlying this circadian regulation is an ultradian rhythm of pulsatile CORT secretion throughout the day that is independent of SCN or stress-inputs. This pulsatility is crucial for stress responsiveness and can vary in frequency and amplitude as a consequence of feedback latency (31, 32)

The circadian rise in GCs occurs immediately prior to the onset of the active period in mammals. In humans, this increase occurs in the morning before awakening, while in nocturnal animals CORT rise occurs in the evening before their active period begins (33–35). This awakening rise is CORT may promote mobilization of necessary energy to begin the activity necessary to carry out daily function (7). In order to mediate its functions, GCs act on two major receptors, type 1 high affinity receptors, mineralocorticoid receptors (MR) and type 2 low affinity receptors, GC receptors (GR) (36). These receptors have different spatial distribution in the brain and periphery, with GR being more broadly distributed and serving as the main receptor in times of stress (37). Stress activation of the HPA axis serves to prepare an organism to respond to a perceived threat and then to permit adaption that reinstates homeostasis (10). This latter function is especially important because chronic HPA axis activation leads to constantly elevated GC levels, which is detrimental to an organism. Negative feedback of GCs on the HPA axis is therefore essential to inhibit the damaging effects of CORT.

GR MECHANISM OF ACTION

Since steroid hormones such as CORT are highly lipophilic, they are able to cross the plasma membrane and enter the cytoplasm of a cell through simple diffusion. Once CORT is inside a cell, it binds to GRs that are initially inactively bound to heat shock protein 90 (HSP90). The binding of CORT to GR results in a conformational change that releases GR from inactivation and causes two major modes of GR action. One mode of GR action occurs through non-genomic signaling pathways, that produce fast-acting effects in the order of seconds to a few minutes (38, 39). This occurs through GC action on membrane bound receptors or cytoplasmic GR that activate rapid signaling of second messenger pathways (13, 40–43). The other mode is the classical GR function which occurs when activated GR translocates into the nucleus and functions as a transcription factor to modulate genomic signaling pathways, which can take a period of 15 minutes to several hours. GR can act as a homodimer, monomer, or heterodimer (with MR or other transcription factors) to regulate gene transcription. Direct binding of GR to positive or negative GC-response elements (GRE) on target genes serves to activate or repress transcription respectively (44). In certain cases some genes require DNA-bound GR to physically interact with another transcription factor adjacently bound to the gene of interest for transcriptional activity to occur. Indirect DNA binding by GR monomers through tethering in a protein-protein interaction to DNA-bound transcription factors, such as AP-1, STAT5, and NF-KB also regulates gene expression (44, 45). In general, dimerization of GR and its binding to GRE results in transactivation GR-responsive genes, while monomeric GR interactions to other transcription factors leads to transrepression of genes (46). Indeed, mutations in the DNA-binding domain of GR that

prevents its ability to dimerize, disrupts GR's transactivation function but not its transrepressive function (47). There is also evidence of a GR trimer bound to POMC that results in transrepression of the POMC gene (48). Heterodimers of GR with MR have been identified to bind GRE and regulate gene transactivation with different kinetics than GR homodimers (49–51). Elevated levels of GC have been shown to increase the likelihood of GR-MR heterodimerization (52). This implies that variable levels of GC may result in differences in homodimer and heterodimer formation to contribute to the fine-tuning of regulation of GC-responsive genes (53). The variety in GR signaling, as a monomer, homodimer, heterodimer, or trimer, is demonstrative of the ability of GC signaling to modulate diverse functions in an organism (54).

While GCs are important for the treatment of many disorders, their chronic use, especially systemic administration**,** can result in mood disorders, osteoporosis, obesity, metabolic syndrome, and reduced immune system function (55–58).Therefore, regulation of the HPA axis by CORT is extremely important in preventing maladaptive phenotypes and maintaining normal mammalian function. Disrupted GC regulation is observed in a number of psychiatric and metabolic disorders. Major sites of negative feedback regulation are the PVN and anterior pituitary, although the hippocampus and prefrontal cortex also mediate GC feedback.

CLINICAL EFFECTS OF IMPAIRED GC SIGNALING

The human GR gene, NR3C1, is located on the short arm of chromosome 5, 5q31–32 and undergoes alternative RNA splicing and translation initiation to yield multiple functionally distinct isoforms. The two most abundant isoforms generated are GRα and GRβ, with GRα being expressed at higher levels in most tissues (44). Alternative translation start sites of the human GR mRNA produce translational isoforms of GRα, GR-A, -B, -C1, -C2, -C3, -D1, -D2, and -D3, also each have their own unique distribution patterns and gene targets (44, 59). This selectivity may underlie the varied GC responses amongst tissues and patients. The multiple GR isoforms, alternative processing, tissue and cell-specific expression patterns and post-translational modifications demonstrate how the GR gene can be modified to produce a maximum range of functions. In the rodent studies described in prior sections, GR disruption along negative feedback sites reduces GR expression in specific brain regions and is associated with abnormal HPA axis regulation that affects anxiety, despair, and metabolic phenotypes. In this section, we briefly explore studies demonstrating that GR disruption and its effects are translatable to human psychiatric and metabolic diseases.

Psychiatric Disorders

As the end products of the HPA axis, GCs bind to GRs at the level of the hypothalamus and anterior pituitary to exert negative feedback effects and maintain homeostasis in response to stress. Increased cortisol levels and an inability to suppress cortisol secretion in the DST are known hallmarks of a number of psychiatric disorders including schizophrenia, bipolar disorder, generalized anxiety, and major depressive

disorders (60–66). Individuals with major psychiatric illness often display hypercortisolemia (67, 68) and a down-regulation in GR mRNA in a network of regions including the hippocampus, amygdala, and temporal cortex (69–71).

Major depressive disorder (MDD) is characterized by increased release of GCs and hyperactivity of the HPA axis (72, 73). A primary feature of this hyperactivity is non-suppression of cortisol secretion in response to administration of the synthetic glucocorticoid, dexamethasone. Patients with MDD display a reduction in the number and function of GR (69, 74–78). Many studies have addressed the role of GR abnormalities in the pathophysiology of MDD by examining the effects of antidepressants on GR expression and function and HPA axis activity (79). Indeed, impaired GR function has been suggested to be causal for HPA axis hyperactivity in depression, as GCs typically regulate the HPA axis through negative feedback inhibition and thereby reduce the production of GCs themselves. This effect is thought to be mediated in part by GR. Therefore, hyperactivity of the HPA axis has been explained by an impaired feedback inhibition of GCs, possibly due to an impaired or dysfunctional GR ("glucocorticoid resistance") (80). In support of this, data in numerous studies of antidepressant treatment on rodents have demonstrated increased GR expression in the hypothalamic and hippocampal brain regions, which may indicate a mode for antidepressant mediated HPA axis normalization (81–84).

The non-coding exon 1 of GR contains a 5' untranslated region (UTR) that determines tissue specific production of GR. Epigenetic modifications such reduced DNA methylation of the GR promoter in this 5' UTR have been observed in rodent models of low maternal care and are associated with increased risk for addiction,

depression, diabetes and obesity (85, 86). The mechanism of action due to increased methylation of NGF1-A disrupts its binding to $hGR1_7$ promoter and thus causes a reduction of GR transcription (87, 88). Moreover, expression of other variants, decreased $hGR1_B$ and $hGR1_C$ but increased $hGR1_H$, due to increased methylation have also been identified (86, 89). Specifically, these GR variants are reduced and correlate with decreased total GR in the hippocampus of suicide victims only when they have a history of childhood abuse. The idea that GC excess early in life can reprogram HPA axis reactivity in part through decreasing GR expression in specific brain regions that are responsible for mediating negative feedback. In support of this concept, fetal GC excess in humans has been found to increase the risk for schizophrenia in adulthood (90). Moreover increased fetal GCs due to maternal obesity or malnutrition also increases the risk of schizophrenia when the child grows into an adult (91–94). Post mortem analysis shows GR mRNA is decreased in the brain of patients with schizophrenia and depression (95). These data represent a convergence between psychiatric and metabolic diseases both associated with increased GC levels, potentially the result of disrupted GR expression hindering negative GC feedback.

A number of single nucleotide polymorphisms (SNPs) have been identified in GR, namely Bcl1, N363S, and ER22/23EK, that cause changes in GC sensitivity (Review (96, 97)). Bcl1 and N363S are associated with increased GC sensitivity, while ER22/23EK associates with reduced GC sensitivity. Moreover some of these SNPs are associated with HPA axis disruption, including altered cortisol and ACTH secretion after stress, and may modify the risk of depression (97). SNPs in genes that mediate GR function have also been observed to associate with psychiatric disorders. For instance,

FKBP5, is a GR chaperone protein that prevents nuclear translocation and reduced GR affinity to GC (98). An increase in FKBP5 due to a SNP results in impaired GR-mediated negative feedback and GC resistance and is found to be associated with PTSD and MDD (99–101). In patients with MDD, decreased hippocampal volume and increased inflammatory cytokines are associated with decreased expression of genes targeted by GR activation (102). These data point to a prime role of regulation of GR function in mediating neuroendocrine and histopathology that affect the development of psychiatric disorders.

Metabolic Disorders

GC activation of GR regulates a variety of important cardiovascular, metabolic and immunologic functions. Synthetic GCs, including prednisone/prednisolone and dexamethasone, are widely prescribed anti-inflammatory drugs used to treat a number of immune and inflammatory diseases including allergies, asthma, lupus and rheumatoid arthritis. Although therapeutic benefits of GCs are vast, long-term use of GCs results in negative side effects including muscle wasting, abdominal fat deposition, and insulin resistance. In addition, some patients do not respond to GC therapy and those that do may develop GC resistance. Moreover, discontinuation of long-term GC therapy has been shown to result in increased risk of delirium and depression in 10% of users (103). Tissue-specific GC resistance has been associated with rheumatoid arthritis, osteoarthritis, Crohn's disease, ulcerative colitis and asthma (104). Impaired negative feedback of GC along the HPA axis that leads to excess circulating GCs may contribute to the development of metabolic disorders. Excess GCs have been observed in metabolic

syndrome cluster diseases including Cushing's syndrome, obesity, type II diabetes, and insulin resistance.

Bioactive concentrations of plasma CORT are regulated, not only by negative feedback, but also by the expression of the corticosteroid binding globulin (CBG) and 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11β-HSD 1/2) (105). These proteins have tissue-specific expression that govern tissue-specific activity of GCs. CBG binding to plasma CORT and sequester its diffusion into cells to facilitate transport through circulation, thus governing the bioavailability of CORT. Low levels of CBG have been identified in stress states as well as adipose tissue of obese rats (106–108). 11β-HSD1 is the enzyme that converts the precursor 11β-deoxycortisol or 11β-deoxycorticosterone to cortisol and corticosterone, respectively. 11β-HSD2 on the other hand, mediates the reverse reaction of CORT to inactive metabolites (105). Disruption of the availability of these regulatory proteins have been linked to obesity and insulin resistance (109–114). To understand how disruption of GR along the negative feedback sites contribute to metabolic disorders, future studies in the previously described genetic models will be needed to evaluate CBG, 11β-HSD1/2, and proteins involved in metabolic activity in more detail.

CONCLUSIONS

Circadian regulation of GC signaling is necessary for maintenance of normal physiological function while stress regulation of GC signaling represents a pathway to manage disruptions in homeostasis. The paradox of stress regulation is that it can impart beneficial effects but it can also be detrimental when not turned off. We've briefly discussed some clinical effects of GCs on psychological and metabolic function in humans. The understanding of molecular activity in humans is largely governed by understanding in animal models. The next chapters will discuss the detrimental effects of stress due to impaired negative regulation of GCs in a number of genetically altered rodent models. We strive to clarify the importance of glucocorticoids and the HPA axis in mediating stress reactivity in terms of endocrine output, behavioral activity, and energy balance.

REFERENCES

1. Hartman FA, Brownell KA. 1930. The hormone of the adrenal cortex. Science 72:76.

2. DeFremery P, Laqueur E, Reichstein T. 1937. Corticosteron, a crystallized compound with the biological activity of the adrenalcortical hormone. Nature 139:26.

3. Reichstein T, Verzar F, Laszt L. 1937. Activity of Corticosteron in the Glucose Test in Rats. Nature 139:331.

4. Hench PS, Kendall EC. 1949. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc. Staff Meet. Mayo Clin. 24:181–97.

5. Kendall EC. 1951. The development of cortisone as a therapeutic agent. Indian Med. J. 45:239–41.

6. Raju TN. 1999. The Nobel Chronicles. Lancet 353:1370.

7. Munck A, Guyre PM, Holbrook NJ. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. 5:25–44.

8. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

9. McEwen BS. 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol. Rev. 87:873–904.

10. De Kloet ER, Joëls M, Holsboer F. 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6:463–75.

11. Joëls M, Sarabdjitsingh RA, Karst H. 2012. Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes. Pharmacol. Rev. 64:901–38.

12. Herman JP, Cullinan WE. 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. 20:78–84.

13. Spies CM, Strehl C, van der Goes MC, Bijlsma JWJ, Buttgereit F. 2011. Glucocorticoids. Best Pract. Res. Clin. Rheumatol. 25:891–900.

14. Miller WL. 2007. StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. Mol. Endocrinol. 21:589–601.

15. Payne AH, Hales DB. 2004. Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones. Endocr. Rev. 25:947–70.

16. Michelsohn a M, Anderson DJ. 1992. Changes in competence determine the timing of two sequential glucocorticoid effects on sympathoadrenal progenitors. Neuron 8:589–604.

17. Goto M, Piper Hanley K, Marcos J, Wood PJ, Wright S, Postle AD, Cameron IT, Mason JI, Wilson DI, Hanley NA. 2006. In humans, early cortisol biosynthesis provides a mechanism to safeguard female sexual development. J. Clin. Invest. 116:953–60.

18. Lightman SL. 2008. The neuroendocrinology of stress: a never ending story. J. Neuroendocrinol. 20:880–4.

19. Young EA, Abelson J, Lightman SL. 2004. Cortisol pulsatility and its role in stress regulation and health. Front. Neuroendocrinol. 25:69–76.

20. Kalsbeek a, van Heerikhuize JJ, Wortel J, Buijs RM. 1996. A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1 antagonist. J. Neurosci. 16:5555–65.

21. Tonsfeldt KJ, Chappell PE. 2012. Clocks on top: the role of the circadian clock in the hypothalamic and pituitary regulation of endocrine physiology. Mol. Cell. Endocrinol. 349:3–12.

22. Krieger DT, Hauser H, Krey LC. 1977. Suprachiasmatic nuclear lesions do not abolish food-shifted circadian adrenal and temperature rhythmicity. Science 197:398–9.

23. Moore RY, Eichler VB. 1972. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. 42:201–6.

24. Raisman G, Brown-Grant K. 1977. The “suprachiasmatic syndrome”: endocrine and behavioural abnormalities following lesions of the suprachiasmatic nuclei in the female rat. Proc. R. Soc. Lond. B. Biol. Sci. 198:297–314.

25. Szafarczyk a, Ixart G, Alonso G, Malaval F, Nouguier-Soulé J, Assenmacher I. 1983. CNS control of the circadian adrenocortical rhythm. J. Steroid Biochem. 19:1009–15.

26. Cascio CS, Shinsako J, Dallman MF. 1987. The suprachiasmatic nuclei stimulate evening ACTH secretion in the rat. Brain Res. 423:173–8.

27. Bittman EL, Doherty L, Huang L, Paroskie A. 2003. Period gene expression in mouse endocrine tissues. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285:R561–9.

28. Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nakahara D, Tsujimoto G, Okamura H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell Metab. 2:297–307.

29. Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW, Eichele G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab. 4:163–73.

30. Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ, Kalsbeek A. 1999. Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur. J. Neurosci. 11:1535–44.

31. Walker JJ, Terry JR, Lightman SL. 2010. Origin of ultradian pulsatility in the hypothalamic-pituitary-adrenal axis. Proc. Biol. Sci. 277:1627–33.

32. Waite EJ, McKenna M, Kershaw Y, Walker JJ, Cho K, Piggins HD, Lightman SL. 2012. Ultradian corticosterone secretion is maintained in the absence of circadian cues. Eur. J. Neurosci. 36:3142–50.

33. Schulz P, Kirschbaum C, Prüßner J, Hellhammer D. 1998. Increased free cortisol secretion after awakening in chronically stressed individuals due to work overload. Stress Med. 14:91–97.

34. Pruessner JC, Wolf OT, Hellhammer DH, Buske-Kirschbaum A, von Auer K, Jobst S, Kaspers F, Kirschbaum C. 1997. Free Cortisol Levels after Awakening: A Reliable Biological Marker for the Assessment of Adrenocortical Activity. Life Sci. 61:2539–2549.

35. Clow A, Hucklebridge F, Stalder T, Evans P, Thorn L. 2010. The cortisol awakening response: more than a measure of HPA axis function. Neurosci. Biobehav. Rev. 35:97–103.

36. Kellendonk C, Gass P, Kretz O, Schütz G, Tronche F. 2002. Corticosteroid receptors in the brain: gene targeting studies. Brain Res. Bull. 57:73–83.

37. Reul JM, de Kloet ER. 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505–11.

38. De Kloet ER, Karst H, Joëls M. 2008. Corticosteroid hormones in the central stress response: quick-and-slow. Front. Neuroendocrinol. 29:268–72.

39. Groeneweg FL, Karst H, de Kloet ER, Joëls M. 2011. Rapid non-genomic effects of corticosteroids and their role in the central stress response. J. Endocrinol. 209:153–67.

40. Cato ACB, Nestl A, Mink S. 2002. Rapid actions of steroid receptors in cellular signaling pathways. Sci. STKE 2002:re9.

41. Gametchu B, Watson CS, Pasko D. 1991. Size and steroid-binding characterization of membrane-associated glucocorticoid receptor in S-49 lymphoma cells. Steroids 56:402–410.

42. Zhang Y, Sheng H, Qi J, Ma B, Sun J, Li S, Ni X. 2012. Glucocorticoid acts on a putative G protein-coupled receptor to rapidly regulate the activity of NMDA receptors in hippocampal neurons. Am. J. Physiol. Endocrinol. Metab. 302:E747–58.

43. Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester G-R, Lauster R, Scheffold A, Buttgereit F. 2004. Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. FASEB J. 18:70–80.

44. Oakley RH, Cidlowski J a. 2013. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J. Allergy Clin. Immunol. 132:1033–44.

45. Polman JAE, Welten JE, Bosch DS, de Jonge RT, Balog J, van der Maarel SM, de Kloet ER, Datson NA. 2012. A genome-wide signature of glucocorticoid receptor binding in neuronal PC12 cells. BMC Neurosci. 13:118.

46. Kumar R, Thompson EB. 2005. Gene regulation by the glucocorticoid receptor: structure:function relationship. J. Steroid Biochem. Mol. Biol. 94:383–94.

47. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schütz G. 1998. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93:531–41.

48. Drouin J, Sun YL, Chamberland M, Gauthier Y, De Léan a, Nemer M, Schmidt TJ. 1993. Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. EMBO J. 12:145–56.

49. Liu W, Wang J, Sauter NK, Pearce D. 1995. Steroid receptor heterodimerization demonstrated in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A. 92:12480–4.

50. Trapp T, Rupprecht R, Castrén M, Reul JMHM, Holsboer F. 1994. Heterodimerization between mineralocorticoid and glucocorticoid receptor: A new principle of glucocorticoid action in the CNS. Neuron 13:1457–1462.

51. Trapp T, Rupprecht R, Castrén M, Reul JM, Holsboer F. 1994. Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS. Neuron 13:1457–62.

52. Nishi M, Tanaka M, Matsuda K, Sunaguchi M, Kawata M. 2004. Visualization of glucocorticoid receptor and mineralocorticoid receptor interactions in living cells with GFP-based fluorescence resonance energy transfer. J. Neurosci. 24:4918–27.

53. Nishi M. 2011. Dynamics of corticosteroid receptors: lessons from live cell imaging. Acta Histochem. Cytochem. 44:1–7.

54. Trapp T, Holsboer F. 1996. Heterodimerization between mineralocorticoid and glucocorticoid receptors increases the functional diversity of corticosteroid action. Trends Pharmacol. Sci. 17:145–149.

55. Goldstein MF. 1999. Chronic Glucocorticoid Therapy-Induced Osteoporosis in Patients With Obstructive Lung Disease*. Chest 116:1733–1749.

56. Wolkowitz OM, Burke H, Epel ES, Reus VI. 2009. Glucocorticoids. Mood, memory, and mechanisms. Ann. N. Y. Acad. Sci. 1179:19–40.

57. Biddie SC, Conway-Campbell BL, Lightman SL. 2012. Dynamic regulation of glucocorticoid signalling in health and disease. Rheumatology (Oxford). 51:403–12.

58. Marques AH, Silverman MN, Sternberg EM. 2009. Glucocorticoid dysregulations and their clinical correlates. From receptors to therapeutics. Ann. N. Y. Acad. Sci. 1179:1–18.

59. Oakley RH, Webster JC, Sar M, Parker CR, Cidlowski JA. 1997. Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. Endocrinology 138:5028–38.

60. Gibbons JL. 1964. Cortisol secretion rate in depressive illness. Arch. Gen. Psychiatry 10:572–5.

61. Carroll BJ, Feinberg M, Greden JF, Haskett RF, James NM, Steiner M, Tarika J. 1980. Diagnosis of endogenous depression. Comparison of clinical, research and neuroendocrine criteria. J. Affect. Disord. 2:177–94.

62. Beck-Friis J, Ljunggren JG, Thorén M, von Rosen D, Kjellman BF, Wetterberg L. 1985. Melatonin, cortisol and ACTH in patients with major depressive disorder

and healthy humans with special reference to the outcome of the dexamethasone suppression test. Psychoneuroendocrinology 10:173–86.

63. Lesch KP, Laux G, Schulte HM, Pfüller H, Beckmann H. 1988. Corticotropin and cortisol response to human CRH as a probe for HPA system integrity in major depressive disorder. Psychiatry Res. 24:25–34.

64. López JF, Chalmers DT, Little KY, Watson SJ. 1998. A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biol. Psychiatry 43:547–73.

65. Rubin RT, Phillips JJ, McCracken JT, Sadow TF. 1996. Adrenal gland volume in major depression: relationship to basal and stimulated pituitary-adrenal cortical axis function. Biol. Psychiatry 40:89–97.

66. Owens MJ, Nemeroff CB. 1993. The role of corticotropin-releasing factor in the pathophysiology of affective and anxiety disorders: laboratory and clinical studies. Ciba Found. Symp. 172:296–308; discussion 308–16.

67. Linkowski P, Kerkhofs M, Van Onderbergen A, Hubain P, Copinschi G, L'Hermite-Balériaux M, Leclercq R, Brasseur M, Mendlewicz J, Van Cauter E. 1994. The 24-hour profiles of cortisol, prolactin, and growth hormone secretion in mania. Arch. Gen. Psychiatry 51:616–24.

68. Mück-Seler D, Pivac N, Jakovljević M, Brzović Z. 1999. Platelet serotonin, plasma cortisol, and dexamethasone suppression test in schizophrenic patients. Biol. Psychiatry 45:1433–9.

69. Webster MJ, Knable MB, O'Grady J, Orthmann J, Weickert CS. 2002. Regional specificity of brain glucocorticoid receptor mRNA alterations in subjects with schizophrenia and mood disorders. Mol. Psychiatry 7:985–94, 924.

70. Perlman WR, Webster MJ, Kleinman JE, Weickert CS. 2004. Reduced glucocorticoid and estrogen receptor alpha messenger ribonucleic acid levels in the amygdala of patients with major mental illness. Biol. Psychiatry 56:844–52.

71. Sinclair D, Tsai SY, Woon HG, Weickert CS. 2011. Abnormal glucocorticoid receptor mRNA and protein isoform expression in the prefrontal cortex in psychiatric illness. Neuropsychopharmacology 36:2698–709.

72. Pariante CM, Lightman SL. 2008. The HPA axis in major depression: classical theories and new developments. Trends Neurosci. 31:464–8.

73. Holsboer F. 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23:477–501.

74. Whalley LJ, Borthwick N, Copolov D, Dick H, Christie JE, Fink G. 1986. Glucocorticoid receptors and depression. Br. Med. J. (Clin. Res. Ed). 292:859–61.

75. Yehuda R, Boisoneau D, Mason JW, Giller EL. 1993. Glucocorticoid receptor number and cortisol excretion in mood, anxiety, and psychotic disorders. Biol. Psychiatry 34:18–25.

76. Sallee FR, Nesbitt L, Dougherty D, Hilal R, Nandagopal VS, Sethuraman G. 1995. Lymphocyte glucocorticoid receptor: predictor of sertraline response in adolescent major depressive disorder (MDD). Psychopharmacol. Bull. 31:339–45.

77. Gormley GJ, Lowy MT, Reder AT, Hospelhorn VD, Antel JP, Meltzer HY. 1985. Glucocorticoid receptors in depression: relationship to the dexamethasone suppression test. Am. J. Psychiatry 142:1278–84.

78. Lowy MT, Reder AT, Gormley GJ, Meltzer HY. 1988. Comparison of in vivo and in vitro glucocorticoid sensitivity in depression: relationship to the dexamethasone suppression test. Biol. Psychiatry 24:619–30.

79. Pariante CM, Miller AH. 2001. Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. Biol. Psychiatry 49:391–404.

80. Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TWW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, Binder EB. 2013. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat. Neurosci. 16:33–41.

81. Reul JM, Stec I, Söder M, Holsboer F. 1993. Chronic treatment of rats with the antidepressant amitriptyline attenuates the activity of the hypothalamic-pituitary-adrenocortical system. Endocrinology 133:312–20.

82. Reul JM, Labeur MS, Grigoriadis DE, De Souza EB, Holsboer F. 1994. Hypothalamic-pituitary-adrenocortical axis changes in the rat after long-term treatment with the reversible monoamine oxidase-A inhibitor moclobemide. Neuroendocrinology 60:509–19.

83. Heydendael W, Jacobson L. 2008. Differential effects of imipramine and phenelzine on corticosteroid receptor gene expression in mouse brain: potential relevance to antidepressant response. Brain Res. 1238:93–107.

84. Seckl JR, Fink G. 1992. Antidepressants increase glucocorticoid and mineralocorticoid receptor mRNA expression in rat hippocampus in vivo. Neuroendocrinology 55:621–6.

85. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7:847–54.

86. Zhang TY, Labonté B, Wen XL, Turecki G, Meaney MJ. 2013. Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. Neuropsychopharmacology 38:111–23.

87. Weaver IC, D'Alessio A. 2007. The Transcription Factor Nerve Growth Factor-Inducible Protein A Mediates Epigenetic Programming: Altering Epigenetic Marks by Immediate-Early Genes. J. Neurosci. 27:1756–1768.

88. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, Turecki G, Meaney MJ. 2009. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat. Neurosci. 12:342–8.

89. Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M, Turecki G. 2012. Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. Biol. Psychiatry 72:41–8.

90. Koenig JI, Kirkpatrick B, Lee P. 2002. Glucocorticoid hormones and early brain development in schizophrenia. Neuropsychopharmacology 27:309–18.

91. Khandaker GM, Dibben CRM, Jones PB. 2012. Does maternal body mass index during pregnancy influence risk of schizophrenia in the adult offspring? Obes. Rev. 13:518–27.

92. Lesage J, Blondeau B, Grino M, Bréant B, Dupouy JP. 2001. Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary adrenal axis in the newborn rat. Endocrinology 142:1692–702.

93. Reynolds RM, Labad J, Buss C, Ghaemmaghami P, Räikkönen K. 2013. Transmitting biological effects of stress in utero: implications for mother and offspring. Psychoneuroendocrinology 38:1843–9.

94. Sasaki A, de Vega WC, St-Cyr S, Pan P, McGowan PO. 2013. Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood. Neuroscience 240:1–12.

95. Knable MB, Torrey EF, Webster MJ, Bartko JJ. 2001. Multivariate analysis of prefrontal cortical data from the Stanley Foundation Neuropathology Consortium. Brain Res. Bull. 55:651–9.

96. DeRijk, R., de Kloet ER. 2005. Corticosteroid receptor genetic polymorphisms and stress responsivity. Endocrine 28:263–70.

97. Zobel A, Jessen F, von Widdern O, Schuhmacher A, Höfels S, Metten M, Rietschel M, Scheef L, Block W, Becker T, Schild HH, Maier W, Schwab SG. 2008. Unipolar depression and hippocampal volume: impact of DNA sequence variants of the glucocorticoid receptor gene. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 147B:836–43.

98. Binder EB. 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology 34 Suppl 1:S186–95.

99. Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Pütz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Künzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Köhnlein O, Dabitz H, Brückl T, Müller N, Pfister H, Lieb R, Mueller JC, Lõhmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B. 2004. Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat. Genet. 36:1319–25.

100. Tatro ET, Everall IP, Masliah E, Hult BJ, Lucero G, Chana G, Soontornniyomkij V, Achim CL. 2009. Differential expression of immunophilins FKBP51 and FKBP52 in the frontal cortex of HIV-infected patients with major depressive disorder. J. Neuroimmune Pharmacol. 4:218–26.

101. Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, Ressler KJ. 2008. Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA 299:1291–305.

102. Frodl T, Carballedo a, Hughes MM, Saleh K, Fagan a, Skokauskas N, McLoughlin DM, Meaney J, O'Keane V, Connor TJ. 2012. Reduced expression of glucocorticoid-inducible genes GILZ and SGK-1: high IL-6 levels are associated with reduced hippocampal volumes in major depressive disorder. Transl. Psychiatry 2:e88.

103. Fardet L, Nazareth I, Whitaker HJ, Petersen I. 2013. Severe neuropsychiatric outcomes following discontinuation of long-term glucocorticoid therapy: a cohort study. J. Clin. Psychiatry 74:e281–6.

104. Kino T, Chrousos G. 2001. Glucocorticoid and mineralocorticoid resistance / hypersensitivity syndromes. J. Endocrinol. 437–445.

105. Wang M. 2005. The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. Nutr. Metab. (Lond). 2:3.

106. Tinnikov AA, Legan M V, Sheveluk NA, Cvetovskaya GA, Naumenko SE, Sidelnikov SG. 1996. Corticosteroid and immune responses to cardiac surgery. Steroids 61:411–5.

107. Bernier J, Jobin N, Emptoz-Bonneton A, Pugeat MM, Garrel DR. 1998. Decreased corticosteroid-binding globulin in burn patients: relationship with interleukin-6 and fat in nutritional support. Crit. Care Med. 26:452–60.

108. Grasa MM, Cabot C, Balada F, Virgili J, Sanchis D, Monserrat C, Fernández-López JA, Remesar X, Alemany M. 1998. Corticosterone binding to tissues of adrenalectomized lean and obese Zucker rats. Horm. Metab. Res. 30:699–704.

109. Fernandez-Real J-M, Pugeat M, López-Bermejo A, Bornet H, Ricart W. 2005. Corticosteroid-binding globulin affects the relationship between circulating adiponectin and cortisol in men and women. Metabolism. 54:584–9.

110. Barat P, Duclos M, Gatta B, Roger P, Mormede P, Moisan MP. 2005. Corticosteroid binding globulin gene polymorphism influences cortisol driven fat distribution in obese women. Obes. Res. 13:1485–1490.

111. Kannisto K, Pietiläinen KH, Ehrenborg E, Rissanen A, Kaprio J, Hamsten A, Yki-Järvinen H. 2004. Overexpression of 11beta-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: studies in young adult monozygotic twins. J. Clin. Endocrinol. Metab. 89:4414–21.

112. Stimson RH, Andersson J, Andrew R, Redhead DN, Karpe F, Hayes PC, Olsson T, Walker BR. 2009. Cortisol release from adipose tissue by 11beta-hydroxysteroid dehydrogenase type 1 in humans. Diabetes 58:46–53.

113. Atalar F, Gormez S, Caynak B, Akan G, Tanriverdi G, Bilgic-Gazioglu S, Gunay D, Duran C, Akpinar B, Ozbek U, Buyukdevrim AS, Yazici Z. 2012. The role of mediastinal adipose tissue 11β-hydroxysteroid d ehydrogenase type 1 and glucocorticoid expression in the development of coronary atherosclerosis in obese patients with ischemic heart disease. Cardiovasc. Diabetol. 11:115.

114. Rask E, Walker BR, Söderberg S, Livingstone DEW, Eliasson M, Johnson O, Andrew R, Olsson T. 2002. Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. J. Clin. Endocrinol. Metab. 87:3330–6.

CHAPTER II

DISRUPTING HYPOTHALAMIC GLUCOCORTICOID RECEPTORS CAUSES HPA AXIS HYPERACTIVITY AND EXCESS ADIPOSITY.

Gloria Laryea, Günther Schütz and Louis J. Muglia

INTRODUCTION

The paraventricular nucleus of the hypothalamus (PVN) is a key regulator of metabolism, stress responses, and physiologic homeostasis. The glucocorticoid receptor (GR) is of specific importance in these varied functions of the PVN. GR functions as the primary mediator of feedback regulation in hypothalamic-pituitary-adrenal (HPA) axis stress response systems. The parvocellular neurons of the PVN synthesize and secrete corticotropin-releasing hormone (CRH) and vasopressin (AVP) in response to stress. CRH is secreted into the hypophyseal portal system and acts on the anterior pituitary where it stimulates the synthesis and secretion of adrenocorticotrophic hormone (ACTH). ACTH is released into the blood stream and stimulates adrenal cortex secretion of cortisol in humans or corticosterone (CORT) in rodents. CORT acts on peripheral tissues and central nervous system GRs to mediate normal circadian function as well as the stress response. CORT then negatively feeds back at the level of the pituitary to inhibit ACTH secretion, as well as at the level of the PVN to inhibit CRH secretion and restore the system to homeostasis. CORT binding to GR in the PVN, pituitary, and other CNS sites in the forebrain, mediates the negative feedback inhibition of CORT. Dysregulation of the HPA axis, possibly due to impaired GR signaling, has been implicated in the pathogenesis of affective disorders, such as major depression, in humans (1-5).

Several genetically altered rodent models of GR disruption have demonstrated a role for GR in mediating neuroendocrine, physiological, and behavioral responses (See (6-8) for review). Global GR deletion in mice targeting Exon 2 of GR, GR^{hypo} (9), or Exon 3, GR^{null} (10, 11) leads to perinatal and fetal mortality due to improper lung maturation. Interestingly, 20% of GR^{hypo} mice survive into adulthood, whereas all the GR^{null} mice die within hours after birth, indicating differences in penetrance depending on the specific nature of targeting the GR locus (12). The fetal lethality limited further studies on stress-related behaviors in these mice, leading to the development of region-specific deletion models.

Conditional deletion of the floxed GR exon 3 allele in the whole brain using *Nestin*-Cre results in mice with HPA axis hyperactivity and consequent Cushing syndrome phenotypes (growth impairment, altered fat distribution, obesity), as well as reduced anxiety-like behaviors (13). When GR disruption is limited to adult forebrain neurons using the *CaMKIIα*-Cre transgene (FB-GRko), mild HPA axis dysregulation occurs, as well as increased anxiety and despair-like behaviors that can be reversed with antidepressant treatment (14, 15). In comparison, GR disruption in embryonic forebrain neurons, PVN and pituitary neurons (using BAC *CaMKIIα*-iCre transgenic and floxed exon 3 transgenic mice ($GR^{CaMKCre}$)) leads to severe HPA axis hyperactivation, growth impairments, and death before post-natal day 10 (16). Disrupting GR in the pituitary, $GR^{POMCCre}$, results in elevated basal CORT levels, changes in PVN CRH and AVP mRNA, reduced body weight, all of which normalize in adulthood, and more active stress-coping behavior in the forced swim test (17). As one of the primary sites of GR

feedback regulation, the PVN's specific role during HPA-axis development and stress reactivity still needs to be analyzed.

We set out to isolate the specific contribution of PVN GR in mediating the aforementioned changes in regulation of glucocorticoid synthesis. We used Sim1-Cre BAC transgenic mice (18) to recombine floxed GR alleles, one removing exon 1C-2, and the other removing exon 3. These mice drive Cre expression during embryonic and post-natal development in the PVN, as well as the medial amygdala and nucleus of the lateral olfactory tract (18). This model has the unique potential to address several important questions in HPA-axis regulation and behavior. For instance, can other extrahypothalamic sites of GR negative feedback modulate glucocorticoid production and compensate for GR disruption in the PVN? Does PVN loss lead to upregulation of CRH expression, as it remains unclear whether a functional glucocorticoid response element exists in CRH gene locus. Additionally, does loss of GR in the PVN affect stress-related behaviors? The two major conventional GR knockouts, described earlier, differ in their phenotypes. Therefore, in addition to understanding these unresolved questions regarding PVN GR, we investigate whether there would be any differences in phenotype depending on what domain of GR was conditionally disrupted. To investigate this, we used mice that had GR floxed for the exon 1C-2 region (GRe1C-2), containing promoter elements, translation start site, and the transcriptional activation domain or the exon 3 region (GRe3), containing the zinc finger of the DNA-binding domain (19, 20), both in combination with the Sim1-Cre BAC transgenic mice.

Our physiological data indicate that loss of the exon 3 region of GR in the PVN results in fat accumulation due to glucocorticoid excess and a more severe HPA axis

dysregulation phenotype than loss of exon 2. Our behavioral data indicate that PVN GR alone does not mediate basal changes in anxiety and despair-related behaviors. We find that conditional deletion of GRe3 in the PVN leads to increased PVN CRH mRNA, increased plasma ACTH and glucocorticoid excess. Unlike GR deletion in the pituitary (17), we do not find compensation, but persistent HPA hyperactivation in adulthood, where circadian rhythm of glucocorticoid secretion is retained. In contrast, deletion of GRe1C-2 region is associated with intact HPA axis regulation. These differences may be contributed to by altered deletion efficiency or residual production of truncated activity-retaining GR fragments.

MATERIALS AND METHODS

Animals

All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Vanderbilt University (Nashville, TN) and Cincinnati Children's Medical Center (Cincinnati, OH). Mice were housed on a 12 h/12 h (Vanderbilt) or 14 h/10 h (Cincinnati Children's Medical Center) light/dark cycle with ad libitum access to rodent chow and water. Mice used for studies were between 3-6 months old.

Sim1Cre-GRe3Δ mice: We mated Sim1Cre mice (18) to floxed GR exon 3 mice (13).

Sim1Cre-GRe2Δ mice: We bred Sim1Cre mice to floxed GR exon 1C-2 mice (21).

Controls: Littermates that were Cre negative but had GR exon 1C-2 or exon 3 floxed alleles were used as controls. In figures displaying a comparison of Sim1Cre-GRe2Δ and

Sim1Cre-GRe3Δ mice, controls represent littermates from both types of matings. Sim1Cre mice were the kind gift of Dr. Brad Lowell, Beth Israel Deaconess Medical Center, Boston, MA. Sim1Cre, floxed GR exon 3, and floxed GR exon 1C-2 lines have been maintained on an inbred C57BL/6 background.

Immunohistochemistry

We anesthetized mice with 2.5% Avertin, perfused them with DEPC 1x PBS followed by 4% DEPC PFA, and post-fixed the brains and pituitaries in 4% DEPC PFA overnight. We then immersed the brains in 70% ethanol and processed them for paraffin embedding. Paraffin embedded brains were sectioned, on a microtome, at 8 μm while the pituitaries were sectioned at 5 μm onto Superfrost+ slides (VWR). The slides were placed at 60ºC overnight then stored at room temperature until later use. The slides were deparaffinized and rehydrated in decreasing concentrations of ethanol. Next, we performed antigen reconstitution of sections in 10mM Citrate Buffer (pH 6.0), followed by PBS washes and 1 hour blocking in 3% normal goat serum/ 0.25% Triton-X-100 in PBS to prevent non-specific binding. *GR*: Primary antibodies were 1:200 rabbit m20-anti-GR, directed against the N-terminus of GR, (Santa Cruz Biotech. Cat# sc-1004) and 1:200 mouse anti-NeuN; Secondary antibodies were 1:250 biotinylated goat anti-rabbit IgG and 1:200 goat anti-mouse Alexa 488 IgG; Washed slides were incubated in avidin/biotin complex (Vectastain), Cyanine-3 tyramide amplified (Perkin Elmer) and coverslipped with DAPI (Vector Laboratories). We quantified the number of GR positive cells that colocalized with Dapi as a percentage of total Dapi positive cells in the PVN. *ACTH*: Primary antibodies were 1:200 rabbit m20-anti-GR (Santa Cruz Biotech.) and

1:200 guinea pig anti-ACTH (Bachem Cat# S-3029); Secondary antibodies were 1:250 biotinylated goat anti-rabbit IgG and 1:100 goat anti-guinea pig FITC IgG; Slides were incubated with ABC, Cyanine-3 tyramide amplified and coverslipped with DAPI as above. We quantified the number of GR positive cells that colocalize with ACTH positive nuclei as a percentage of total ACTH positive cells in the anterior pituitary.

Body weight, gonadal fat, and adrenal gland measurements

We weighed mice weekly starting on weaning day, postnatal day 21 (P21) to 22 weeks of age. We dissected and weighed intra-gonadal fat pads and adrenal glands of mice 3-6 months old.

Corticosterone radioimmunoassay

Mice were single housed for a week and submandibular bleeds were performed in mice at nadir, peak, and 20 minutes after restraint stress, with two days between bleeds. The blood was centrifuged at 14000 rpm for 6 minutes and the plasma was removed and stored at -80ºC until a radioimmunoassay (RIA) was performed. RIA was performed using the Corticosterone Double Antibody - 125I RIA Kit (MP Biomedicals, Solon, OH).

Plasma glucose concentration

We used the AVIVA plus glucose meter to measure plasma glucose levels

Dexamethasone suppression test

Mice were given saline or dexamethasone (0.1mg/kg) injections in the morning and bleeds were performed 1 hour before lights out. The blood was centrifuged at 14000 rpm for 6 minutes and the plasma was removed and stored at -80ºC until the CORT RIA was performed.

CRH *in situ* hybridization

We anesthetized mice with 2.5% Avertin, perfused them with DEPC 1x PBS followed by 4% DEPC PFA, and post-fixed the brains in 4% DEPC PFA overnight. Brains were immersed in 20% DEPC sucrose solution for 48 hours, then embedded in OCT. 16um sections were obtained from brains at -15ºC in a cryostat and mounted onto Superfrost+ slides (VWR), vacuum-dried overnight, and stored at -80ºC until *in situ* hybridization was performed. P-33 UTP was used to label an RNA probe complementary to a 0.32 KB fragment on exon 2 of the CRH mRNA. Sections were hybridized to the labeled probe at 65ºC for 16 hours and washed in 0.1X SSC at 60ºC for 30 min. Slides were exposed to Maximum resonance film (Kodax BioMAx, Rochester N.Y.) for 4 days. We scanned the autoradiographic images at 3200 dots/inch into Photoshop on an Epson Perfection V600 scanner and quantified using NIH Image J software.

Behavioral tests

All behavioral tests were performed between 8:00 a.m. and 12:00 p.m. Mice were allowed to acclimate in the testing room for an hour prior to behavioral testing. Male mice between 12 and 20 weeks old were used in tests. All behavioral tests were

performed in the Vanderbilt Murine Neurobehavioral lab at the Vanderbilt University Medical Center and the Cincinnati Children's Hospital behavior core.

We evaluated anxiety using the open field (OF) and the elevated zero maze. Both tests pit the innate aversion of rodents to open unsecure spaces against their natural curiosity to explore the environment. *Open Field*: The OF activity was measured in chambers (Med Associates, 27×27×20.5 cm) that were contained within a light- and air-controlled environment. Each trial lasted 30 minutes per mouse. During that time, the Activity Monitor v5.10 program (Med Associates Inc, St. Albans, VT) was used to measure behaviors such as time spent, distance travelled, and number of entries into the inner and outer zones of the chamber. *Elevated Zero Maze:* This maze (34 cm inner diameter, 46 cm outer diameter, placed 40 cm off the ground on 4 braced legs) consists of a round maze with opposing open and closed arms. Mice are placed in the center of the open arm and activity is scored for 5 minutes using ANY-Maze software (San Diego Instruments, San Diego, CA). Time spent in the open and closed areas, as well as number of entries into the open and closed areas were measured.

Forced swim and tail suspension tests are measures of learned helplessness and despair (due to their predictive validity). *Forced swim test:* This test utilizes a 2L beaker, filled to 1.5L with water at room temperature (25°C). The mouse was placed in the beaker for 5 minutes. Every 5 seconds, the mouse's activity was recorded by an observer blind to genotype. We quantified the total time inactive during the trial, where more inactivity is indicative of increased despair. *Tail suspension tests*: The tail suspension test employed a platform from which mice are suspended by their tails; motion was scored with either an automated system with user-defined thresholds or manually with the

observer blinded to genotype. Motion was scored for 5 minutes manually or for 7 minutes in the automated system. Time spent below a specified lower threshold was interpreted as inactivity. Animals excluded from analysis after behavioral testing were those unable to be scored due to falling off the zero maze apparatus or sinking in the forced swim. These test failures did not differ in frequency between the different genotypes.

Statistical analysis

Results are presented as mean ± SEM. Data were subjected to Student t-test, one- or two-way ANOVA test (Prism 5.0 software; GraphPad Software, Inc., San Diego, CA). Statistical significance was analyzed using the Tukey or Bonferroni post hoc test. Significant difference was defined at a p value ≤ 0.05.

RESULTS

Deletion of GRe1C-2 and GRe3 in the PVN neurons expressing Sim1

We bred Sim1-Cre BAC transgenic mice (18) to mice that have *loxP* sites flanking exons 1C and 2 (21) or exon 3 of the GR gene (13). We thus generated mice with GRe1c-2 deleted in the PVN (Sim1Cre-GRe2Δ) or mice with GRe3 deleted in the PVN (Sim1Cre-GRe3Δ). Control mice were negative for Cre-recombinase. We evaluated the specificity and efficiency of GR deletion by fluorescent immunohistochemistry and quantified the percent of GR positive nuclei in the PVN. We demonstrated a 43% reduction in PVN GR expression in Sim1Cre-GRe2Δ mice (p=0.03). In contrast, we

found 87% decrease in PVN GR in Sim1Cre-GRe3Δ mice (p=0.002) (Figure 2.1.) when compared to control mice (One-way ANOVA F(2,8)=15.47, p=0.0018).

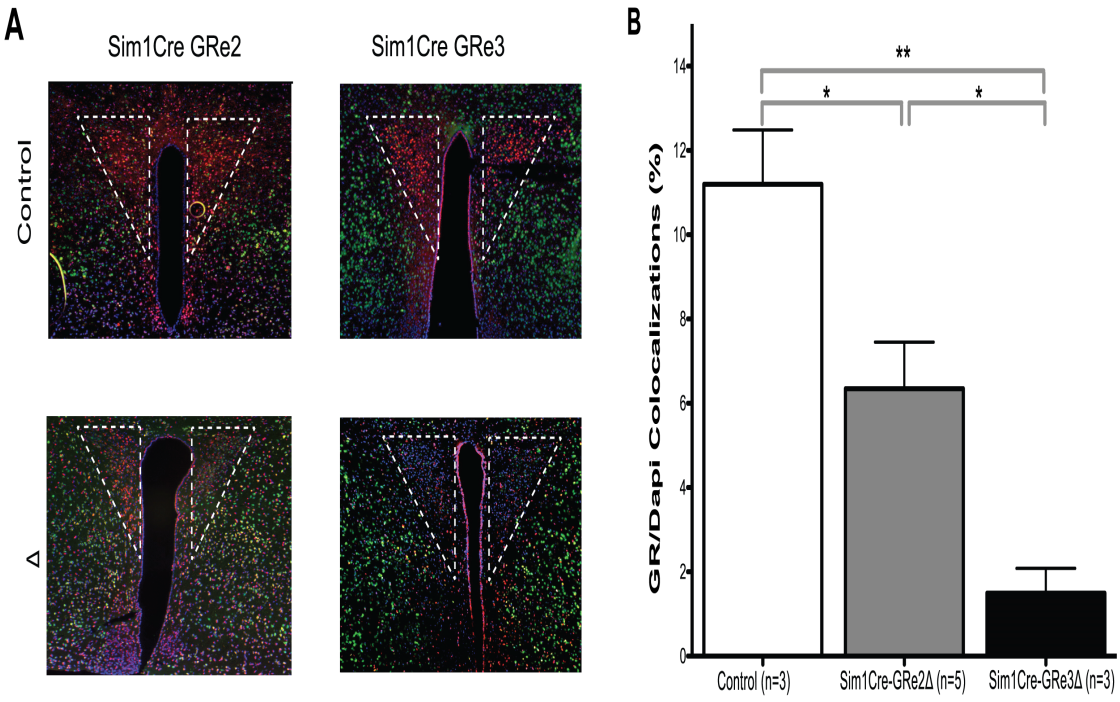

Figure 2.1. GR immunoreactivity is reduced in the PVN (outlined area) of mice with disrupted GR. (A) Immunofluorescent staining for GR protein in the PVN of controls, Sim1Cre-GRe2Δ, and Sim1Cre-GRe3Δ mice. GR (red), NeuN (green), Dapi (blue). (B) Quantification of the amount of GR in the PVN that co-localized with Dapi as a percent of total Dapi. The bars represent mean + SEM. Sim1Cre-GRe2Δ mice have a 43% reduction in GR protein, while Sim1Cre-GRe3Δ mice have an 87% reduction compared to controls (ctrl). *p-value < 0.05* and **p<0.01.

Sim1Cre-GRe3Δ, but not Sim1Cre-GRe2Δ, mice have increased body weight and fat mass in adulthood.

In terms of body weight, Sim1Cre-GRe2Δ mice are phenotypically indistinguishable from control mice. On the other hand, Sim1Cre-GRe3Δ mice display characteristics of a glucocorticoid excess (Cushingoid) phenotype. At birth, Sim1Cre-GRe3Δ mice are smaller than controls ($p < 0.05$), but in adulthood they display increased weight gain, and obesity (Figure 2.2.). We observed this in females ($p < 0.05$) (Figure 2.2. A) and some male Sim1Cre-GRe3Δ mice, although there was enough variability between the males such that differences were not statistically different (Figure 2.2. B).

To determine contributors to increased body weight, we dissected and weighed perigonadal fat pads from female and male mice. Female Sim1Cre-GRe3Δ mice had a 14-fold increase in fat pad mass, while male Sim1Cre-GRe3Δ mice had a 3-fold increases in fat pad mass compared to control littermates ($p=0.0006$, females, $p=0.001$ males) (Figure 2.2. D,E). Due to this observed increase in adiposity and predicted high CORT levels, we tested serum glucose concentrations at baseline and following restraint stress. We observed that baseline blood glucose levels did not vary between genotypes in either gender ($p=0.12$ females, $p=0.48$ males) (Figure 2.2. F,G). However, Sim1Cre-GRe3Δ mice fail to exhibit the stress-induced elevations in blood glucose levels ($p=0.22$ females, $p=0.3$ males) observed in controls ($p=0.0007$ females, $p=0.002$ males) and Sim1Cre-GRe2Δ mice ($p=0.0005$ females, $p=0.03$ males) (Figure 2.2. F,G).

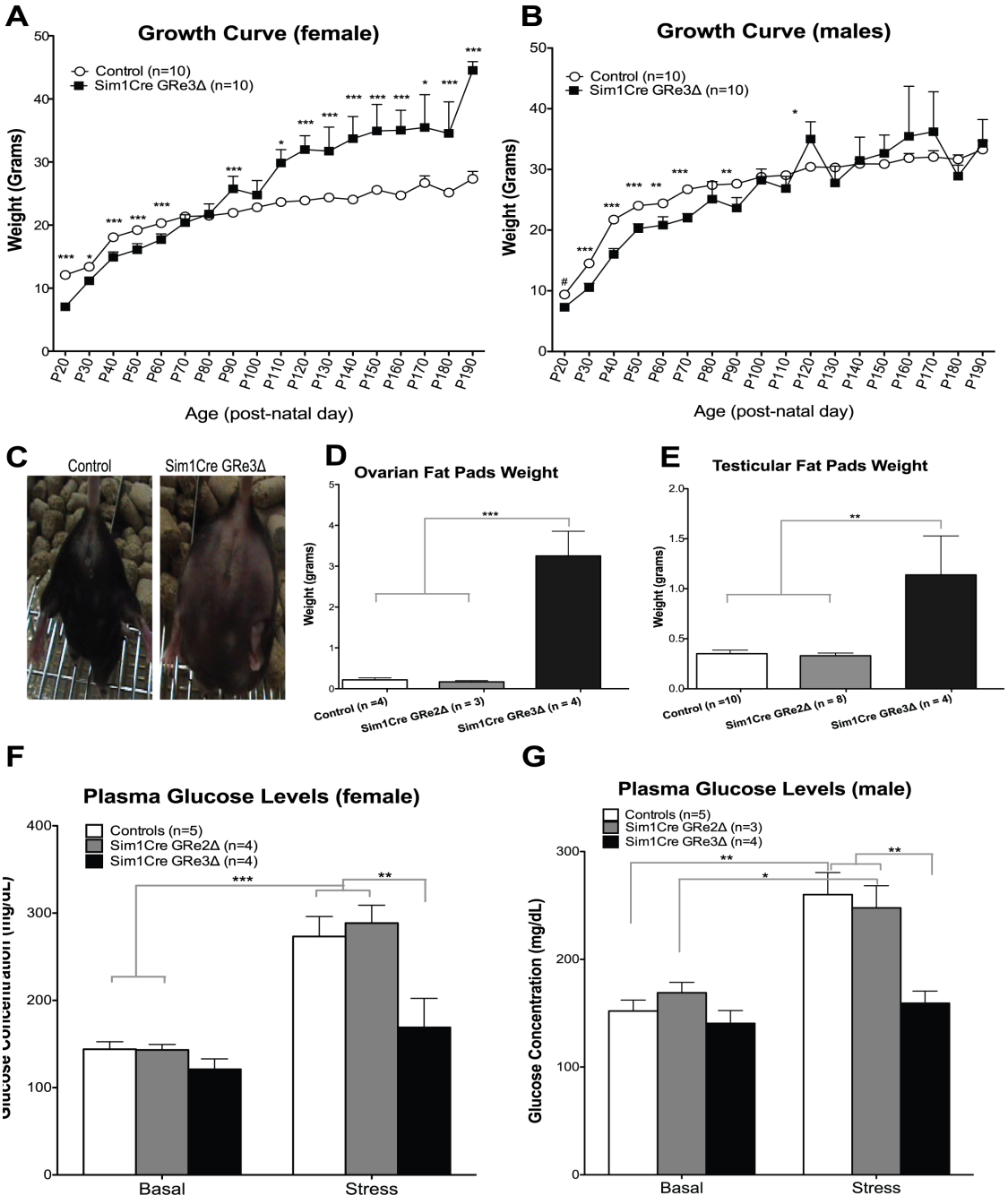

Figure 2.2. Sim1Cre-GRe3Δ mice have altered growth and fat accumulation. The mean (±S.E.M.) body weights of control and Sim1Cre-GRe3Δ mice over a 6-month period, females (A) and males (B). Images depicting phenotype differences between female control and Sim1Cre-GRe3Δ mice at 4 months of age (C). Ovarian (D) and Testicular (E) fat pad weight in each group of mice. (F,G) Plasma glucose levels measured at nadir, peak, and after 20-minutes of restraint stress. Control and Sim1Cre-GRe2Δ mice display increase plasma glucose after stress, ***p< 0.001, whereas Sim1Cre-GRe3Δ mice do not, p >0.05, females (F) and males (G). p-value < 0.05* and **p<0.01, *** p < 0.001, **** p<0.0001.

Sim1Cre-GRe3Δ mice, but not Sim1Cre-GRe2Δ mice, display increased HPA axis activity

To evaluate the cause of the Cushing-like phenotype, we measured serum CORT levels. We found significantly elevated basal plasma CORT levels in both female ($p = 0.07$ nadir, $p = 0.0005$ peak) and male ($p < 0.0001$, nadir and peak) Sim1Cre-GRe3Δ mice that maintained circadian rhythmicity (Figure 2.3. A,B). Twenty minutes of restraint stress further elevated CORT levels (Figure 2.3. A,B) in Sim1Cre-GRe3Δ mice ($p = 0.04$ females, $p < 0.0001$ males). Moreover Sim1Cre-GRe3Δ mice fail to suppress CORT secretion in the dexamethasone suppression test ($p = 0.45$ females, $p = 0.27$ males) while their littermate controls suppressed CORT secretion by approximately 50% after dexamethasone administration ($p = 0.02$ females, $p = 0.05$ males) (Figure 2.3. C,D).

To determine the mechanism by which loss of GR in the PVN leads to increased CORT, we evaluated CRH mRNA and protein expression in the PVN of control, Sim1Cre-GRe2Δ, and Sim1Cre-GRe3Δ mice. We observed a 3-fold increase in CRH mRNA expression in Sim1Cre-GRe3Δ ($p = 0.01$) but not Sim1Cre-GRe2Δ mice ($p = 0.9$) compared to controls (one-way ANOVA $F(2,9) = 30.22$, $p = 0.0001$) (Figure 2.4. A,B). Interestingly, we found a significant effect of genotype on amygdala CRH mRNA expression (One-way ANOVA $F(2,9) = 6.202$, $p = 0.0203$), where CRH mRNA in the central nucleus of the amygdala increased in Sim1Cre-GRe3Δ mice compared to Sim1Cre-GRe2Δ mice (t-test, $p = 0.016$), with a similar trend in SimCre-GRe3Δ mice compared to control mice that did not achieve statistical significance (Figure 2.5. A). Despite the significant increase in PVN CRH expression, pituitary ACTH immunoreactivity did not differ and GR protein in ACTH producing cells of the pituitary

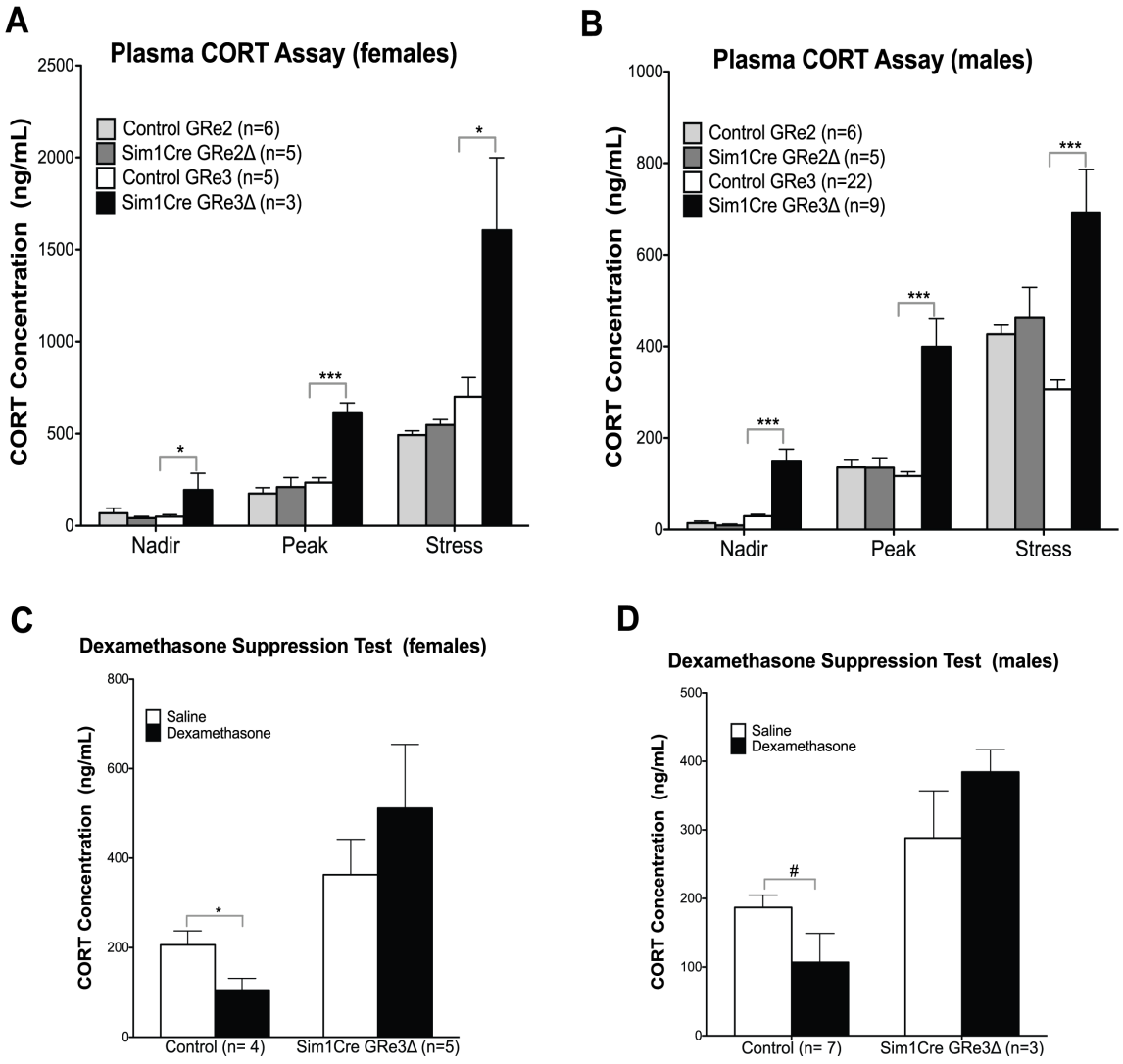

Figure 2.3. Loss of PVN GR results in hypercorticosterolemia and impaired negative feedback in female (A, C) and male (B, D) mice. (A,B) Plasma CORT levels measured at nadir, peak, and after 20-minutes of restraint stress. Sim1Cre-GRe3Δ mice display increased plasma CORT at nadir, peak and after stress compared to controls and Sim1Cre-GRe2Δ mice. (C,D) Dexamethasone suppression test was used to evaluate negative feedback. Control female mice show a 50% suppression of CORT after dexamethasone $p<0.05$ (C), while males show a trend towards CORT suppression (D). Sim1Cre-GRe3Δ mice do not show suppression of CORT after dexamethasone administration. $^{\#}p = 0.053$ $*p<0.05$, $***p<0.001$.

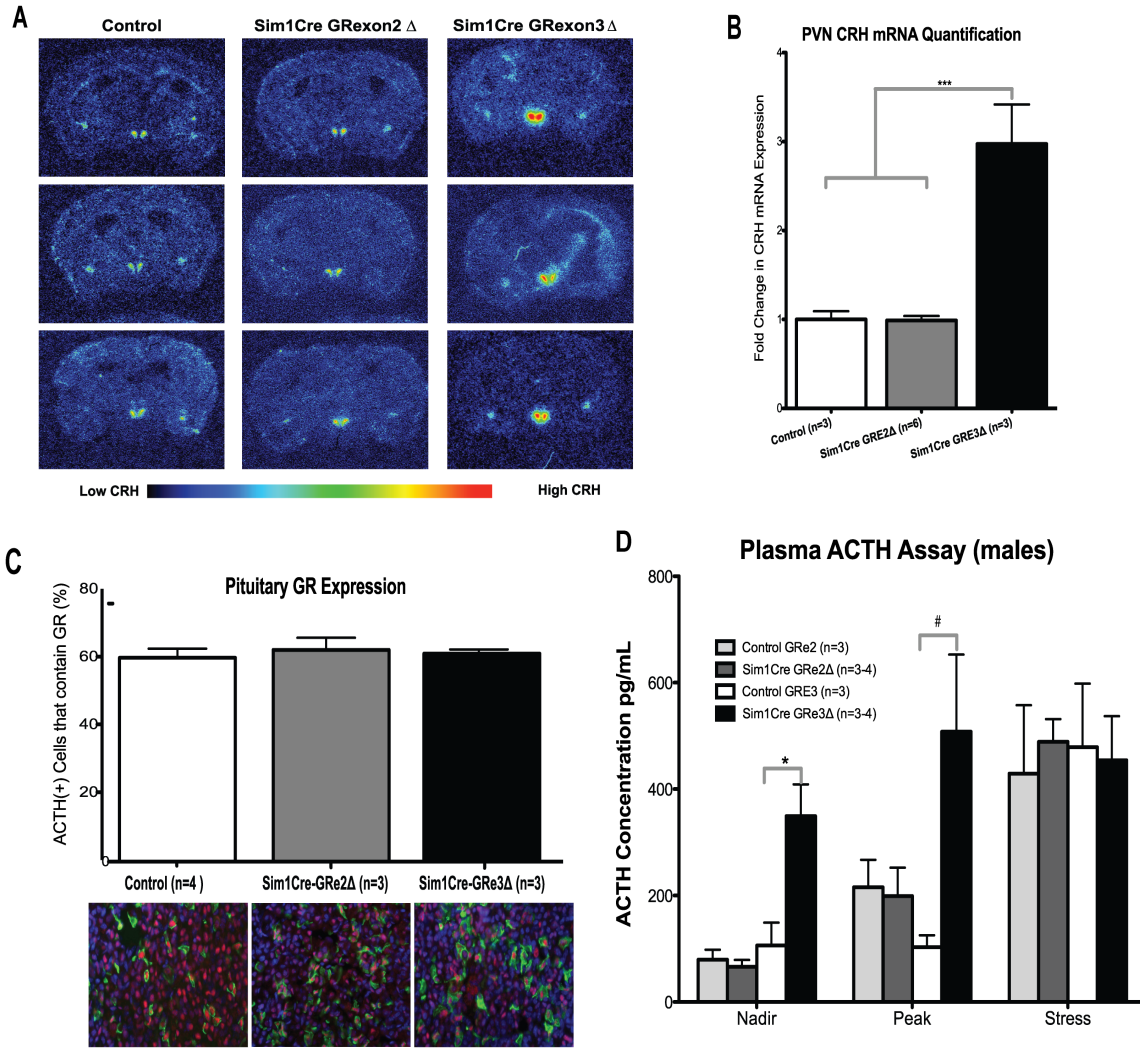

Figure 2.4. HPA axis hyperactivity is observed in Sim1Cre-GRe3Δ mice. (A,B) CRH mRNA expression is increased in the PVN of Sim1Cre-GRe3Δ mice. (A) Representative images of pseudo-colored CRH *in situ* hybridization in control mice (left), Sim1Cre-GRe2Δ mice (middle), and Sim1Cre-GRe3Δ mice (right). (B) Quantification of pixel density showed a 3-fold increase in PVN CRH mRNA in Sim1Cre-GRe3Δ mice compared to controls and Sim1Cre-GRe2Δ mice. (C) Expression of GR in ACTH+ nuclei was quantified as a percentage of total ACTH+ nuclei (top panel) in the anterior pituitary. As visualized in the immunofluorescent images in the bottom panel, quantification revealed no differences between groups, implying intact GR in ACTH-producing cells GR (red), ACTH (green), Dapi (blue). (D) Plasma ACTH levels measured at nadir, peak, and after 20-minutes of restraint stress. Sim1Cre-GRe3Δ mice display increased plasma ACTH at nadir. They trend towards increased peak ACTH levels with no change in stress levels of ACTH compared to controls and Sim1Cre-GRe2Δ mice. $^{\#}$p=0.06, *p=0.03, ***p=0.0001.

remained intact (Figure 2.4. C). Circulating nadir ACTH levels were significantly elevated while peak levels had a trend towards increase in male Sim1Cre-GRe3Δ mice compared to controls (two tailed t-test p=0.03-nadir, p=0.06 peak) but not in Sim1Cre-GRe2Δ mice compared to controls (Figure 2.4. D). Stress levels of ACTH (Figure 2.4. D) do not differ between genotypes. Female Sim1Cre-GRe3Δ mice show a trend towards increased adrenal weights (p = 0.059) (Figure 2.5. B), whereas male adrenal weights in Sim1Cre-GRe3Δ mice are significantly increased (p=0.009) compared to controls and Sim1Cre-GRe2Δ mice (Figure 2.5. C).

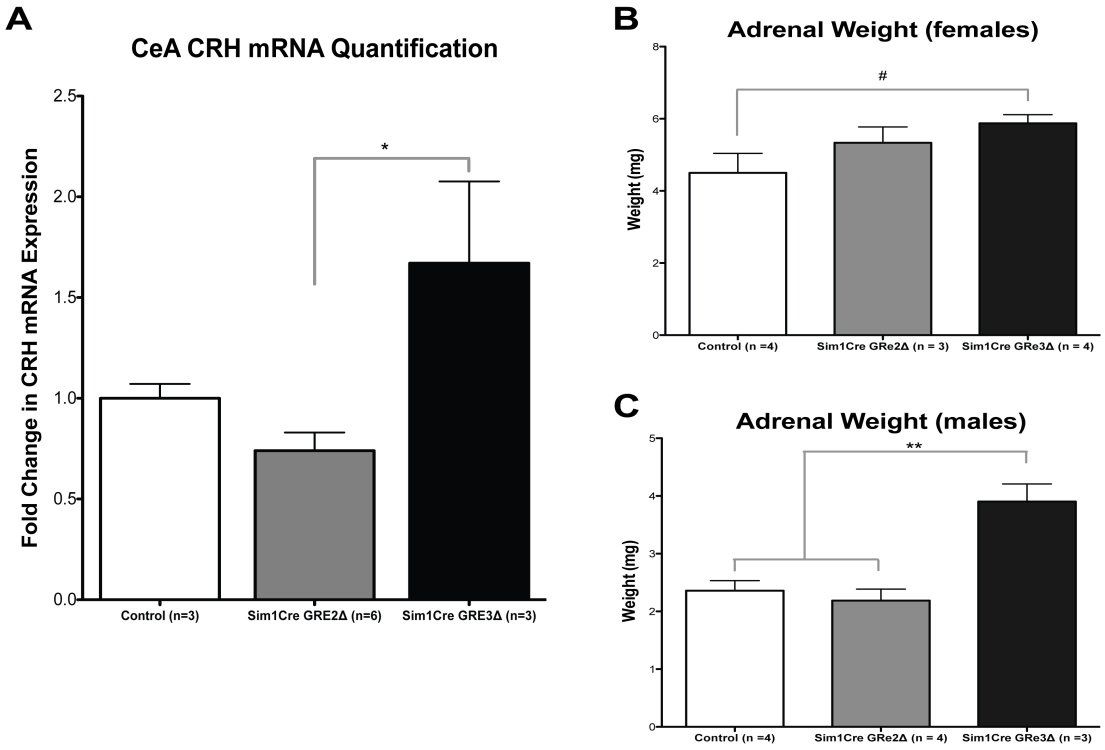

Figure 2.5. Amygdala CRH mRNA expression and adrenal gland weights. (A**)** Sim1Cre-GRe3Δ mice show significantly increased CRH mRNA compared to Sim1Cre-GRe2Δ mice (p = 0.016) and a similar non-significant trend compared to controls. (B,C) There is a trend towards increased adrenal weights in Sim1Cre-GRe3Δ female mice compared to controls (B). Male Sim1Cre-GRe3Δ mice show increased adrenal gland weights compared to controls and Sim1Cre-GRe2Δ mice (C). $^{\#}$p <0.06, *p<0.05, **p<0.01.

Sim1Cre-GRe3Δ mice exhibit reduced locomotor activity

Due to the role of brain GR in mediating anxiety and despair-related activity, we tested mice in different behavioral paradigms. Behaviorally, locomotor activity is reduced in male Sim1Cre-GRe3Δ mice (in terms of distance travelled in the open field, $p = 0.02$) (Figure 2.6. A,B). However, changes in anxiety- (OF and elevated zero maze) (Figure 2.7. A,B) and despair-like behaviors (tail suspension test and FST) (Figure 2.7. C,D) were not observed in Sim1Cre-GRe3Δ mice. Comparatively, Sim1Cre-2Δ mice have normal locomotor activity and display no anxiety- or despair –like behavior (Figure 2.8. A-D).

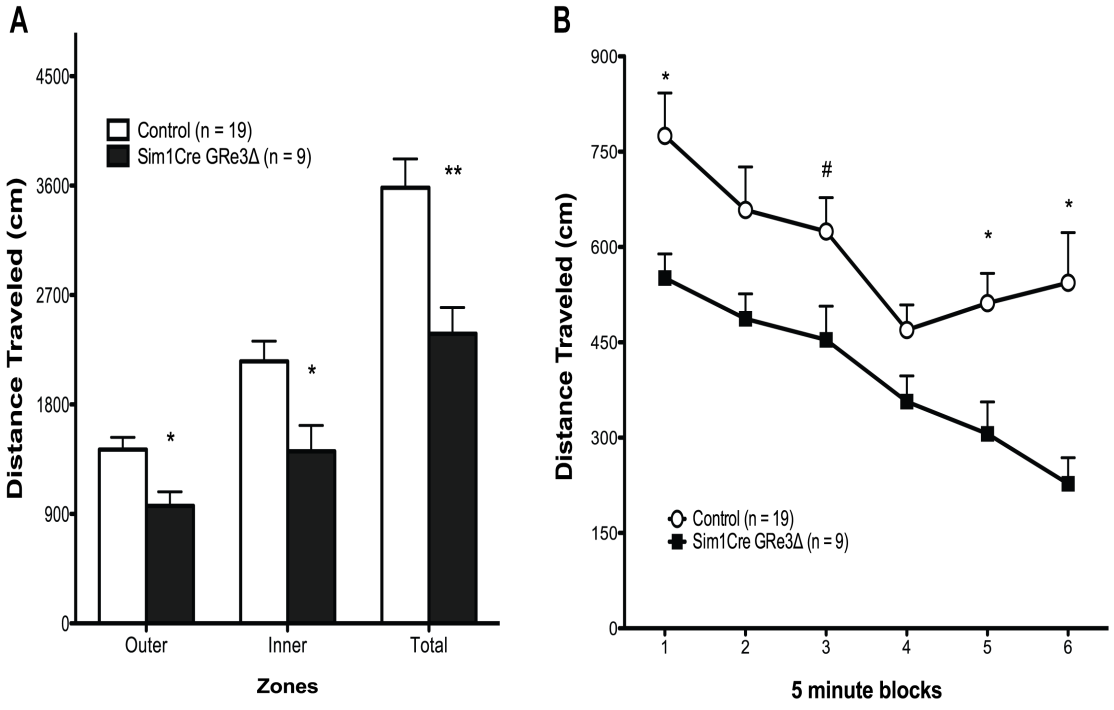

Figure 2.6. Altered locomotor activity of Sim1Cre-GRe3Δ mice in the OF test. Sim1Cre-GRe3Δ mice show reduced distance traveled in outer zone and total distance (A) as well as distance travelled in 5-minute blocks (B). $^{\#}p<0.09$, $^{*}p<0.05$, $^{**}p<0.01$.

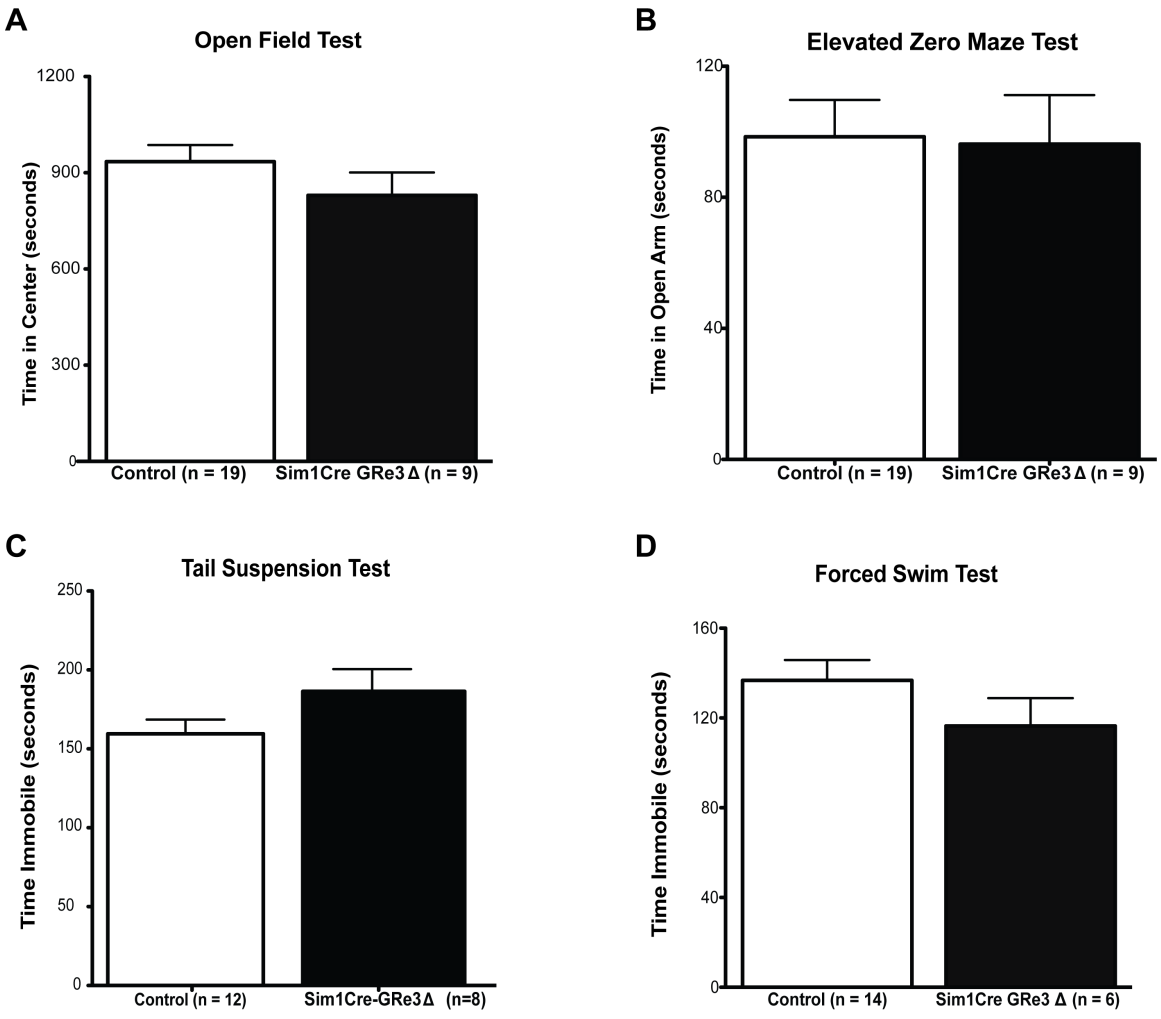

Figure 2.7. **Sim1Cre-GRe3Δ mice do not exhibit anxiety or despair-like behavior.** Anxiety behavior is measured as the mean (±S.E.M.) time spent in the center zone of the OF test (A) or in the open arm of the elevated zero maze (B). Despair-behavior is measured as the time immobile during the tail suspension test (C) and the FST (D). In all tests, Sim1Cre-GRe3Δ mice (n= 6-9) show similar activity compared with control mice (n=12-19). Student's t test; $p>0.05$.

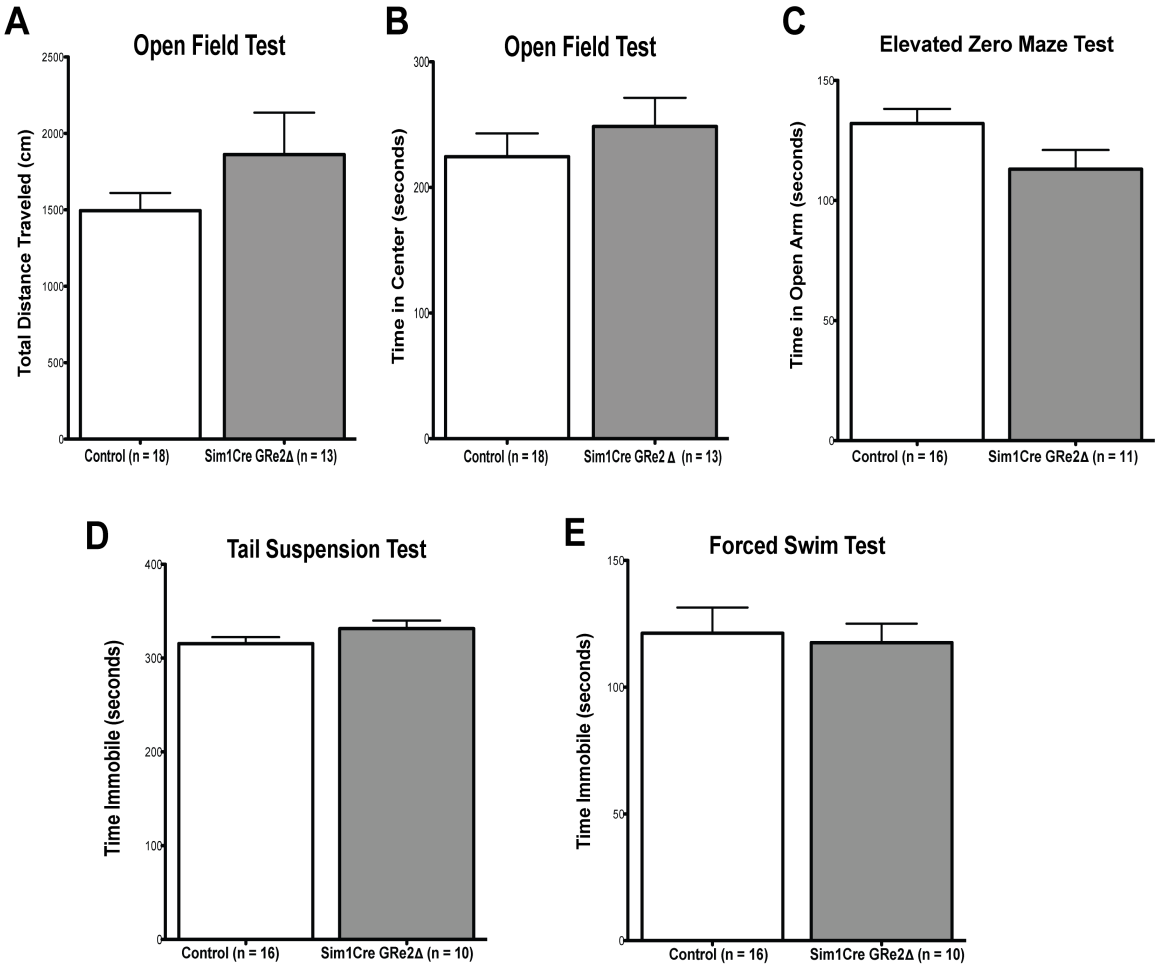

Figure 2.8. Sim1Cre-GRe2Δ mice exhibit normal locomotor behavior and do not exhibit anxiety or despair-like behavior. (A) Sim1Cre-GRe2Δ show distance traveled in the open field similar to control mice. Anxiety behavior is measured as the mean (±S.E.M.) time spent in the center zone of the OF test (B) or in the open arm of the elevated zero maze (C). Despair-behavior is measured as the time immobile during the tail suspension test (D) and the FST (E). In all tests, Sim1Cre-GRe2Δ mice (n= 10-13) show similar activity compared with control mice (n=16-18). Student's t test; $p>0.05$.

DISCUSSION

In this study, we investigate the neuroendocrine, metabolic, and behavioral consequences of disruption of GR in the PVN. Surprisingly, we find that the phenotype of Sim1-Cre-mediated deletion of floxed GR gene substrates depends upon the specific floxed GR allele targeted. Sim1Cre-GRe3Δ mice manifest elevated serum concentrations of corticosterone throughout the circadian cycle and in association with a stressor, compared with either Sim1Cre-GRe2Δ or non-deleted control mice. Because of the greater extent of deletion in the PVN of Sim1Cre-GRe3Δ compared with Sim1Cre-GRe2Δ mice, together with the potential for truncated carboxy-terminal products in Sim1Cre-GRe2Δ mice, the Sim1Cre-GRe3Δ line reflects a more complete phenotype for PVN GR loss. Despite elevated glucocorticoid concentrations and metabolic derangements, we did not find evidence for behavioral alterations in the Sim1Cre-GRe3Δ line. While we did not observe differences between controls and Sim1Cre-GRe2Δ mice in HPA axis regulation under the conditions we analyzed, it is possible that a more extensive time series or different stress exposures could reveal a more subtle aspect of glucocorticoid feedback dysregulation.

While the Sim1Cre line also expresses Cre-recombinase in the supraoptic nucleus, nucleus of the lateral olfactory tract, and other hypothalamic and medial amygdala nuclei (18), these sites are unlikely to influence the HPA axis regulation phenotype we report here. These Sim1Cre transgenics are the most selective mice that have been generated thus far for evaluating PVN-related functions of genes.

Differential HPA Axis Phenotype Between Sim1Cre-GRe2Δ and Sim1Cre-GRe3Δ Mice

In mice, the GR gene, *Nr3c1*, is greater than 110kb in length and is the splice product of 9 exons, of which several alternative, non-coding first exons contribute (20). Exon 2 of GR encodes the usual translational start sites and the transactivation domain while exon 3 and 4 contain the zinc-finger of the DNA binding domain. The five C-terminal exons compose the ligand-binding domain of the receptor (20). Studies of GR disruption have mainly targeted the exon 2 and 3 regions.

The differences we observe in Sim1Cre-GRe2Δ compared with Sim1Cre-GRe3Δ mice may be the result of the level of GR deletion between the two lines. We measured a 43% reduction in GR immunoreactive cells in Sim1Cre-GRe2Δ PVN, whereas Sim1Cre-GRe3Δ mice display an 87% reduction in PVN GR. This difference in deletion extent as being a contributor to the observed phenotypic differences is supported by previous findings in FBGRKO mice that at 2 months of age, when 50% of GR immunoreactive neurons in the hippocampus are lost, demonstrate no significant differences in HPA axis activity or behavior, but at 4-6 months when there is 90-100% deletion, have impaired HPA axis activity and increased anxiety and despair-like behaviors (14). These data suggest a critical threshold for GR, that when breached disrupts normal functions.

An alternative, or additional contributor, to the differences between floxed lines may result from Sim1Cre-GRe2Δ mice having the capacity to produce truncated, function-retaining GR products. Should antibody-mediated detection of possible truncated carboxy-terminal products in Sim1Cre-GRe2Δ mice arise, the transcriptional activation abilities of these products may still be uncertain. While conventional

knockouts targeting exon 2, GR^{hypo}, (9) or exon 3, GR^{null}, (11) both result in fetal lethality, there is survival of 20% of the GR^{hypo} mice into adulthood. Evidence suggests that insertion of a neomycin-resistance cassette into the second exon of GR results in incomplete gene inactivation due to the synthesis of a truncated C-terminal GR protein that maintains residual binding activity (9, 10, 19, 22). Truncated products were not revealed when GR exon 2 was disrupted by Cre-mediated excision of exon 1C to 2 of GR in thymocytes (21). Given the complexity of GR alternative exon 1 utilization and distal splicing, we cannot discount the existence of tissue-dependent alternative splicing that might yield activity-retaining GR products. In contrast to conventional GR exon 2 deletion, disruption of exon 3 results in complete inactivation of the GR gene due to out of frame splicing and premature translation termination (11, 13). We have been unsuccessful in identifying or generating antibodies against the C-terminus of GR suitable for immunohistochemistry to directly demonstrate residual GR immunoreactivity in the exon 1C-2 deleted lines.

A recent study that used the same Sim1Cre transgenic mice crossed with floxed GR allele starting at an MfeI site 420-bp upstream of exon 2 region found that the mutant homozygotes die at birth(23). The difference between our PVN GR exon 2 deleted mice and theirs was the inclusion of additional promoter regulatory regions in our line. Given the lack of mortality in either of our lines, the lethal phenotype observed by Jeannetteau et al. does not arise from PVN deletion effects, but rather deletion in the lung, which was not observed in either of our two lines.

Consequences of Glucocorticoid Excess in Sim1Cre-GRe3Δ Mice

Previous generation and characterization of GR^{NesCre} mice, with GR deletion in all brain regions including the PVN, revealed a glucocorticoid excess, Cushingoid phenotype (13). More selective GR inactivation in the PVN of our Sim1Cre-GRe3Δ mice also results in a Cushingoid phenotype. Sim1Cre-GRe3Δ, compared to control and Sim1Cre-GRe2Δ, mice had reduced weight from weaning (P21) to the first two months of age. This pattern changed to increased weight gain throughout adulthood and an obesity phenotype, with thinning of the hair on the abdominal areas and areas of the face and back. We observed sexual dimorphism in dysregulation of adult body weight, with female Sim1Cre-GRe3Δ mice consistently weighing more than control mice. In comparison, thirty percent of male Sim1Cre-GRe3Δ mice did not weigh more than control mice, but maintained the truncal obesity phenotype. The obesity and increased body mass phenotype in Sim1Cre-GRe3Δ mice was largely contributed to by increased adiposity as reflected by perigonadal fat pad weights. Female and male Sim1Cre-GRe3Δ mice had approximately 14-fold and 3-fold increases in pelvic fat pad weight, respectively, compared to controls and Sim1Cre-GRe2Δ mice. This differential accumulation of adipose tissue may explain the differences observed in the growth curves of adult female and male mice. Given that males and females typically have altered patterns of fat distribution and accumulation, the influence of excess glucocorticoids interacting with sex hormones may account for the difference in adiposity. Similar to the growth phenotype in Sim1Cre-GRe3Δ mice, GR^{NesCre} mice also demonstrate reduced body weight in the early period of life (24). However, while Sim1Cre-GRe3Δ mice display increased fat accumulation, adult GR^{NesCre} mice exhibit significantly reduced

body fat mass. Since CRH acts to inhibit food intake and drive energy expenditure, it was suggested that increased PVN CRH in the GR^{NesCre} mice drives the reduced fat mass (24). As we also find substantially increased PVN CRH in Sim1Cre-GRe3Δ mice, an alternative explanation may be that GR deletion in other brain regions regulating metabolism or sympathetic tone in GR^{NesCre} mice account for the differences. Consistent with the phenotypic evidence of glucocorticoid excess, Sim1Cre-GRe3Δ mice displayed greater than 2.5-fold increases in nadir and peak levels of CORT with maintained circadian rhythmicity. The levels of CORT in Sim1Cre-GRe3Δ mice were further increased after restraint stress.

Interestingly, we found that Sim1Cre-GRe3Δ mice did not increase glucose secretion after restraint stress. This was quite surprising since high circulating glucocorticoids are known to induce hyperglycemia and insulin resistance in organisms (25, 26). It is possible that in Sim1Cre-GRe3Δ mice, the high basal levels of glucocorticoids have resulted in resistance to effects of further increases in glucocorticoids due to existing high occupation of receptors or receptor desensitization.

Consequences of GR Disruption on Hypothalamic and Pituitary Regulation

CRH has been implicated as the initiator of circadian and stress induced increases in glucocorticoid production, and is subject to glucocorticoid feedback modulation. Consistent with this role, CRH mRNA in the PVN of Sim1Cre-GRe3Δ mice, in which we find high glucocorticoid levels, is 3 times higher than levels in control and Sim1Cre-GRe2Δ mice.

We found no difference in GR expression in ACTH immunoreactive cells in the anterior pituitary between control, Sim1Cre-GRe2Δ, or Sim1Cre-GRe3Δ mice. This intact pituitary GR, however, was not sufficient to drive feedback inhibition in the dexamethasone suppression test or prevent the elevated PVN CRH from increasing basal plasma ACTH concentrations in Sim1Cre-GRe3Δ mice. Although Sim1Cre-GRe3Δ mice exhibit higher basal ACTH levels than controls, they do not demonstrate the stress-induced elevations in ACTH observed in control and Sim1Cre-GRe2Δ mice. This is in divergence with CORT levels, where we observe both a circadian and a stress-induced rise in CORT. We tested whether adrenal hypertrophy had developed that would exaggerate smaller changes in ACTH in Sim1Cre-GRe3Δ mice. We found that female mice have trends towards increased adrenal weight compared to controls. Adrenal hypertrophy did occur in male Sim1Cre-GRe3Δ mice, demonstrating a 65% increase in adrenal weights compared to control and Sim1Cre-GRe2Δ mice.

PVN GR Disruption and Behavioral Indices of Anxiety and Despair

In the current study, despite marked HPA axis dysregulation, we found no significant differences in anxiety-related behaviors measured in the OF or elevated zero maze tests. Neither did we observe significant differences in despair-like behavior in the tail suspension or forced swim tests. GR disruption in the entire brain, GR^{NesCre}, (13) and forebrain only, FBGRKO mice (14, 15), result in behavioral maladaptations in mice. The lack of behavioral changes in our PVN GR disrupted mice supports the interpretation that altered GR signaling in limbic areas mediates the genesis of behavioral dysregulation. We did find that Sim1Cre-GRe3Δ mice have reduced locomotor behavior in the distance

travelled in the OF test compared to control mice. This locomotor deficit may arise from the sustained effects of elevated glucocorticoids on muscle function.

In conclusion, we report here that PVN GR is needed for feedback inhibition of HPA axis function, but is not required for circadian rhythmicity of adrenal activity. Surprisingly, GR loss in the PVN, with chronically elevated circulating glucocorticoid levels, does not lead to heightened anxiety or despair-like behavior. In many circumstances of glucocorticoid dysregulation with Cushing's syndrome, behavioral changes, particularly anxiety and depression-like symptoms are common. One possibility our results may suggest is that loss of PVN GR in some manner prevents the induction of stress-related behaviors in the context of normal extrahypothalamic GR and elevated corticosterone. The whole brain knockout of GR using Nestin-Cre demonstrates that loss of GR in the context of loss of GR in other brain regions does lead to altered anxiety-related behaviors (13), as does intact PVN GR with disrupted forebrain GR (14, 15). Given our findings, the most parsimonious explanation synthesizing this information would be that PVN GR does not play a direct role in mediating anxiety and despair behavior. One intriguing possibility is that the extrahypothalamic brain regions in Sim1Cre- GRe3Δ mice are able to accommodate the elevated glucocorticoid levels since they still undergo a qualitatively similar pattern of circadian regulation.

Interesting new areas of investigation arise as a result of these studies. For example, given the normal anxiety and despair-related behaviors observed in Sim1Cre-GRe3Δ mice, can the brain compensate for chronically excess glucocorticoid exposure by altering CORT metabolism or functional GR signaling? Potential mechanisms include down-regulation of GR in extrahypothalamic regions, alteration of expression of co-

activators or co-repressors, or increases in 11β-HSD type 2 in other brain regions such that normalization of functional signaling occurs. Another area of interest is to investigate how PVN GR loss and excess glucocorticoid attenuate stress-induced increases in blood glucose levels. Future studies designed to answer these questions should prove informative for understanding normal glucocorticoid physiology, the pathogenesis of affective disorders, and the consequences of pharmacological glucocorticoid therapy.

REFERENCES

1. Barden N, Reul JM, Holsboer F. 1995. Do antidepressants stabilize mood through actions on the hypothalamic-pituitary-adrenocortical system? Trends in neurosciences 18:6-11.

2. Holsboer F. 1995. The role of clinical research. Nature medicine 1:492-493.

3. Holsboer F. 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 23:477-501.

4. Holsboer F, Barden N. 1996. Antidepressants and hypothalamic-pituitary-adrenocortical regulation. Endocrine reviews 17:187-205.

5. Zobel AW, Yassouridis A, Frieboes RM, Holsboer F. 1999. Prediction of medium-term outcome by cortisol response to the combined dexamethasone-CRH test in patients with remitted depression. The American journal of psychiatry 156:949-951.

6. Erdmann G, Berger S, Schutz G. 2008. Genetic dissection of glucocorticoid receptor function in the mouse brain. Journal of neuroendocrinology 20:655-659.

7. Gass P, Reichardt HM, Strekalova T, Henn F, Tronche F. 2001. Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: models for depression and anxiety? Physiology & behavior 73:811-825.

8. Kolber BJ, Wieczorek L, Muglia LJ. 2008. Hypothalamic-pituitary-adrenal axis dysregulation and behavioral analysis of mouse mutants with altered glucocorticoid or mineralocorticoid receptor function. Stress 11:321-338.

9. Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G. 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes & development 9:1608-1621.

10. Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, Beier K, Metz J, Garcia-Arraras JE, Roig-Lopez JL, Monaghan P, Schmid W, Cole TJ, Kellendonk C, Tronche F, Schutz G, Unsicker K. 1999. Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. Development 126:2935-2944.

11. Tronche F, Kellendonk C, Reichardt HM, Schutz G. 1998. Genetic dissection of glucocorticoid receptor function in mice. Current opinion in genetics & development 8:532-538.

12. Kellendonk C, Gass P, Kretz O, Schutz G, Tronche F. 2002. Corticosteroid receptors in the brain: gene targeting studies. Brain research bulletin 57:73-83.

13. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature genetics 23:99-103.

14. Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ. 2005. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc Natl Acad Sci U S A 102:473-478.

15. Boyle MP, Kolber BJ, Vogt SK, Wozniak DF, Muglia LJ. 2006. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. J Neurosci 26:1971-1978.

16. Erdmann G, Schutz G, Berger S. 2008. Loss of glucocorticoid receptor function in the pituitary results in early postnatal lethality. Endocrinology 149:3446-3451.

17. Schmidt MV, Sterlemann V, Wagner K, Niederleitner B, Ganea K, Liebl C, Deussing JM, Berger S, Schutz G, Holsboer F, Muller MB. 2009. Postnatal glucocorticoid excess due to pituitary glucocorticoid receptor deficiency: differential short- and long-term consequences. Endocrinology 150:2709-2716.

18. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmquist JK, Lowell BB. 2005. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493-505.

19. Mittelstadt PR, Ashwell JD. 2003. Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression. Mol Endocrinol 17:1534-1542.

20. Strahle U, Schmidt A, Kelsey G, Stewart AF, Cole TJ, Schmid W, Schutz G. 1992. At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc Natl Acad Sci U S A 89:6731-6735.

21. Brewer JA, Khor B, Vogt SK, Muglia LM, Fujiwara H, Haegele KE, Sleckman BP, Muglia LJ. 2003. T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation. Nature medicine 9:1318-1322.

22. Cole TJ, Myles K, Purton JF, Brereton PS, Solomon NM, Godfrey DI, Funder JW. 2001. GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant. Molecular and cellular endocrinology 173:193-202.

23. Jeanneteau FD, Lambert WM, Ismaili N, Bath KG, Lee FS, Garabedian MJ, Chao MV. 2012. BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. Proc Natl Acad Sci U S A 109:1305-1310.

24. Kellendonk C, Eiden S, Kretz O, Schutz G, Schmidt I, Tronche F, Simon E. 2002. Inactivation of the GR in the nervous system affects energy accumulation. Endocrinology 143:2333-2340.

25. Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, Walker BR. 1998. Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? The Journal of clinical endocrinology and metabolism 83:757-760.

26. Reynolds RM, Walker BR. 2003. Human insulin resistance: the role of glucocorticoids. Diabetes, obesity & metabolism 5:5-12.

CHAPTER III

ONTOGENY OF HYPOTHALAMIC GLUCOCORTICOID RECEPTOR-MEDIATED INHIBITION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

INTRODUCTION

Glucocorticoid receptors (GR) mediate many organismal functions including developmental, metabolic and stress responses. GR in the paraventricular nucleus of the hypothalamus (PVN) is one of the sites of negative feedback for hypothalamic-pituitary-adrenal (HPA) axis activity. During the stress response, HPA axis activity returns to baseline through negative feedback of corticosterone (CORT) on GR at the level of the PVN and anterior pituitary to turn off the stress response. In the previous chapter, we demonstrated in adult mice, that loss of GR exon 3 but not GR exon 2 in the PVN causes HPA axis hyperactivity with increased PVN CRH mRNA and elevated plasma ACTH and CORT. The hypercorticosterolemia subsequently led to a Cushingoid phenotype in the mutant mice. Moreover, we determined that despite this HPA axis hyperactivity, PVN GR loss did not affect anxiety or despair behavior under basal conditions in adult mice.

During the adolescent period in humans and other animals, the brain is still undergoing development. There are morphological and connectivity changes in a number of brain regions such as the cortex, amygdala, and hippocampus that are still maturing during adolescence(1–6). This makes the adolescent brain more malleable to the effects of environmental experiences including stress, drugs and alcohol (7–11) and number of mental illnesses in adults have their onset in childhood or adolescence (12–15). Studies indicate that during development, the HPA axis in adolescent rodents undergoes

reorganization (16–18) thus making this developmental period of time sensitive to the effects of stress. Due to the propensity of the developing adolescent brain to be influenced by stimuli, we hypothesized that adolescent mice will be more susceptible to the effects of PVN GR disruption. Here we aim to define the developmental trajectory of PVN GR function by evaluating the effects of PVN GR loss during early adolescence (postnatal days 30-50) and late adolescence (postnatal days 51-70) on neuroendocrine and behavioral activity.

MATERIALS AND METHODS

Animals

The animal protocols were in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees of Cincinnati Children's Research Foundation (Cincinnati, OH).

Mice were housed on a 14 h/10 h light/dark cycle with ad libitum access to rodent chow and water. Mice were generated as previously described (19). Briefly, Sim1Cre-GRe3Δ mice were generated by mating Sim1Cre mice (20) to floxed GR exon 3 mice (21), Sim1Cre-GRe2Δ mice were generated by mating Sim1Cre mice to floxed GR exon 1C-2 mice (22), and controls are littermates that were Cre negative but floxed GR exon 1C-2 or exon 3 positive. Floxed GR exon 3 mice were obtained from Dr. Günther Schütz, German Cancer Research Center, Heidelberg, Germany. Sim1Cre mice were the gift of Dr. Brad Lowell, Beth Israel Deaconess Medical Center, Boston, MA.

Immunohistochemistry

We used 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) to anesthetize mice at P2 and perfused them with DEPC 1x PBS followed by 4% DEPC PFA. We post-fixed the brains in 4% DEPC PFA overnight and immersed the brains in 70% ethanol to process for paraffin embedding. A microtome was used to section paraffin embedded brains at 8 μm onto Superfrost+ slides (VWR). We placed the slides at 60ºC overnight and stored at room temperature until later use. We deparaffinized the slides, rehydrated in decreasing concentrations of ethanol, reconstituted the antigen in 10mM Citrate Buffer (pH 6.0). Slides were washed in PBS and blocked for 1 hour in 3% normal goat serum/ 0.25% Triton-X-100 in PBS to prevent non-specific binding. *GR*: Primary antibodies were 1:200 rabbit m20-anti-GR, directed against the N-terminus of GR, (Santa Cruz Biotech. Cat# sc-1004) and 1:200 mouse anti-NeuN; Secondary antibodies were 1:250 biotinylated goat anti-rabbit IgG and 1:200 goat anti-mouse Alexa 488 IgG. We washed the slides, incubated in avidin/biotin complex (Vectastain), Cyanine-3 tyramide amplified (Perkin Elmer) and coverslipped with DAPI (Vector Laboratories).

Corticosterone radioimmunoassay

Mice between P30–50 (early adolescence) and P51–70 (late adolescent) were double housed for a week. Submandibular bleeds were performed in mice at nadir, peak, and 20 minutes after restraint stress with two days between bleeds. We centrifuged the blood at 14000 rpm for 6 minutes and stored the plasma at -80ºC until a

radioimmunoassay (RIA) was performed. The Corticosterone Double Antibody - ^{125}I RIA Kit (MP Biomedicals, Solon, OH) was used to perform the RIA.

CRH *in situ* hybridization

Mice were anesthetized mice with a 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) solution, perfused with DEPC 1x PBS followed by 4% DEPC PFA, and the brains were post-fixed overnight in 4% DEPC PFA. After 48-hour immersion in 20% DEPC sucrose solution at 4ºC, brains were embedded in OCT. 16um sections were obtained from brains at -15ºC in a cryostat and mounted onto Superfrost+ slides (VWR), vacuum-dried overnight, and stored at -80ºC until *in situ* hybridization was performed. P-33 UTP was used to label an RNA probe complementary to a 0.32 KB fragment on exon 2 of the CRH mRNA. Sections were hybridized to the labeled probe at 65ºC for 16 hours and washed in 0.1X SSC at 60ºC for 30 min. Slides were exposed to Maximum resonance film (Kodax BioMAx, Rochester N.Y.) for 4 days. We scanned the autoradiographic images at 3200 dots/inch into Photoshop on an Epson Perfection V600 scanner and quantified using NIH Image J software.

Behavioral tests

Behavioral tests were performed between 8:00 a.m. and 12:00 p.m. in mice ages P30–50 and adult mice 3-4 months old. Mice acclimated in a room adjacent to the testing room for an hour before behavioral testing. Behavioral tests were performed in the Cincinnati Children's Hospital behavior core.

Open field (OF) test: Locomotor exploration/activity was measured in a 41 cm × 41cm chamber (Accuscan Instruments, Columbus, OH). The chamber was equipped with a 16 LED-photocells 2 cm above the floor in x- and y-planes 2.5cm apart. Each trial lasted for a period of 30 minutes, and was analyzed in 5-minute interval. During that time, the PAS Activity program (San Diego Instruments, CA) was used to measure behaviors such as time spent, distance travelled, and number of entries into the inner and outer zones of the chamber.

Light dark (LD) test: The same apparatus used in the OF test was used in this LD test. A rectangular black acrylic (40 cm x 20 cm) insert with an opening was placed into the box and provided a half light, half dark box. Mice were placed a corner of the dark side and allowed to explore for 10 minutes. We recorded latency to enter the light zone, number of entries and time spent in the different zones.

Elevated Zero Maze (EZM) test: This maze (50cm inner diameter, 5cm lane width, 15cm wall height, placed 50 cm off the ground on 4 braced legs) consists of a round maze with opposing open and closed arms. Mice are placed in the center of the open arm and activity is scored for 5 minutes. Time spent in the open and closed areas, as well as latency and number of entries into the open and closed areas was measured.

Tail suspension test (TST): This test used a rectangular tabletop platform with a hole through which the mouse's tail was suspended. Latency to immobile and time spent immobile were measured manually with the use of a computer software.

Forced swim test (FST): This test utilizes a clear 25 cm x 20 cm acrylic cylinder filled ¾ full with water at room temperature (25°C). Mice are placed in the beaker for 1 minute to acclimate to the chamber, after which we tested the mice for 5 minutes.

Latency to immobile and time spent immobile were measured. Mice were placed in a warm cage and toweled dried.

Statistical analysis

The results are presented as mean ± SEM after data were subjected to Student t-test, one- or two-way ANOVA test (Prism 5.0 software; GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as a p value ≤ 0.05 and was analyzed using the Tukey or Bonferroni post hoc test.

RESULTS

Deletion of GRe1C-2 and GRe3 in the PVN neurons expressing Sim1

Sim1Cre-GRe3Δ and Sim1Cre-GRe2Δ transgenic mice were generated as previously described (19). To determine the when deletion of GR first occurs, we performed fluorescent immunohistochemistry in P2 brains. We observed a reduction in PVN GR expression in Sim1Cre-GRe3Δ mice compared to controls at age P2 (Figure 3.1.), but no changes in Sim1Cre-GRe2Δ mice (data no shown) when compared to control mice.

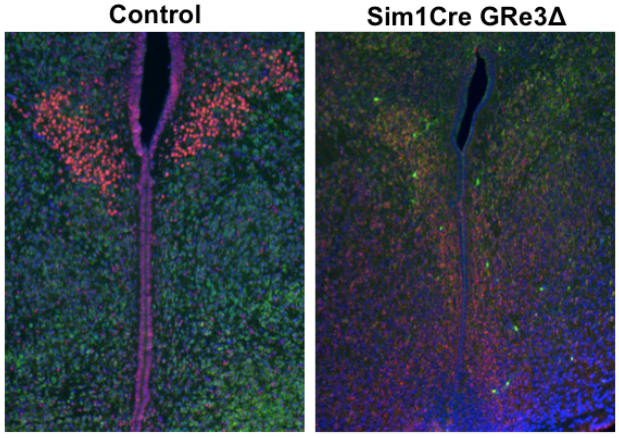

Figure 3.1. Decreased GR expression in Sim1Cre-GRE3Δ at postnatal day 2. Immunofluorescent images of the PVN showing GR (red), NeuN (green), and Dapi (blue) in mice at postnatal day 2 (P2). Representative of 3 mice.

Adolescent Sim1Cre-GRE3Δ mice display developmental HPA Axis dysregulation in a gender-specific manner

We measured serum CORT levels in controls, Sim1Cre-GRe2Δ and Sim1Cre-GRe3Δ mice early in life to determine the developmental consequences of PVN GR deletion. During early adolescent period, P30–P50, we found that male Sim1Cre-GRe3Δ mice had significantly elevated nadir ($p=0.001$) and peak ($p=0.005$) plasma CORT levels compared to controls, but stress CORT levels did not differ ($p=0.299$) (Figure 3.2.A). In late adolescence P51–P70, male Sim1Cre-GRe3Δ mice show elevated plasma CORT at nadir ($p=0.001$), peak ($p<0.0001$) and after 20 minutes of restrain stress ($p<0.0001$) compared to controls (Figure 3.2.B), similar to what was observed in adult male mice (19). On the other hand, early adolescence in female Sim1Cre-GRe3Δ mice was characterized by no changes in basal and stress-induced plasma CORT levels compared to control mice (Figure 3.2.C). In late adolescence, nadir plasma CORT levels in female Sim1Cre-GRe3Δ mice were significantly higher than controls ($p=0.011$) but peak and stress-induced CORT did not differ (Figure 3.2.D). Interestingly, adolescent male Sim1Cre-GRe2Δ mice (P30 – P50) have elevated plasma CORT levels at peak compared to controls ($p=0.001$), but no differences at nadir and stress. This increase is transient and does not last in late adolescence (Figure 3.2.B) or adulthood (19). The circadian rhythm of CORT secretion was apparent by P30-50 in all groups, controls, Sim1Cre-GRe3Δ and Sim1Cre-GRe2Δ and both genders, and was maintained in the transgenic mice (Figure 3.2.).

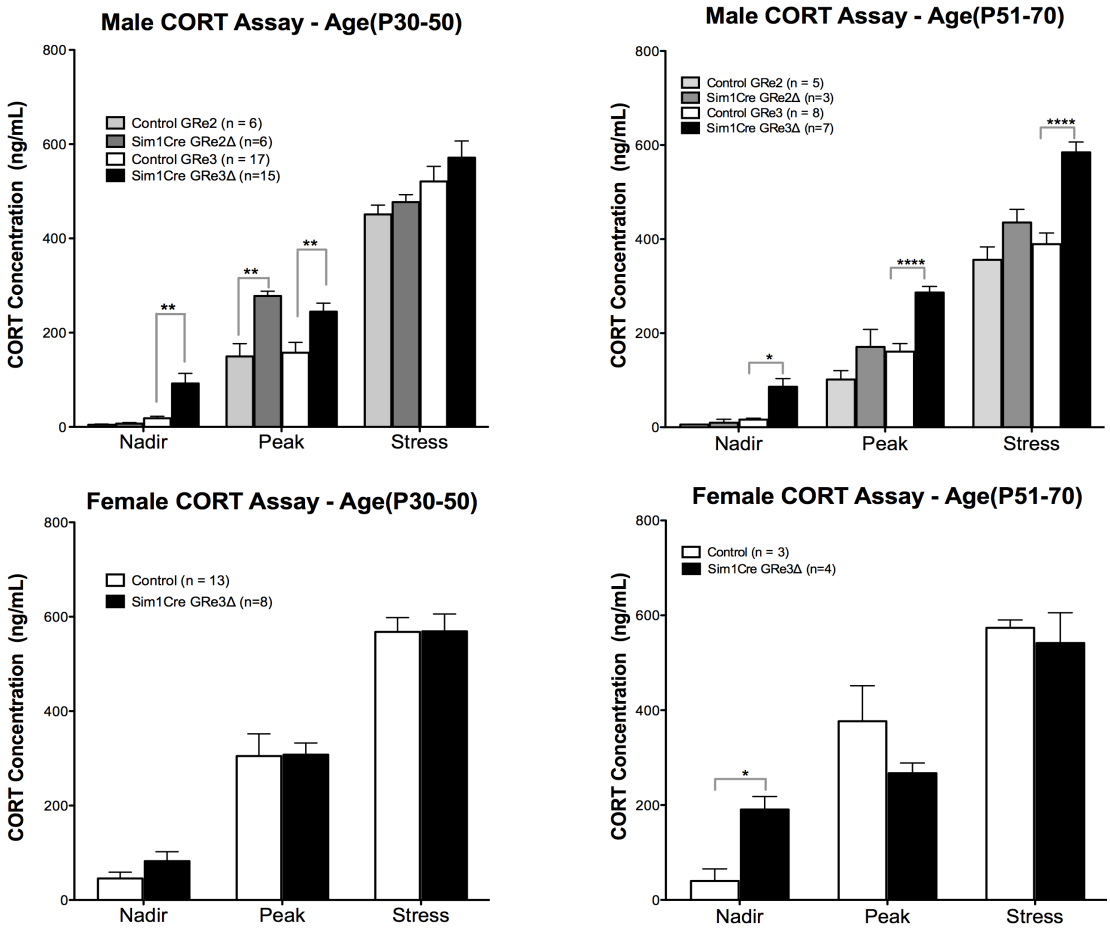

Figure. 3.2. Loss of PVN GR results developmental and gender-specific differences in HPA axis regulation. (A,B) Plasma CORT levels measured at nadir, peak, and after 20-minutes of restraint stress. Sim1Cre-GRe3Δ mice ages P30-P50 display increased plasma CORT at nadir and peak and after stress compared to controls and Sim1Cre-GRe2Δ mice. (C,D) Dexamethasone suppression test was used to evaluate negative feedback. Control female mice show a 50% suppression of CORT after dexamethasone (C), while males show a trend towards CORT suppression, (D). Sim1Cre-GRe3Δ mice do not show suppression of CORT after dexamethasone administration. $^{\#}p=0.053$ *$p<0.05$, ***$p<0.001$.

Adolescent Sim1Cre-GRE3Δ mice have reduced locomotor activity and increased anxiety behavior

In order to evaluate how HPA axis disruption in adolescence affected stress-related behavior, we evaluated anxiety behavior in the Open field test (OF), the light-dark test (LD) and the Elevated zero maze (EZM). Male mice were tested between the ages of P30–P50, with two days between tests. In the OF test, adolescent Sim1Cre-GRE3Δ mice have reduced locomotor activity identified in the distance traveled in the open field maze (Figure 3.3.A, $p< 0.0001$) and reduced entries into the center of the open field which is characteristic of increased anxiety (Figure 3.3.C, $p= 0.002$). However, the amount of time spent in the center and the latency to enter the center of the open field did not differ in adolescent Sim1Cre-GRE3Δ mice compared to controls (Figure 3.3. B). In the LD tests, there were no differences in the time spent, entries, and latency to enter the light zone (Figure 3.4. A,B). In the EZM test, adolescent Sim1Cre-GRE3Δ mice compared to controls spent less time ($p=0.012$) and made fewer entries ($p=0.004$) into the open arm of the maze (Figure 3.3. E, F), indicative of increased anxiety-like behavior, but there were no differences in latency to enter open arm (Figure 3.3. D).

Adolescent Sim1Cre-GRE3Δ mice have no despair-like behavior

We evaluated the effects of PVN GR loss in adolescence on despair-like behavior. Two major tests of despair are the tail suspension tests (TST) and the forced swim test (FST). In the both the TST and FST, adolescent Sim1Cre-GRE3Δ mice did not differ in the time immobile or latency to immobile compared to control mice (Figure 3.4. B – E).

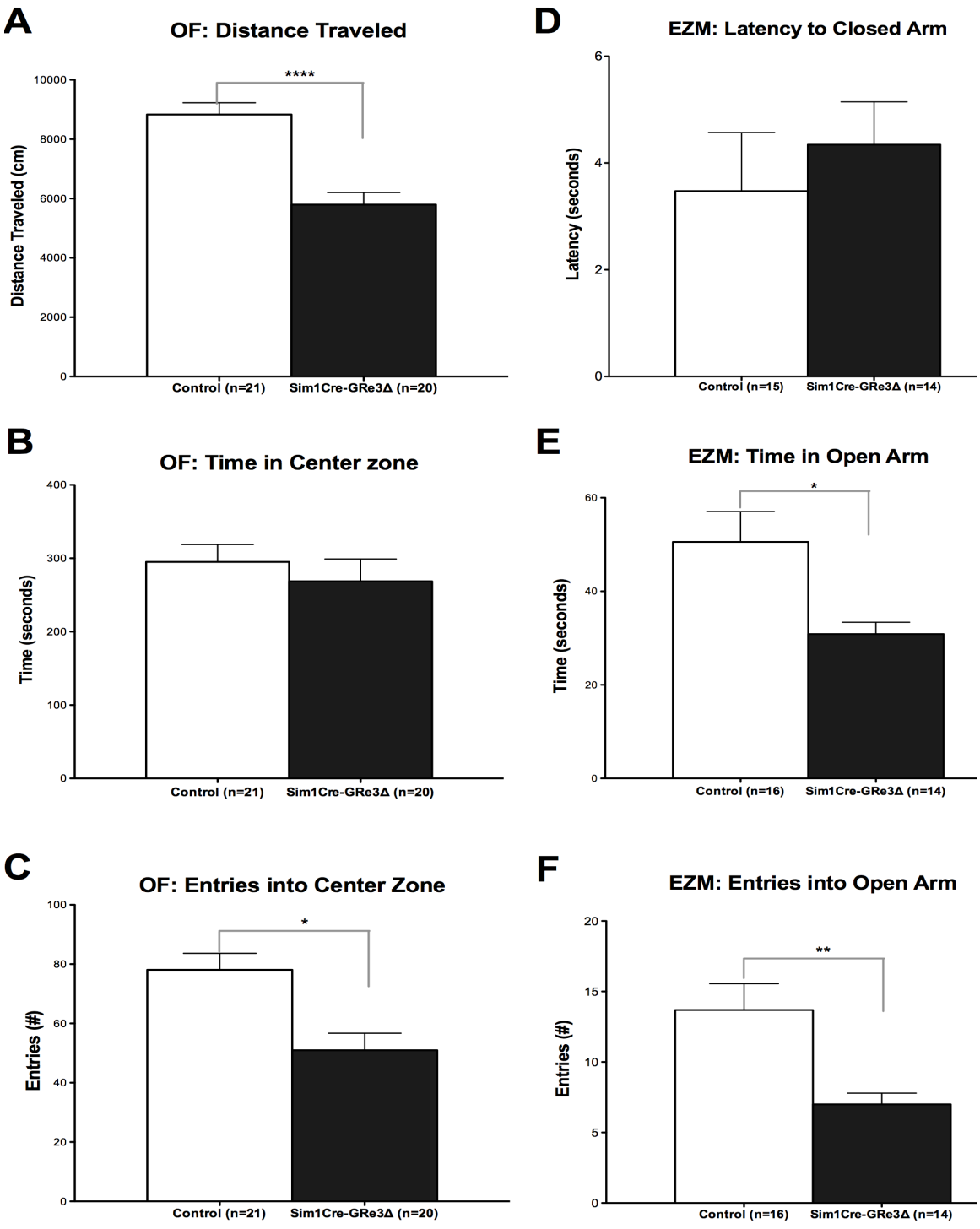

Figure 3.3. Reduced locomotor activity and increased anxiety behavior in adolescent Sim1Cre-GRe3Δ mice in the OF and EZM tests. Sim1Cre-GRe3Δ mice show reduced distance traveled (A), no change in time spent in center (B), and decreased entries into the center of the OF (C). In the EZM tests, Sim1Cre-GRe3Δ mice have no difference in latency to enter the closed arm (D), spend less time spent in the open arm (E), and have reduced number of entries into the open arm (F). *p<0.05, ** p<0.01, ****p<0.0001.

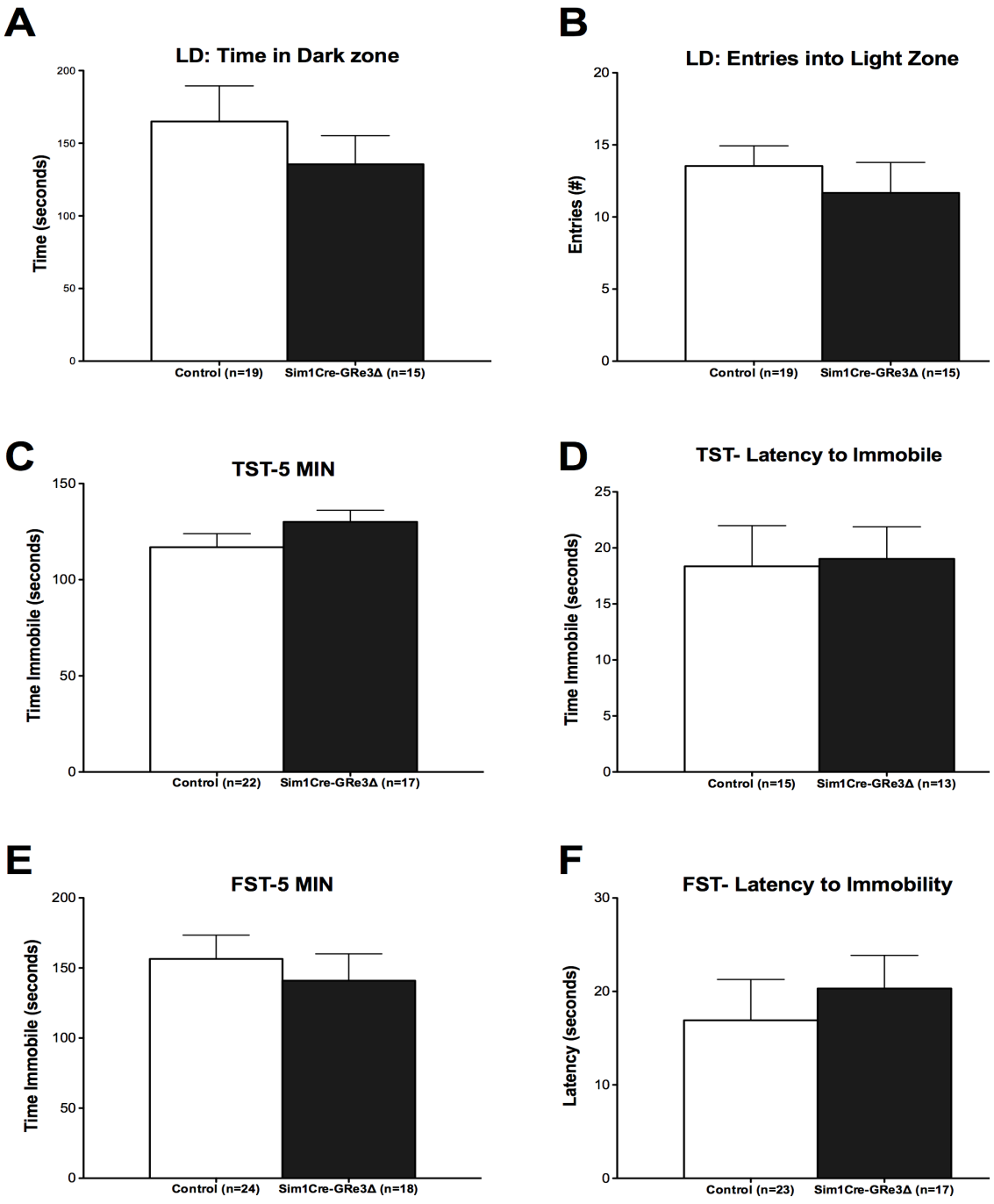

Figure 3.4. Adolescent Sim1Cre-GRe3Δ mice do not exhibit anxiety behavior in the LD test or despair behavior in the TST and FST tests. Sim1Cre-GRe3Δ mice show no change in time spent, (A), or entries, (B), into the light zones. Sim1Cre-GRe3Δ mice show no differences in time spent immobile (C) or latency to immobility, (D), in the TST test. Sim1Cre-GRe3Δ mice show no differences in time spent immobile, (E), or latency to immobility in the FST test, (F).

Retesting in adulthood shows anxiety behavior in Sim1Cre-GRe3Δ mice that was not previously observed

Our previous data demonstrated that adult Sim1Cre-GRe3Δ mice had no anxiety or despair phenotypes (19). Given that we observe anxiety-like behavior in the OF and EZM tests in adolescent mice, we sought to determine whether the behaviors observed in adolescence were transient and lost in adulthood. We therefore retested some of the previously tested adolescent mice again when they were 3-4 months old. In adulthood, the Sim1Cre-GRe3Δ mice have the expected reduction in locomotor activity (Figure 3.5. A, $p< 0.001$), but to our surprise, these Sim1Cre-GRe3Δ mice entered into the center of the OF test significantly less than controls (Figure 3.5. D, $p=0.004$). Moreover the Sim1Cre-GRe3Δ mice retested in adulthood showed a decreased distance traveled in the LD test ($p=0.04$), trend towards reduced entries into the light zone in the LD test ($p=0.09$) (Figure 3.5. E,F). No significant differences were found in the EZM test compared to controls (Figure 3.6. A-C). Furthermore no differences in despair-like behavior in the TST and FST were observed between Sim1Cre-GRE3Δ and control mice (data not shown). Since anxiety behavior was not previously observed in adult Sim1Cre-GRe3Δ mice, we questioned whether the current behavioral results were due to the fact that the mice had been previously tested in adolescence. We thus tested naïve adult mice in the anxiety and behavioral tests.

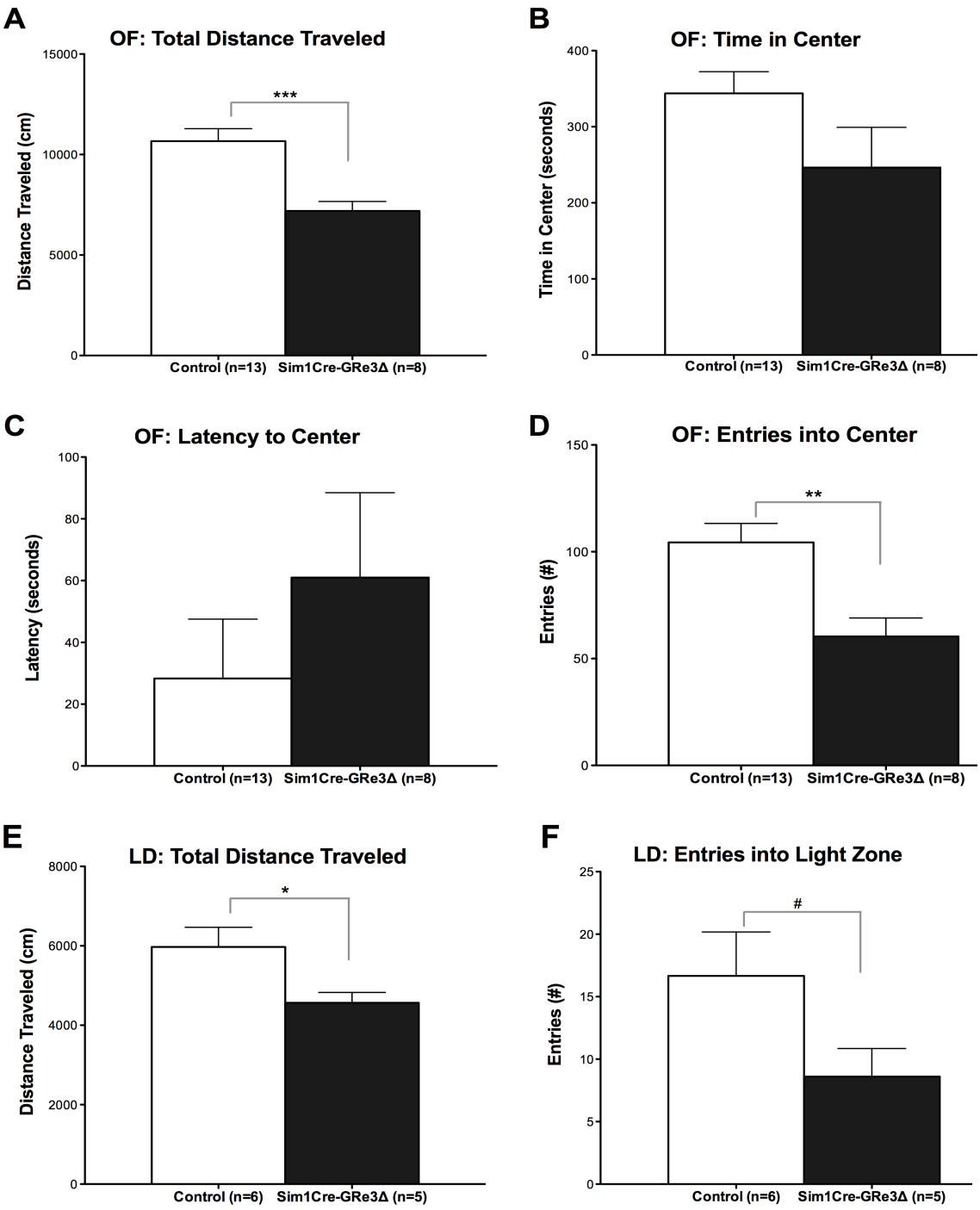

Figure 3.5. OF and LD tests show reduced locomotor activity and increases anxiety behavior in Sim1Cre-GRe3Δ mice retested in adulthood. *OF test* Sim1Cre-GRe3Δ mice show reduced distance traveled (A), no change in time spent in center (B), or latency to enter the center of the OF (C), and decreased entries into the center of the OF (D). *LD test* Sim1Cre-GRe3Δ mice show reduced distance traveled (E), and a trend towards decreased entries into the light zone (F). There was no change in time spent or latency to enter light zone (data not shown). $^{\#}p=0.09$, $*p<0.05$, $**p<0.01$, $***p<0.001$.

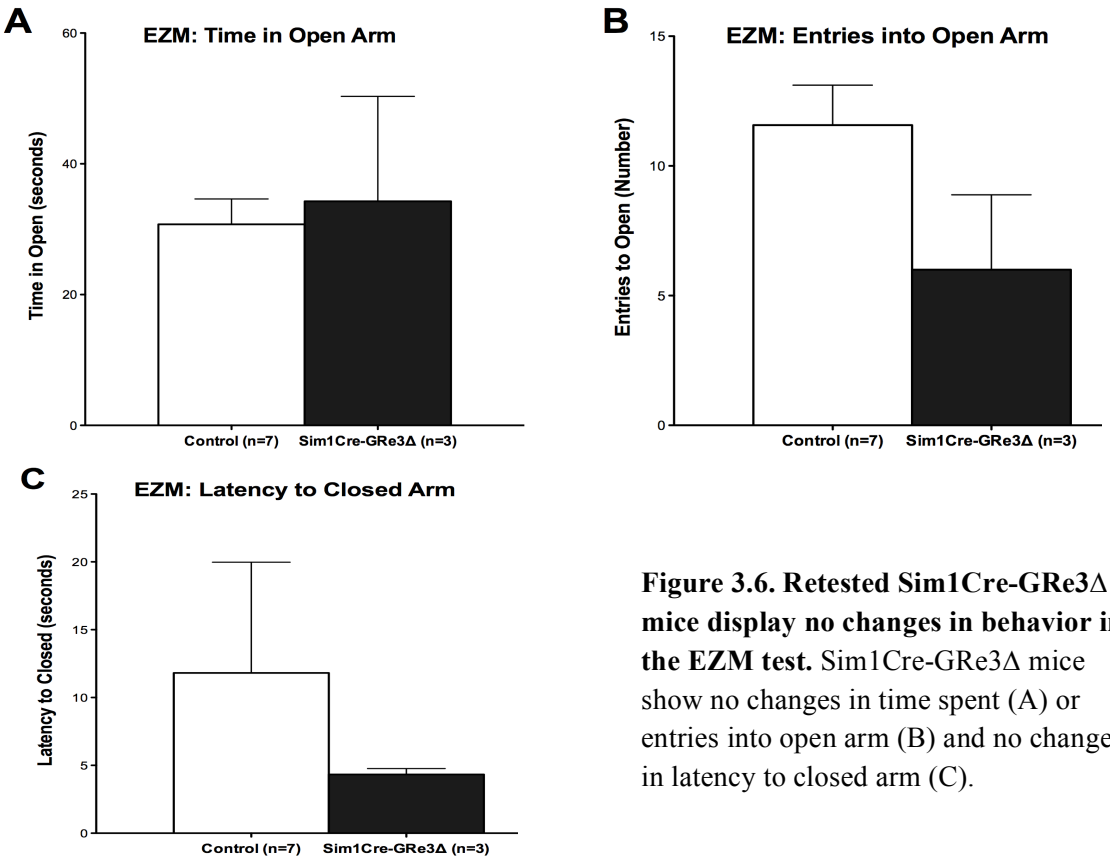

Figure 3.6. Retested Sim1Cre-GRe3Δ mice display no changes in behavior in the EZM test. Sim1Cre-GRe3Δ mice show no changes in time spent (A) or entries into open arm (B) and no changes in latency to closed arm (C).

Naïve adult Sim1Cre-GRE3Δ mice tested in the CCRF behavior core have increased anxiety in the OF and LD, but not the EZM and no despair behavior

In contrast to our previous data in adult mice demonstrating no trace of anxiety behavior (19), this study in adult Sim1Cre-GRE3Δ mice demonstrated increased anxiety behavior in all measures in the OF and LD tests (Figure 3.7.). There were no significant differences in the EZM, TST or FST in these adult mice (Figure 3.8.), similar to previous data in adult Sim1Cre-GRe3Δ mice (19). There are no differences in any of the behaviors measured in Sim1Cre-GRE2Δ mice compared to control mice (data not shown).

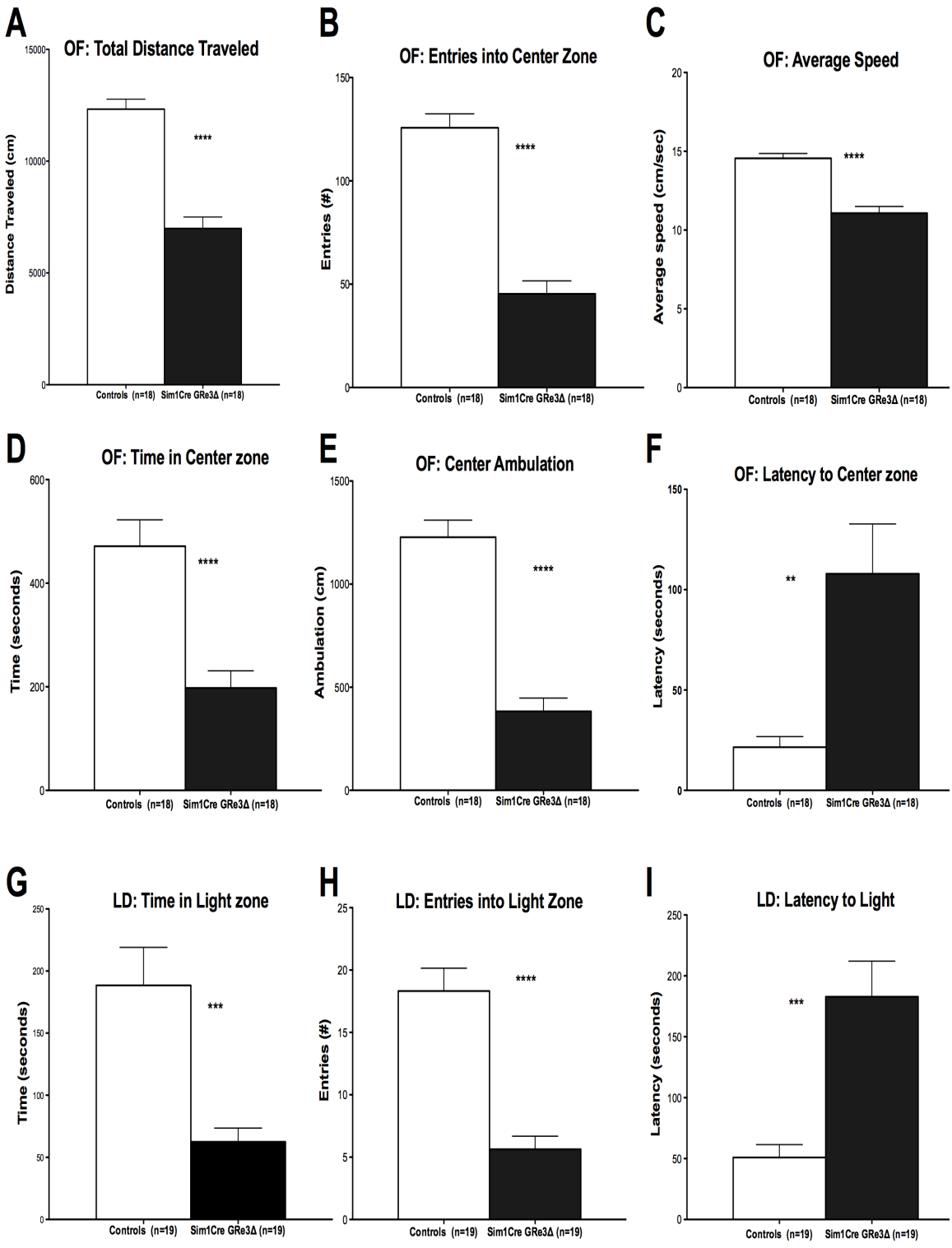

Fig 3.7. Increased anxiety behavior in adult Sim1Cre-GRE3Δ mice in OF and LD tests. Adult male Sim1Cre-GRE3Δ mice tested in the CCHMC behavior core show increased anxiety in the open field (A-F*)* and light-dark (G-I) tests.

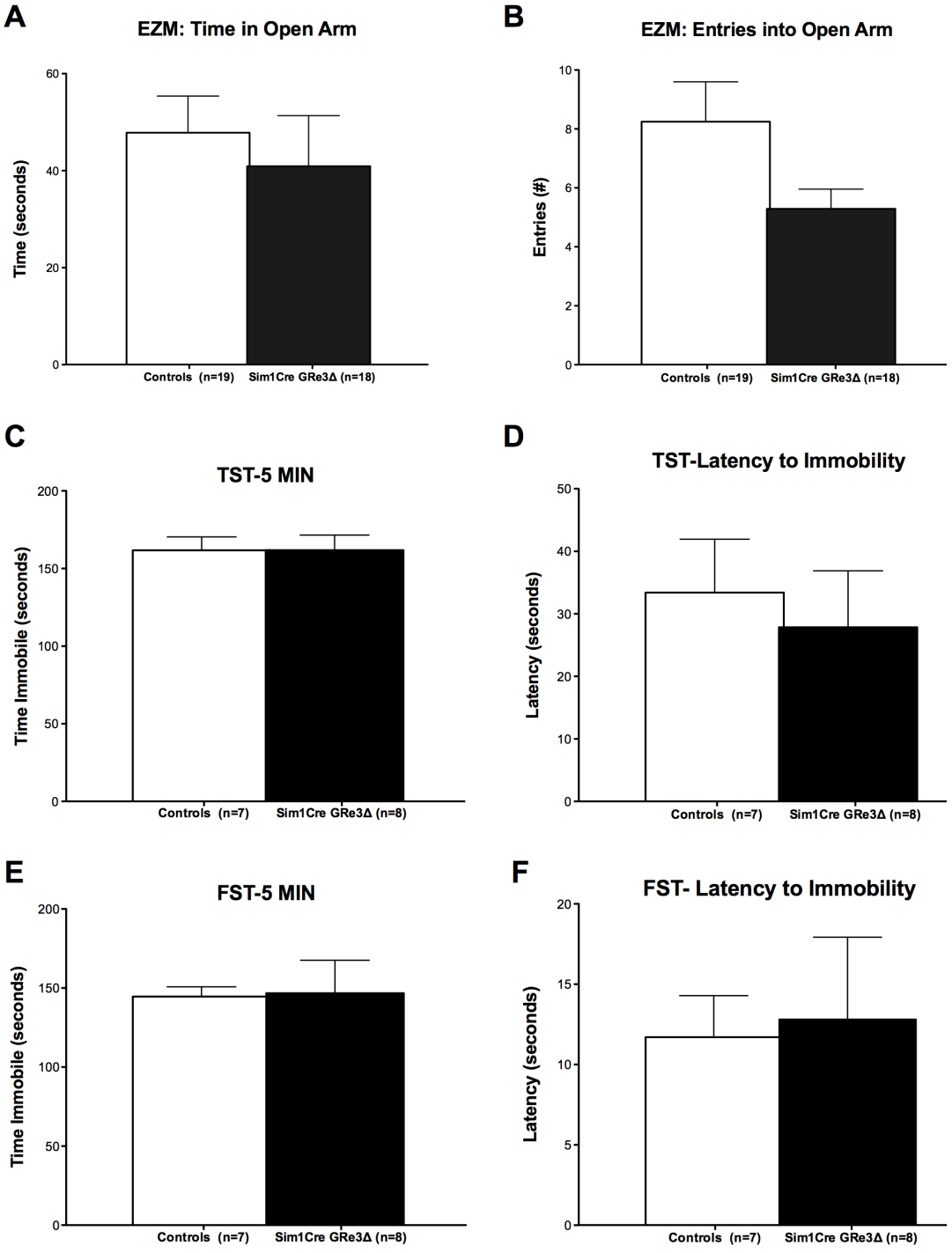

Fig 3.8. Adult Sim1Cre-GRE3Δ mice display no anxiety or despair behavior in the EZM, TST, and FST tests. Adult male Sim1Cre-GRE3Δ mice tested in the CCHMC behavior core show no anxiety behavior in the EZM test (A,B), or despair behavior in the TST (C,D) and FST tests (E,F).

DISCUSSION

HPA axis regulation in adolescence

Early life experiences can result in developmental changes in stress reactivity and behavior. Our data indicates that in Sim1Cre GRe3Δ mice, PVN GR loss occurs by as postnatal day 2. The early disruption of PVN GR leads to elevated nadir and peak plasma CORT levels during early adolescence (P30-P50) in male Sim1Cre GRe3Δ mice. However stress-induced increases in CORT are not different between control and Sim1Cre GRe3Δ mice at that age, and only appear after P51. This indicates that during the early adolescent period of life in males, PVN GR is important for maintaining basal regulation of glucocorticoid secretion, but it is not necessary for regulating stress-induced glucocorticoid secretion until late adolescence/ early adulthood. We propose that early in life glucocorticoid feedback is primarily regulated through pituitary GR and PVN GR feedback dominates later in development.

In females, concentrations of plasma CORT do not vary between control and Sim1Cre GRe3Δ mice in early adolescence. In late adolescence/ early adulthood, nadir levels of plasma CORT are increased in Sim1Cre GRe3Δ, but peak and stress levels do not differ from controls. In contrast to males, PVN GR in female mice is not necessary for maintaining basal regulation of glucocorticoid secretion during adolescence, and does not become necessary until adulthood. The gender difference in CORT secretion between male and female Sim1Cre GRe3Δ mice is suggestive of modulation of GR function by sex hormones. There is mounting evidence for role of estrogen in affecting GR signaling pathways (23–25). Thus differences in GR signaling in females compared to males may account for the gender-specific developmental disparities that we observe in adolescent

Sim1Cre GRe3Δ mice. Our studies suggest that PVN GR mediation of negative feedback occurs earlier in development in males than in females.

Surprisingly, we observe elevated peak plasma CORT levels in male Sim1Cre GRe2 Δ mice in adolescence that is lost in adulthood. This transient developmental increase in CORT indicates that GR loss in Sim1Cre GRe2Δ mice does have transient effects on the levels of glucocorticoid secretion. The differences in that we observe in CORT levels at ages P51-P70 in the Sim1Cre GRe2Δ mice compared to Sim1Cre GRe3Δ mice are similar to what we previously observed in adult mice (19).

Behavioral phenotypes of Sim1Cre GRe3Δ mice throughout development

Many psychiatric disorders have their onset during the adolescent period (14). Similar to adult mice previously tested (19), adolescent mice with loss of GR exon 3 in the PVN display locomotor deficits in the open field and a lack of despair phenotypes. However, we observe increased anxiety behavior in adolescent Sim1Cre GRe3Δ mice in the OF and EZM tests, but not in the LD test. When retested in adulthood, these adolescent mice lose the anxiety phenotype in the EZM test but retain anxious phenotypes observed in the OF, and show trends in anxiety in the LD test. Given that there may be effects of prior testing in the behavior we observed, we evaluated behavior in a cohort of adult mice not previously tested. Surprisingly, these adult Sim1Cre GRe3Δ mice display increased anxiety behavior in all measures of anxiety in the OF and LD tests, completely the opposite of our previously published data. Similar to our previously published data, no anxiety behavior was observed in the EZM however, and no despair behaviors were observed in the TST and FST tests. One possible reason for the

differences between the studies is the different in institutions that behavioral testing occurred. The Vanderbilt neurobehavioral core was used in the studies where we saw no anxiety phenotypes in adult mice. This behavior core has separate testing facilities for mice and rats. The behavioral tests in this current study were performed in the Cincinnati Children's research foundation's (CCRF) behavior core. The CCRF core is for the testing of both rats and mice. In the case of the EZM, TST, and FST tests, separate equipment exist for mice and rats. In the case of the OF and LD tests, the same apparatus is used for testing both mice and rats. The OF maze is rat-sized, and there is a possibility that there is residual rat odor when the mice are tested. Since there is a predator-prey relationship between rats and mice, this testing environment, as well as the size of the maze may factor into the anxiety behavior of adult Sim1Cre GRe3Δ mice tested at CCRF. This is supported by the lack of anxiety behavior of Sim1Cre GRe3Δ mice in the EZM test, which is used only for testing in mice. Since Sim1Cre GRe2Δ mice show no anxiety behavior in this study (data not shown), the data implies that Sim1Cre Gre3Δ mice have a predisposition to anxiety that is environment dependent. The effect of this testing environment in adult Sim1Cre Gre3Δ mice makes it difficult to interpret the anxiety behavior in OF test observed in adolescent Sim1Cre Gre3Δ mice. We cannot discount other factors that may be influencing the differences in anxiety phenotypes between mice tested at the different institutions. A more controlled experiment would enable more accurate delineation of the effects of a gene x environment interaction in causing behavioral disruptions in Sim1Cre Gre3Δ mice.

The adolescent period is a time of increased susceptibility to affective and anxiety disorders in human and (14, 17, 26–28). In our studies, the EZM test shows increased

anxiety during adolescence in Sim1Cre GRe3Δ mice that is lost during adulthood. This may indicate that during adolescence, PVN GR disruption leading to HPA axis hyperactivation mice increases susceptibility to anxiety. As the mice grow into adulthood, however, they habituate to their constantly elevated levels of glucocorticoid and the anxiety phenotype in the EZM test is lost. Since normal behavior is observed in the TST and FST tests, GR in the PVN is likely a mediator of pathways involved in anxiety modulation.

Taken together, our data indicate that PVN GR is important in maintaining basal glucocorticoid levels from early adolescence onward and starts mediating stress-induced glucocorticoid secretion later during adulthood. This finding suggests that pituitary GR mediates stress-induced negative feedback from birth through early adolescence and then PVN GR picks up the task and mediates it through adulthood.

REFERENCES

1. McAnarney ER. 2008. Adolescent brain development: forging new links? J. Adolesc. Health 42:321–3.

2. Giedd JN. 2008. The teen brain: insights from neuroimaging. J. Adolesc. Health 42:335–43.

3. Lenroot RK, Giedd JN. 2006. Brain development in children and adolescents: insights from anatomical magnetic resonance imaging. Neurosci. Biobehav. Rev. 30:718–29.

4. Sowell ER, Trauner DA, Gamst A, Jernigan TL. 2002. Development of cortical and subcortical brain structures in childhood and adolescence: a structural MRI study. Dev. Med. Child Neurol. 44:4–16.

5. Durston S, Hulshoff Pol HE, Casey BJ, Giedd JN, Buitelaar JK, van Engeland H. 2001. Anatomical MRI of the developing human brain: what have we learned? J. Am. Acad. Child Adolesc. Psychiatry 40:1012–20.

6. Spear LP. 2000. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. 24:417–463.

7. Schindler AG, Tsutsui KT, Clark JJ. 2014. Chronic Alcohol Intake During Adolescence, but not Adulthood, Promotes Persistent Deficits in Risk-Based Decision Making. Alcohol. Clin. Exp. Res. 38:1622–1629.

8. Wheeler AL, Lerch JP, Chakravarty MM, Friedel M, Sled JG, Fletcher PJ, Josselyn SA, Frankland PW. 2013. Adolescent cocaine exposure causes enduring macroscale changes in mouse brain structure. J. Neurosci. 33:1797–803a.

9. Carcoba LM, Orfila JE, Natividad LA, Torres O V, Pipkin JA, Ferree PL, Castañeda E, Moss DE, O'Dell LE. 2014. Cholinergic Transmission during Nicotine Withdrawal Is Influenced by Age and Pre-Exposure to Nicotine: Implications for Teenage Smoking. Dev. Neurosci.

10. Adriani W, Laviola G. 2000. A unique hormonal and behavioral hyporesponsivity to both forced novelty and d-amphetamine in periadolescent mice. Neuropharmacology 39:334–346.

11. Steinberg L. 2005. Cognitive and affective development in adolescence. Trends Cogn. Sci. 9:69–74.

12. Lewinsohn PM, Seeley JR, Klein DN. 2003. Bipolar disorders during adolescence. Acta Psychiatr. Scand. 108:47–50.

13. Van Nimwegen L, de Haan L, van Beveren N, van den Brink W, Linszen D. 2005. Adolescence, schizophrenia and drug abuse: a window of vulnerability. Acta Psychiatr. Scand. Suppl. 35–42.

14. Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. 2005. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch. Gen. Psychiatry 62:593–602.

15. Kessler RC, Avenevoli S, Costello EJ, Georgiades K, Green JG, Gruber MJ, He J, Koretz D, McLaughlin KA, Petukhova M, Sampson NA, Zaslavsky AM, Merikangas KR. 2012. Prevalence, persistence, and sociodemographic correlates of DSM-IV disorders in the National Comorbidity Survey Replication Adolescent Supplement. Arch. Gen. Psychiatry 69:372–80.

16. Meaney MJ, Sapolsky RM, McEwen BS. 1985. The development of the glucocorticoid receptor system in the rat limbic brain. I. Ontogeny and autoregulation. Brain Res. 350:159–64.

17. Arnsten AFT, Shansky RM. 2004. Adolescence: vulnerable period for stress-induced prefrontal cortical function? Introduction to part IV. Ann. N. Y. Acad. Sci. 1021:143–7.

18. Sowell ER, Thompson PM, Holmes CJ, Batth R, Jernigan TL, Toga AW. 1999. Localizing age-related changes in brain structure between childhood and adolescence using statistical parametric mapping. Neuroimage 9:587–97.

19. Laryea G, Schütz G, Muglia LJ. 2013. Disrupting hypothalamic glucocorticoid receptors causes HPA axis hyperactivity and excess adiposity. Mol. Endocrinol. 27:1655–65.

20. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang C, Mountjoy K, Kishi T, Elmquist JK, Lowell BB. 2005. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493–505.

21. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schütz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23:99–103.

22. Brewer JA, Khor B, Vogt SK, Muglia LM, Fujiwara H, Haegele KE, Sleckman BP, Muglia LJ. 2003. T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation. Nat. Med. 9:1318–22.

23. Weiser MJ, Foradori CD, Handa RJ. 2010. Estrogen receptor beta activation prevents glucocorticoid receptor-dependent effects of the central nucleus of the amygdala on behavior and neuroendocrine function. Brain Res. 1336:78–88.

24. Weiser MJ, Handa RJ. 2009. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. Neuroscience 159:883–95.

25. Burgess LH, Handa RJ. 1992. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. Endocrinology 131:1261–9.

26. Niwa M, Jaaro-Peled H, Tankou S, Seshadri S, Hikida T, Matsumoto Y, Cascella NG, Kano S, Ozaki N, Nabeshima T, Sawa A. 2013. Adolescent stress-induced epigenetic control of dopaminergic neurons via glucocorticoids. Science 339:335–9.

27. Kovacs M, Feinberg TL, Crouse-Novak MA, Paulauskas SL, Finkelstein R. 1984. Depressive disorders in childhood. I. A longitudinal prospective study of characteristics and recovery. Arch. Gen. Psychiatry 41:229–37.

28. Loman MM, Gunnar MR. 2010. Early experience and the development of stress reactivity and regulation in children. Neurosci. Biobehav. Rev. 34:867–76.

CHAPTER IV

GLOBAL BUT NOT BRAIN-SPECIFIC BMAL1 DEFICIENCY LEADS TO HYPOTHALAMIC-PITUITARY-ADRENAL AXIS HYPOFUNCTION

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INTRODUCTION

Most biological processes in an organism are regulated in a circadian fashion using external environmental cues to synchronize daily oscillations. These regulated biological processes range from molecular events such as gene transcription and hormone secretion to functional changes in metabolic activity, reproduction and sleep (1-5). The main environmental stimulus regulating circadian rhythms is light, but temperature has also been show to regulate periodicity (6, 7). Light stimuli are transmitted through the retino-hypothalamic tract to the master circadian synchronizer, the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus (8-10). As the master regulator, SCN controls rhythms of cells in central nervous system and the periphery. SCN lesions completely abolish circadian rhythmicity of locomotor behavior and plasma corticosterone (CORT) (11-15) and disrupt rhythms of sleep, feeding, and drinking (12, 16, 17).

Clock genes underlie the oscillatory patterns of circadian rhythms. Bmal1 and Clock are two transcription factors that heterodimerize and induce the transcriptions of Period (Per1,2,3) and Cryptochrome (Cry1,2) genes. As the period and cryptochrome genes accumulate, they negatively feedback on the Clock on Bmal1 genes to repress their transcription. This negative feedback loop of clock genes causes transcription or

repression of other genes and thus entrain them to a diurnal rhythmic pattern thereby, entraining biological processes that they govern to have circadian rhythms (See (4, 18) for review).

Disruption of circadian rhythmicity is associated with a number of pathologies including infertility, metabolic syndrome, abnormal apoptosis, retinal degeneration, altered sleep, and impaired memory (19-22). Animal models with different clock genes disrupted have proven informative regarding the individual role of these genes in multiple aspects of behavioral rhythms such as length of free-running periods and light-induced phase shifts (23, 24). Of all the clock genes however, only isolated disruption of Bmal1 produces the complete behavioral arrhythmicity and abolished glucocorticoid rhythms observed in SCN-lesioned animals (25). This is mainly due to the fact that the other core clock genes are not exclusive in function. CLOCK mutants maintain rhythmicity because NPAS2 (a neuronal PAS domain protein) can substitute and heterodimerize with Bmal1. The individual PER and CRY genes can partially compensate for loss of related family members (20, 26-29). Due to this, Bmal1 represents a target crucial to the understanding of circadian rhythms and the pathways it regulates, one of which is the glucocorticoid system.

Glucocorticoid secretion is the major output of the hypothalamic-pituitary adrenal (HPA) axis. Corticotropin-releasing hormone in the periventricular nucleus of the hypothalamus signals the pituitary to synthesize and secrete adrenocorticotropic hormone (ACTH). ACTH then acts to cause the synthesis and secretion of cortisol (humans) or corticosterone (rodents). The synthesis and secretion of the hormones, CRH, ACTH, and CORT occur in a rhythmic fashion driven by SCN clock machinery (30). Glucocorticoids

are important in development and through life mediating functions of many pathways including organogenesis, inflammation, metabolism, learning and memory, stress, and behavior. Thus disruption of circadian glucocorticoid rhythms has profound effects on these pathways and disease states associated with them. Investigating the effects of circadian activity on the HPA axis provides an important avenue to understand biological processes.

Global Bmal1 deletion in mice disrupts a number of biological processes leading to arrhythmic locomotor activity, reduced total activity levels, decreased CORT production and hyposensitivity to ACTH, among other metabolic and reproductive phenotypes (25, 31). In order to determine the role of the central nervous system in these phenotypes, nervous-system-specific Bmal1 deleted mouse models have been developed. These mice demonstrate normal light-entrained locomotor rhythms but impaired food-entrained locomotor activity as well as neurodegeneration (8, 32).

Here we further explore the consequences of global and brain-specific Bmal1 deletion on HPA axis signaling and behavioral activity. We find similar to other studies that global Bmal1 loss disrupts that circadian rhythms of CORT. However we also identify here that Bmal1 is necessary for the maintenance of rhythms of adrenocorticotropic hormone (ACTH) and acute steroidogenic protein (StAR). We also demonstrate that loss of brain Bmal1 does not affect diurnal CORT rhythms or circadian locomotor activity, but does affect the period of free-running activity.

MATERIAL AND METHODS

Animals

All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Cincinnati Children's Medical Center (Cincinnati, OH). Mice were housed on a 14 h/10 h (Cincinnati Children's Medical Center) light/dark cycle with ad libitum access to rodent chow and water. Mice used for studies were between 2-7 months old.

Bmal1 null mice were generated as previously described (33). Bmal1 wild-type and heterozygotes mice were used as controls. NestinCre-Bmal1 ko Mice were generated by mating NestinCre-positive mice (The Jackson Laboratory, Bar Harbour, ME, USA) to Bmal1 floxed mice. Bmal1 floxed mice generated as previously described (33) were used as controls.

Tissue Harvest

Mice were anesthetized with carbon dioxide and cervically dislocated. Adrenal tissue was harvested at nadir and peak from control and Bmal1 null mice. Brains were harvested to obtain hypothalamus and cortex tissue, kidneys were harvested and rapidly frozen on dry ice from control and NestinCre-Bmal1 ko mice. All tissue was stored at -80°C until processing.

Western Blot

Harvested tissue was homogenized for protein extraction in a lysis buffer comprised of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, protease Inhibitor,

phosphatase Inhibitors I and II, 1 mM DTT, and 1% NP-40. 60ug of tissue extracts were resolved in NuPAGE 4-12% Bis-Tris gels (Life Technologies, Carlsbad CA). Proteins were transferred onto nitrocellulose membranes (Perkin Elmer, Hebron KY) using semidry transfer method. Membranes were blocked in 5% milk (Lab Scientific Inc) in PBS and 0.1% Tween-20 (Sigma-Aldrich, St. Louis MO). Blots were probed with rabbit anti-Bmal1 primary antibody (Bethyl labs, Montgomery TX) and horseradish peroxidase labeled goat anti rabbit IgG (Santa Cruz Biotech, Dallas TX) secondary. After stripping with Stripping buffer (Thermo Scientific, Florence KY), membranes were reprobed with anti-actin (Sigma-Aldrich, St. Louis MO) primary and goat anti-rabbit IgG HRP (Santa Cruz Biotech, Dallas TX) secondary antibodies. Blots were processed with Super Signal West Chemiluminescent Substrate (Thermo Scientific, Florence KY, Florence KY) and visualized on Hyblot CL Autoradiography film (Denville Scientific, South Plainfield NJ).

Radioimmunoassays (RIA)

Control and NestinCre-Bmal1 Ko male and female mice were double housed for a week and bled by the submandibular method into EDTA-treated tubes. Samples were taken at nadir, peak, and 20 minutes after restraint stress, with two days between bleeds. The blood was centrifuged at 14000 rpm for 10 minutes and the plasma was removed and stored at -80ºC until a radioimmunoassay (RIA) was performed. Corticosterone and ACTH RIA were performed using the Corticosterone and hACTH Double Antibody - 125I RIA Kit respectively (MP Biomedicals, Solon, OH).

Real-time quantitative PCR

RNA was extracted from adrenal tissue harvested at nadir or peak from control (Bmal1 floxed mice) and Bmal1 null mice using the RNeasy Micro Kit (Qiagen, Valencia CA) and transcribed to cDNA using the Quantitect Reverse Transcription kit (Qiagen, Valencia CA). Real time PCR was performed for Steroidogenic acute regulatory protein (StAR) using Express SYBR Green Supermix with ROX (Life Technologies, Grand Island NY). Previously reported StAR forward and reverse primers (34) were used: forward 5'-CTGGCAGGCATGGCCACACA-3' and reverse 5' GGCAGCCACCCCTTGAGGTC 3'. StAR mRNA expression was normalized to GAPDH expression with primers generated using NIH Primer Blast: forward 5'-TCAAGCTCATTTCCTGGTATGAC-3' and reverse 5'-TCTTGCTCAGTGTCCTTGCTG-3'.

Applied Biosystems StepOnePlus RealTime PCR System and software (Life Technologies, Grand Island NY) was used for of cDNA amplification. PCR reaction conditions: one cycle at 50°C for 2 minutes, one cycle at 95°C for 5 minutes, and 35 cycles at 95°C for 15 seconds and once cycle at 60°C for 1 minute. The ΔΔCT method was used to quantify fold change in mRNA expression normalized to control nadir samples.

Circadian Locomotor activity

Mice were individually housed in circadian activity chambers equipped with running wheels (Coulbourn Instruments, Whitehall PA). The wheels in this chamber are connected to a wiring system that record activity any time the mouse makes a revolution

on the wheel. Animals where maintained under a 12:12 hr light:dark cycle for eight days to measure baseline activity. The animals were then switched to constant darkness for 28 days. Activity was recorded by Actimetrics Clocklab software (Wilmette, IL). Actograms and free running period data were generated using MathWorks Matlab software (Natick, MA). Animal care was conducted in accordance with Animal Care and Use Committees guidelines of Cincinnati Children's Medical Center, with periodic equipment maintenance and ad libitum access to food and water.

Statistical analysis

Data were subjected to two-tailed Student t-test or two-way ANOVA with Bonferroni post hoc analysis (Prism 5.0 software; GraphPad Software, Inc., San Diego, CA). Results are presented as mean ± SEM. P value ≤ 0.05 was used to identify significant differences

RESULTS

Bmal1 Null Mice Display Impaired Plasma CORT and adrenal StAR Rhythms

Bmal1 null mice have been previously reported to be arrhythmic in constant darkness and have disrupted circadian rhythms in CORT secretion (25, 31, 35). We find that Control mice have elevated CORT at peak compared to nadir ($p< 0.0001$). No circadian rise was observed in Bmal1 null mice. Despite the lack of circadian rise in CORT secretion, the Bmal1 null mice still retain the ability for stress-induced elevations in plasma CORT (Figure 4.1.A).

Steroidogenic acute regulatory protein is a rate-limiting step in adrenal steroidogenesis. StAR functions as a transport protein to allow cholesterol into the mitochondria where it is converted to the precursor of various steroid hormones including CORT. Previously reported data identified StAR as a clock-controlled gene whose transcription is regulated by the CLOCK:Bmal1 heterodimer and thus has diurnal variation in its expression (36). Moreover, the diurnal expression of adrenal StAR mRNA and protein correlate with the biosynthesis of CORT in rhythmic fashion (36). To verify that the cause of lack of circadian CORT rise in the Bmal1 null mice was due to its biosynthesis, we performed real-time quantitative PCR for adrenal StAR mRNA.

Levels of StAR mRNA in the Bmal1 null mice had a tendency to be lower than in control mice at nadir ($p=0.06$), and were decreased significantly at peak ($p=0.03$) (Figure 4.1.B). We confirm here that the significant circadian rise of StAR mRNA in control mice ($p=0.009$) is lost in Bmal1 null mice ($p=0.8$) (Figure 4.1.B). Indicating that the blunted circadian rise of CORT in Bmal1 null mice was due to disruption in diurnal StAR mRNA expression.

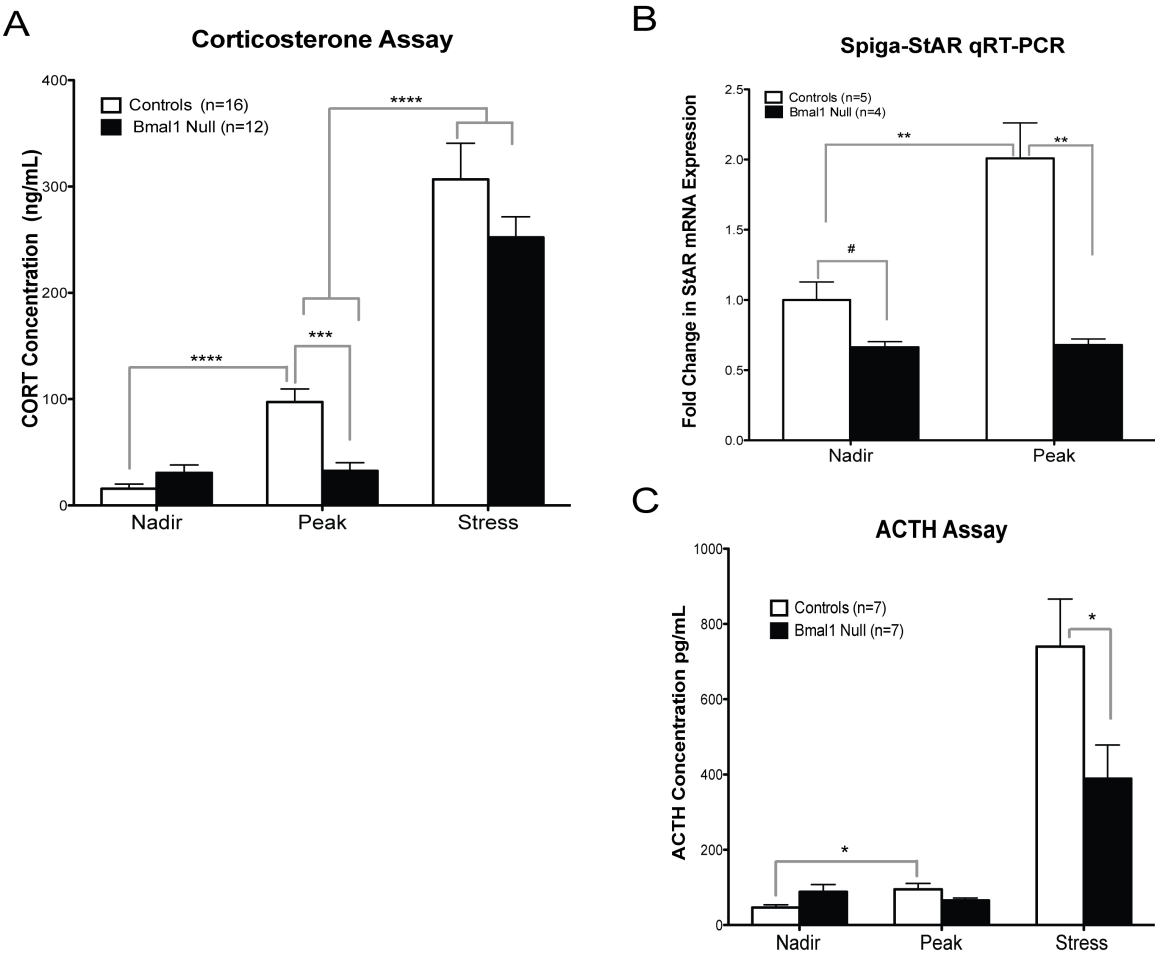

Figure 4.1. Effects of complete Bmal1 loss on HPA axis activity. (A) Plasma CORT levels measured at nadir, peak, and after 20-minutes of restraint stress. Bmal1 null mice lack circadian rise plasma CORT at peak but display normal stress induced increased in CORT. (B) Bmal1 null mice have a trend towards reduced adrenal StAR mRNA expression and lack circadian rise in StAR mRNA compared to controls. (C) Plasma ACTH levels measured at nadir, peak, and after 20-minutes of restraint stress. Bmal1 null mice lack circadian rise plasma ACTH and display reduced stress-induced ACTH levels. $^{\#}p< 0.07$, *p<0.05, **p< 0.01, ***p<0.001, ****p<0.0001.

ACTH Regulates Adrenal StAR Expression

In mice with adrenal-specific Bmal1 deletion, adrenal StAR and CORT diurnal rhythms are abolished only under constant dark conditions (36). The fact that under light-dark conditions StAR circadian rhythms are abolished in Bmal1 null mice (Figure 4.1.B), but maintained in adrenal Bmal1-deleted mice suggest the influence of upstream hormonal regulators in its periodicity.

Given that hypothalamus and pituitary regulate CORT secretion, we evaluated ACTH expression from the pituitary. We measured ACTH to identify whether plasma ACTH expression correlated with the abolished circadian rhythm of StAR mRNA in Bmal1 null mice. Indeed control mice showed a circadian rise in ACTH levels ($p=0.013$) that was absent in Bmal1 null mice (Figure 4.1.C). Interestingly, both control and Bmal1 null mice show stress induced increases in ACTH ($p<0.001$, $p=0.006$ respectively), although levels in control mice were elevated to a greater extent ($p=0.04$) (Figure 4.1.C). Thus plasma ACTH is associated with diurnal circulation of adrenal StAR and subsequently plasma CORT.

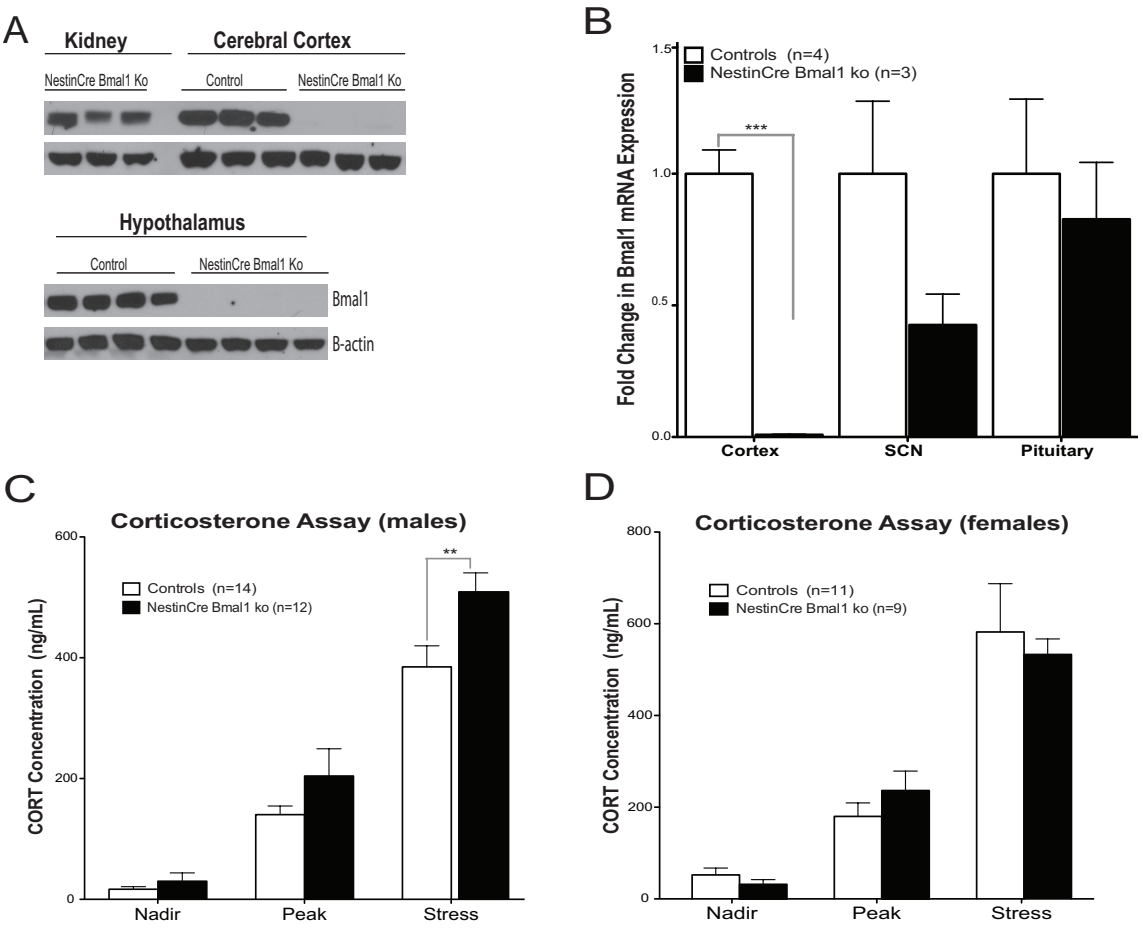

Figure 4.2. Effects of brain Bmal1 loss on Bmal1 expression and HPA axis activity. (A) Western blot demonstrating Bmal1 protein expression in the kidney, cortex, and hypothalamus of control and NestinCre Bmal1 ko mice. We observe undetectable levels of Bmal1 protein in the cortex and hypothalamus of NestinCre Bmal1 ko mice. (B) Quantitative PCR verifies Bmal1 mRNA lost in the cortex of NestinCre Bmal1 ko mice, but there is still residual Bmal1 mRNA in the SCN and no effect in the pituitary. (C,D) Plasma CORT levels measured at nadir, peak, and after 20-minutes of restraint stress shows only a difference in stress-induced CORT levels in males (C), but no differences in females (D). **p< 0.01, ***p<0.001

Intact CORT and Locomotor Circadian Rhythms in Bmal1-deleted Brains

To further examine the role of Bmal1 in the hypothalamus and brain more generally, we generated mice with Bmal1 deleted in the CNS (NestinCre-Bmal1 ko). We verified loss of Bmal1 in the brain using western blot analysis. We find that NestinCre-Bmal1 ko mice had no detectable Bmal1 protein in the cortex or hypothalamus, and this loss of Bmal1 was limited to the brain as the kidney of these mice had normal Bmal1 expression (Figure 4.2.A). Evaluation of Bmal1 mRNA expression demonstrated that NestinCre-Bmal1 ko mice lack Bmal1 mRNA expression it the cortex but retain some residual expression in the SCN (Figure 4.2.B). This residual expression of SCN Bmal1 may account for the normal circadian rhythmicity of CORT secretion in NestinCre-Bmal1 ko mice compared to control mice (Figure 4.2.C,D). Plasma CORT levels in NestinCre-Bmal1 ko mice also did not differ from controls (Figure 4.2. C,D). Interestingly, while both genotypes exhibit significant increases in stress-induced CORT ($p < 0.0001$), levels in male NestinCre-Bmal1 ko mice were elevated to a higher extent compared to controls ($p = 0.016$) (Figure 4.2. B).

Consistent with data in other studies, our NestinCre-Bmal1 ko mice maintain normal circadian locomotor activity (32, 37). We tested this using circadian activity chambers equipped with running wheels. Double plotted actograms confirm that in first 8 days in light-dark cycle (12-12 hours) both groups of mice entrain to the light dark cycle (Figure 4.3. A). In the remaining 28 days of constant darkness, we observe that the NestinCre-Bmal1 ko mice retain locomotor rhythmicity similar to controls (Figure 4.3. A). We, however, do observe significantly reduced free-running period in NestinCre-Bmal1 ko mice compared to control mice ($p=0.048$) (Figure 4.3. B).

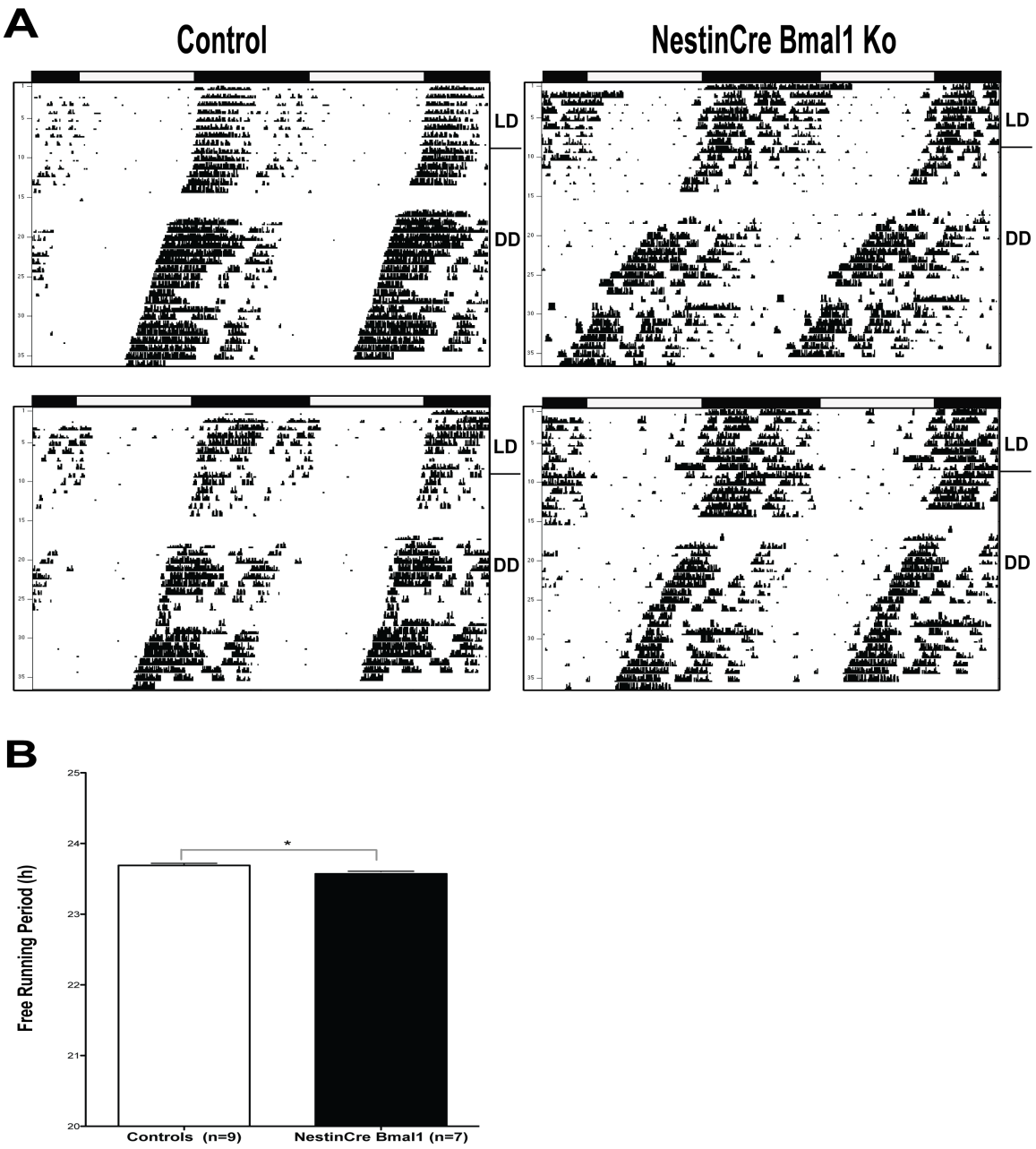

Figure 4.3. NestinCre-Bmal1 ko mice have normal locomotor behavior but reduced free-running period. (A) Actograms of representative control and NestinCre Bmal1 ko mice show no differences in circadian locomotor activity in LD and DD. (B) Evaluation of length of free-running period demonstrates a reduced period in the NestinCre Bmal1 ko mice compared to controls. *$p<0.05$

DISCUSSION

Bmal1 null mice display disrupted circadian rhythms in glucocorticoid secretion (31). We confirm this data in our studies and further demonstrate disruption of diurnal rhythms in plasma ACTH and adrenal StAR mRNA in a normal light-dark cycle. We demonstrate that brain Bmal1 is not necessary for maintenance of behavioral or CORT rhythmicity, but residual Bmal1 expression in SCN neurons are necessary.

Our data show that while ACTH levels do not differ between control and Bmal1 null mice, there is a circadian rise in the control mice, not observed in Bmal1 null mice. This ACTH trend correlates to our plasma CORT data where diurnal rhythm is lost but differs in that peak levels of CORT are significantly reduced in Bmal1 null mice compare to control. These disruptions in ACTH and CORT regulation in combination with the blunted and arrhythmic StAR mRNA suggest that CORT regulation in the Bmal1 null mice is in part due to arrhythmic ACTH signaling and/or reduced adrenal StAR activity. In vitro data from Leliavski et al. demonstrates that the adrenals of Bmal1 null mice have reduced sensitivity to ACTH stimulation (31), which could explain the dissociation between ACTH levels and blunted CORT levels in Bmal1 null compared to controls. Disruption in diurnal CORT rhythm has also been reported in CLOCK mutants (38, 39), PER1 and PER2 mutants (40, 41), as well as CRY1/2 double knockouts (42). Interestingly, our data and data from some these studies demonstrate that CLOCK and Bmal1 disruption dampens CORT secretion (31), whereas PER1, PER2, and CRY1/2 disruption elevates CORT secretion. This indicates the direct control of glucocorticoid secretion by both the positive and negative arms of the clock gene network.

We also observe that despite blunted and arrhythmic CORT and ACTH, Bmal1 null mice still have the ability for stress-induced elevation in both hormones. However while stress-induced CORT levels in Bmal1 null mice rise to the level of control mice, ACTH levels are still blunted in the Bmal1 null mice. One implication of this result is that circadian disruption has no effect on stress-mediated glucocorticoids release. This is substantiated by similar data found in PER2 knockout mice, where basal CORT rhythms are disrupted but stress-induced CORT is intact (41). One possible reason for this may be due to autonomic nervous system stimulation of CORT under stressful conditions, leading to CORT dissociation from ACTH. Interestingly in two days of constant darkness, a mild acute forced swim stressor causes a 3-fold increase in control CORT levels but no increase in Bmal1 null mice (31). Moreover, Bmal1 null mice display similar stress-induced increases in plasma ACTH as do control mice in DD (31). However, in our studies, a 20-minute acute restraint stressor causes greater than 19-fold elevation of CORT in control mice and 8-fold elevation of CORT in Bmal1 null mice. While we observe significant elevation in plasma ACTH levels after 20-minute restraint stress, the elevations in control mice are twice as high as those in Bmal1 null mice. These data indicate that the type of stressor as well as the presence or absence of light cues can influence ACTH and glucocorticoid signaling. In general, the data does point to diminished HPA axis sensitivity in Bmal1 null mice and affirms the notion of different neuroendocrine pathways mediating basal versus stress-mediate glucocorticoid secretion.

Adrenal specific Bmal1 deletion was accomplished by using the ACTH receptor (MC2R) promoter to drive expression of an antisense Bmal1 gene. In these transgenic mice, adrenal StAR expression as well as both plasma and adrenal CORT rhythms are

normal in the presence of light cues (36). In constant darkness, adrenal CORT rhythms are abolished while the plasma CORT rhythms are retained but attenuated (36). This data suggests that adrenal Bmal1 affects the peak levels of CORT secretion but not the circadian rhythm of CORT.

Consistent with other studies (32, 37), we observed in our NestinCre-Bmal1 ko mice that loss of Bmal1 in neurons is associated with normal circadian locomotor activity, however the free-running period is significantly reduced by approximately 7 minutes (Figure 5.3. B). Interestingly, when brain-Bmal1 is rescued in mice on a Bmal1 null background, circadian rhythmicity is restored, but the free-running period is reduced by one hour compared to control mice (35). Reduced free running period when Bmal1 is lost in the brain and when it is expressed only in the brain indicates peripheral Bmal1 also plays a role in determining the period of locomotor activity.

Complete Bmal1 loss in mice abolishes both circadian locomotor and CORT rhythms (at least under normal light/dark conditions). Given that both brain- and adrenal-specific Bmal1 loss do not affect normal CORT rhythms, the findings together suggest that intact Bmal1 expression in the pituitary may compensate to mediate circadian rhythmicity in NestinCre-Bmal1 ko mice. This is not surprising since glucocorticoid pathways at the level of the pituitary and the adrenal cortex have their own endogenous clock systems to regulate in the absence of brain Bmal1 input (43-45). A Cre-mediated Bmal1 deletion targeting the pituitary or hypothalamic neurons may help to elucidate the inherent source of diurnal CORT synthesis in more detail. However, the incomplete Bmal1 deletion in the SCN of NestinCre-Bmal1 ko mice is a potential source for the maintained circadian CORT rhythms. We conclude that SCN, but not brain, Bmal1, is

necessary for maintaining the circadian rhythm of locomotor activity and plasma CORT levels.

REFERENCES

1. Bailey M, Silver R. 2014. Sex differences in circadian timing systems: Implications for disease. Frontiers in neuroendocrinology 35:111-139.

2. Boden MJ, Kennaway DJ. 2006. Circadian rhythms and reproduction. Reproduction 132:379-392.

3. Lowrey PL, Takahashi JS. 2004. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annual review of genomics and human genetics 5:407-441.

4. Orozco-Solis R, Sassone-Corsi P. 2014. Epigenetic control and the circadian clock: Linking metabolism to neuronal responses. Neuroscience 264C:76-87.

5. Moore RY. 2007. Suprachiasmatic nucleus in sleep-wake regulation. Sleep medicine 8 Suppl 3:27-33.

6. Bunning E. 1962. Mechanism in circadian rhythms: functional and pathological changes resulting from beats and from rhythm abnormities. Annals of the New York Academy of Sciences 98:901-915.

7. Mendoza J, Challet E. 2009. Brain clocks: from the suprachiasmatic nuclei to a cerebral network. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 15:477-488.

8. Meijer JH, Schwartz WJ. 2003. In Search of the Pathways for Light-Induced Pacemaker Resetting in the Suprachiasmatic Nucleus. Journal of biological rhythms 18:235-249.

9. Morin LP. 1994. The circadian visual system. Brain research. Brain research reviews 19:102-127.

10. Ralph MR, Foster RG, Davis FC, Menaker M. 1990. Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975-978.

11. Moore RY, Eichler VB. 1972. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain research 42:201-206.

12. Stephan FK, Zucker I. 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proceedings of the National Academy of Sciences of the United States of America 69:1583-1586.

13. Watanabe K, Hiroshige T. 1981. Phase relation between episodic fluctuations of spontaneous locomotor activity and plasma corticosterone in rats with suprachiasmatic nuclei lesions. Neuroendocrinology 33:52-59.

14. Raisman G, Brown-Grant K. 1977. The 'suprachiasmatic syndrome': endocrine and behavioural abnormalities following lesions of the suprachiasmatic nuclei in the female rat. Proc R Soc Lond B Biol Sci 198:297-314.

15. Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nakahara D, Tsujimoto G, Okamura H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell metabolism 2:297-307.

16. Mouret J, Coindet J, Debilly G, Chouvet G. 1978. Suprachiasmatic nuclei lesions in the rat: alterations in sleep circadian rhythms. Electroencephalography and clinical neurophysiology 45:402-408.

17. Angeles-Castellanos M, Salgado-Delgado R, Rodriguez K, Buijs RM, Escobar C. 2010. The suprachiasmatic nucleus participates in food entrainment: a lesion study. Neuroscience 165:1115-1126.

18. Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. Nature 418:935-941.

19. Karatsoreos IN, Bhagat S, Bloss EB, Morrison JH, McEwen BS. 2011. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. Proceedings of the National Academy of Sciences of the United States of America 108:1657-1662.

20. Ko CH, Takahashi JS. 2006. Molecular components of the mammalian circadian clock. Human molecular genetics 15 Spec No 2:R271-277.

21. Ratajczak CK, Boehle KL, Muglia LJ. 2009. Impaired steroidogenesis and implantation failure in Bmal1-/- mice. Endocrinology 150:1879-1885.

22. Zelinski EL, Deibel SH, McDonald RJ. 2014. The trouble with circadian clock dysfunction: Multiple deleterious effects on the brain and body. Neuroscience and biobehavioral reviews 40C:80-101.

23. Debruyne JP, Noton E, Lambert CM, Maywood ES, Weaver DR, Reppert SM. 2006. A clock shock: mouse CLOCK is not required for circadian oscillator function. Neuron 50:465-477.

24. Bae K, Weaver DR. 2003. Light-induced phase shifts in mice lacking mPER1 or mPER2. Journal of biological rhythms 18:123-133.

25. Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

26. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. 2001. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525-536.

27. DeBruyne JP, Weaver DR, Reppert SM. 2007. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nature neuroscience 10:543-545.

28. Thresher RJ, Vitaterna MH, Miyamoto Y, Kazantsev A, Hsu DS, Petit C, Selby CP, Dawut L, Smithies O, Takahashi JS, Sancar A. 1998. Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. Science 282:1490-1494.

29. van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A. 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630.

30. Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E. 2012. Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. Molecular and cellular endocrinology 349:20-29.

31. Leliavski A, Shostak A, Husse J, Oster H. 2014. Impaired glucocorticoid production and response to stress in Arntl-deficient male mice. Endocrinology 155:133-142.

32. Musiek ES, Lim MM, Yang G, Bauer AQ, Qi L, Lee Y, Roh JH, Ortiz-Gonzalez X, Dearborn JT, Culver JP, Herzog ED, Hogenesch JB, Wozniak DF, Dikranian K, Giasson BI, Weaver DR, Holtzman DM, Fitzgerald GA. 2013. Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. The Journal of clinical investigation 123:5389-5400.

33. Ratajczak CK, Asada M, Allen GC, McMahon DG, Muglia LM, Smith D, Bhattacharyya S, Muglia LJ. 2012. Generation of myometrium-specific Bmal1 knockout mice for parturition analysis. Reproduction, fertility, and development 24:759-767.

34. Spiga F, Liu Y, Aguilera G, Lightman SL. 2011. Temporal effect of adrenocorticotrophic hormone on adrenal glucocorticoid steroidogenesis: involvement of the transducer of regulated cyclic AMP-response element-binding protein activity. Journal of neuroendocrinology 23:136-142.

35. McDearmon EL, Patel KN, Ko CH, Walisser JA, Schook AC, Chong JL, Wilsbacher LD, Song EJ, Hong HK, Bradfield CA, Takahashi JS. 2006. Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. Science 314:1304-1308.

36. Son GH, Chung S, Choe HK, Kim HD, Baik SM, Lee H, Lee HW, Choi S, Sun W, Kim H, Cho S, Lee KH, Kim K. 2008. Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. Proceedings of the National Academy of Sciences of the United States of America 105:20970-20975.

37. Mieda M, Sakurai T. 2011. Bmal1 in the nervous system is essential for normal adaptation of circadian locomotor activity and food intake to periodic feeding. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:15391-15396.

38. Kennaway DJ, Owens JA, Voultsios A, Varcoe TJ. 2006. Functional central rhythmicity and light entrainment, but not liver and muscle rhythmicity, are Clock independent. American journal of physiology. Regulatory, integrative and comparative physiology 291:R1172-1180.

39. Takita E, Yokota S, Tahara Y, Hirao A, Aoki N, Nakamura Y, Nakao A, Shibata S. 2013. Biological clock dysfunction exacerbates contact hypersensitivity in mice. The British journal of dermatology 168:39-46.

40. Dallmann R, Touma C, Palme R, Albrecht U, Steinlechner S. 2006. Impaired daily glucocorticoid rhythm in Per1 (Brd) mice. Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 192:769-775.

41. Yang S, Liu A, Weidenhammer A, Cooksey RC, McClain D, Kim MK, Aguilera G, Abel ED, Chung JH. 2009. The role of mPer2 clock gene in glucocorticoid and feeding rhythms. Endocrinology 150:2153-2160.

42. Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW, Downes M, Evans RM. 2011. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature 480:552-556.

43. Becquet D, Boyer B, Rasolonjanahary R, Brue T, Guillen S, Moreno M, Franc JL, Francois-Bellan AM. 2014. Evidence for an internal and functional circadian clock in rat pituitary cells. Molecular and cellular endocrinology 382:888-898.

44. Wunderer F, Kuhne S, Jilg A, Ackermann K, Sebesteny T, Maronde E, Stehle JH. 2013. Clock gene expression in the human pituitary gland. Endocrinology 154:2046-2057.

45. Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Tian J, Hoffmann MW, Eichele G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell metabolism 4:163-173.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY AND CONCLUSIONS

Part 1 of this dissertation determines the consequences of disrupting the glucocorticoid receptor (GR) in the paraventricular nucleus of the hypothalamus (PVN) from adolescence through adulthood. Negative feedback regulation of glucocorticoid (GC) synthesis and secretion by the GR along the hypothalamic-pituitary-adrenal (HPA) axis coordinates basal and stress-induced increases in glucocorticoid secretion. This chapter describes the effects of loss of the GR along major sites of negative feedback in the brain and pituitary, particularly focusing on the PVN. Utilizing genetically-altered mouse models, we evaluate circadian regulation of HPA axis, the stress-stimulated response neuroendocrine and behavioral activity, as well as the integrated response of organism metabolism.

GR disruption in the hypothalamus

In the HPA axis, the PVN is the site of corticotropin-releasing hormone (CRH) synthesis that drives GC synthesis. Circadian regulation of PVN CRH activity is mediated by suprachiasmatic nucleus (SCN) synchronization. Neuroanatomical studies have provided evidence for SCN neuropeptide input to the PVN. For instance, vasopressin (AVP) neurons originating from the SCN project into the dorsal hypothalamic nuclei which then relay information to CRH neurons in the PVN (1).

Physiological data has also demonstrated that the SCN evokes both inhibitory and excitatory postsynaptic potentials in PVN neurons and in this way directly targets PVN CRH neurons (2). This SCN control would therefore drive the canonical HPA axis pathway of GC synthesis and secretion by synchronizing PVN CRH to induce the secretion of pituitary adrenocorticotropic hormone (ACTH) that leads to the synthesis and secretion of corticosterone (CORT). It is of note, however, that both the pituitary and adrenal cortex have endogenous clock pathways that can be activated independently of HPA axis rhythmicity. Furthermore, GC action via GR does not have a direct effect on SCN clock gene expression, but does cause phase shifts in peripheral clock gene-driven circadian functions peripherally, indicating a reciprocal relationship between SCN and GC, in which diurnal GC is able to coordinate SCN driven circadian functions in peripheral oscillators (3).

To specifically investigate the impact of GC feedback at the level of the hypothalamus, we generated mutant mice using a Sim1Cre transgene that expresses Cre-recombinase in Sim-1 neurons, primarily in the PVN, as well as sparsely in other nuclei (4). The Sim1Cre transgene was used to remove exon1C-2 (Sim1Cre-GRe2Δ) or exon 3 (Sim1Cre-GRe3Δ) (Figure 5.1. A) in floxed GR mice. We demonstrated in the resulting mutant mice that loss of 50%-90% of PVN GR protein does not disrupt circadian CORT or ACTH release (5). In control mice, Sim1Cre-GRe2Δ and Sim1Cre-GRe3Δ mice, basal evening CORT and ACTH levels were elevated significantly above morning levels. This retention of diurnal CORT and ACTH rhythms occurred in spite of significantly increased CORT and ACTH secretion in Sim1Cre-GRe3Δ mice. This is evidence that PVN GR is not involved in regulating the qualitative pattern of diurnal secretion of GCs.

Adult Sim1Cre-GRe3Δ mice have augmented HPA axis activity with increased PVN CRH, and increased ACTH and CORT secretion compared control mice. A different PVN GR knockout mouse generated by Jeanneteau and colleagues showed a different phenotype in that the homozygous mutants ($GR^{flox+/+}$;$Sim1Cre^{+}$) are embryonic lethal due to possible residual Cre-recombinase expression in the lung (6). The heterozygous mice ($GR^{flox+/-}$;$Sim1Cre^{+}$), which are hypomorphic for GR in the PVN however, also demonstrate increased plasma CORT ACTH levels. The elevated plasma CORT and ACTH levels are a reflection of increased CRH transcripts in the PVN of $GR^{flox+/-}$;$Sim1Cre^{+}$ and Sim1Cre-GRe3Δ mice (5, 6) (Table 5-1.). Interestingly, none of these HPA axis changes were observed in adult Sim1Cre-GRe2Δ mice, but adolescent males show a transient increase in peak CORT levels. The level of GR loss, 47% in Sim1Cre-GRe2Δ mice vs. 87% in Sim1Cre-GRe3Δ mice, is likely a contributing factor to the difference in phenotype. This is supported by data in GR null heterozygous mice which have 50% loss of GR and display no changes in glucocorticoid secretion under basal conditions (7) as well as in FBGRKO mice, which do not show HPA axis abnormalities when less than 60% GR is loss (8). An alternate explanation for the differences between Sim1Cre-GRe2Δ and Sim1Cre-GRe3Δ mice might be the influence of the distinct exons deleted and the potential for residual production of portions of GR. The exon 3 of GR contains the zinc-finger of the DNA binding domain while the exon 2 contains the transcriptional activation domain. Genetically altered mice with site specific mutations along the GR gene, such as in the GR dimerization-deficient mice (9), may provide more intricate analysis of importance of exon/domain functions. In fact, GR^{dim} mice, have elevated CORT levels at nadir and peak compared to control mice (10),

indicating that the dimerization and/or DNA binding function of GR is necessary for negative feedback regulation of HPA axis activity. The developmental function of PVN GR was exposed in data from adolescent Sim1Cre-GRe3Δ mice. Early in adolescence, male Sim1Cre-GRe3Δ mice display augmented basal CORT levels but not stress-induced CORT, indicating that PVN GR plays a role in mediate negative feedback during adolescence. This role is gender specific since it is not observed in female Sim1Cre-GRe3Δ mice (Table 5-1.). By late adolescence, PVN GR appears to be necessary for negative feedback of CORT at both the basal and stress-induced levels in males, but only at the nadir level in females. Therefore, there is not only gender-specific activity of PVN GR, but also developmental progression of PVN GR function.

Negative feedback in the hypothalamus occurs when CORT binds to GR in the PVN. Activated GR translocates to the nucleus of parvocellular neurons where it may bind to a negative GRE in the CRH promoter gene, causing repression of CRH transcription (11, 12), though this remains controversial. Malkoski and colleagues identified a GC-responsive cis-regulatory element on the CRH promoter, which is stimulated by cAMP. GR was shown to mediate greater than a 50% reduction in CRH promoter activity through direct binding to a negative GRE on the CRH promoter in a region that is highly conserved (12). However, many other studies have found a lack of direct GR interaction with the CRH gene. For instance, studies of GR^{dim} mice, which have a mutation in the DNA-binding domain, demonstrate no changes in CRH expression (9). Moreover, hypothalamic chromatin immunoprecipitation of the CRH promoter demonstrated no GR interaction or GRE detected near the promoter (13). Further exploration of the CRH gene indicated that GCs act on GR to promote DNA methylation

and histone acetylation of the CRH promoter to repress CRH expression (14, 15). In vitro studies show that GR repression of CRH can occur through its disruption of second messenger signaling pathways (16). Studies also indicate that GCs act on G-protein coupled membrane receptors in PVN neurons to increase endocannabinoid signaling. The activated endocannabinoids cause retrograde suppression of glutamate secretion onto PVN neurons, thereby reducing the frequency of miniature excitatory post-synaptic currents (17–19). This results in GC-mediated fast-feedback inhibition of CRH neurons in the PVN and thus inhibition of ACTH and CORT secretion (20–24). These data collectively indicate the existence of multiple mechanisms for GC-mediated repression of PVN CRH activity that does not depend on direct GR interaction with the CRH gene.

Data from our lab demonstrates that loss of ~50% of GR in the PVN does not affect HPA axis activity or behavior in adulthood but causes transient changes in adolescence. When levels of GR are reduced below 20% deleterious effects due to CORT excess are observed (5). Indeed, adult Sim1Cre-GRe3Δ mice show a 3-fold increase in CRH mRNA levels in the PVN as a result of 87% GR loss, whereas Sim1Cre-GRe2Δ mice have only 43% of GR PVN loss. Thus, the level of GR loss in the PVN directly influences alterations in CRH transcripts in the PVN and subsequent plasma ACTH and CORT levels.

In addition to having elevated basal nadir and peak levels of CORT, adult Sim1Cre-GRe3Δ mice respond to restraint stress with greater increases in CORT release than controls (5). Our data indicate that the stress-induced regulatory activity by PVN GR is low or absent during early adolescence, indicating that pituitary GR may be more in control of stress mediation during that time in development. In contrast to CORT, stress-

induced ACTH levels in adult Sim1Cre-GRe3Δ mice did not differ from that of control mice. This dissociation of elevated stress-induced CORT with normal stress-induced ACTH expression implies a possible increase in adrenal cortex sensitivity to ACTH under stressful conditions. Indeed, increased adrenal sensitivity to ACTH is observed in GR^{NesCre} mice (25), but not in FBGRKO, resulting in elevated CORT secretion in response to exogenous ACTH injections (26, 27) (Table 5-1.). A role for PVN GR mediating this increased adrenal sensitivity during stress is therefore possible.

At the onset of a stress response, GCs are known to mediate metabolic activity by enhancing glycogenolysis, lipolysis, and proteolysis to produce substrates of glucose, fatty acids, and amino acids, respectively, that can be diverted for use in specific tissues depending on need (28). Given the involvement of GR in metabolic activity as well as the hypothalamus being a center for regulation of energy metabolism, (Kellendonk 2002, endocrinology), Sim1Cre-GRe3Δ mice present an opportunity to evaluate the influence of loss of PVN GR on several metabolic phenotypes.

At birth, the Sim1Cre-GRe3Δ mutants appear similar to control littermates. During postnatal development, Sim1Cre-GRe3Δ mice show stunted growth (5). Phenotypic differences start manifest at P20, and there is significantly reduced body weight. In contrast to pituitary GR loss, Sim1Cre-GRe3Δ male and female mice begin to catch up to their control littermates beginning around P70 and surpass controls in weight from P100 onward. This increased weight included the accumulation of adipose tissue in the gonadal region of the mutants, and a truncal obesity phenotype. Given the chronically high levels of GCs in the mutants, this result is consistent with Cushing's syndrome in these mice. Compared to Sim1Cre-GRe3Δ mice, the excess GCs in GR^{NesCre} and $GR^{flox+/-}$;

Sim1Cre^{+} mice do not result in increased adiposity; in fact these mice have reduced weight in adulthood. GCs regulate expression of hypothalamic peptides, such as leptin, insulin, and neuropeptide Y, which govern growth and energy homeostasis (29). Leptin, which signals the hypothalamus to decrease hunger and therefore food intake as well as fat storage, is elevated in young GR^{NesCre} mice. Adult GR^{NesCre} mice have reduced levels of insulin-like growth factor 1 (IGF-1) which is involved in stimulating growth in many organs (29). Thus, excess GCs may induce decreased body weight by regulation of leptin and IGF-1. Since Sim1CreGRe3Δ mice become obese and have increased adiposity, this finding is suggestive of a difference in CORT function during early development compared to adulthood in mediating growth signaling. This ramification is supported by rat studies showing that prenatal dexamethasone application causes low birth weight (30), but dexamethasone application into adult rats causes an increase in enzymes responsible for synthesis of lipids, and thus increased visceral adiposity (31). The cause of discordance in weight between adult Sim1Cre-GRe3Δ and GR$^{flox+/-}$; and Sim1Cre^{+} mice is unclear, given that excess GCs also occurs in these lines. The influence of increased PVN CRH in mediating some of the growth phenotypes in these mutants cannot be discounted, as CRH signaling also regulates feeding and energy balance (32, 33).

GR disruption in the pituitary

The anterior lobe is the target of PVN CRH and arginine vasopressin input that stimulates the synthesis and secretion of ACTH. CRH activates the synthesis of ACTH through the function of an orphan nuclear receptor, Nur77 (11). When activated by CRH signaling through its receptors, Nur77 induces the transcription of POMC by binding to

DNA-response elements on the POMC gene. Prohormone convertase 1 and 2 enzymes process the POMC precursor polypeptide to generate ACTH and β-lipoprotein, which can be further processed into α-melanocyte-stimulating hormone, β-endorphin, corticotrophin-like intermediate peptide and γ-lipotrophins (34). It is important to note that Nur77 deficient mice display normal HPA axis signaling, and there is evidence that closely-related members of the Nur77 nuclear receptor family can compensate in its absence (35, 36). Diurnally secreted ACTH binds to type 2 melanocortin receptors (MCR2) on the surface of the adrenal cortex. Activation of MCR2, a G-protein coupled receptors, triggers cAMP second messenger activity that results in immediate and chronic synthesis of StAR and other steroidogenic enzymes (34) to cause CORT synthesis and secretion (37).

GC negative feedback at the level of the pituitary begins at E16.5 (38) when CORT acts on pituitary GR. Rapid and delayed feedback inhibition of pituitary ACTH by GC have been demonstrated to occur through both genomic and non-genomic pathways in a number of studies (39–43). A negative GRE has been previously identified proximal to the POMC gene responsive to dexamethasone suppression in vitro, indicating direct GR genomic effects (44). Indirect effects have also been suggested in that activated GR homodimer can represses Nur77 activity, thereby decreasing POMC mRNA expression and inhibiting ACTH production (11). Studies also indicate that GC-mediated fast-feedback inhibition of ACTH occurs through non-genomic pathways, dependent on endocannabinoid receptor activity (20, 21). To evaluate negative feedback regulation at the level of the pituitary, Schmidt and colleagues generated mice with pituitary GR deletion. This was accomplished by the crossing of GR floxed exon 3 mice to a POMC-

Cre transgenic line ($GR^{POMCCre}$). GR expression was lost in the POMC-expressing cells of the pituitary and arcuate nucleus of these mice (Figure 5.1. B) (45). Morning levels of plasma CORT and ACTH under basal conditions were extremely elevated by P6. This excess GC is particularly interesting because it occurred despite reduced PVN CRH. It could be argued that this excess CORT is actually mediating negative feedback at the level of the PVN, hence the reduced CRH mRNA. However this is unlikely due to the fact that GR mRNA in these mice is also reduced at that age and levels of plasma ACTH are increased. While PVN CRH mRNA is decreased, expression of AVP transcripts in the PVN is increased and may contribute to the increase in plasma ACTH levels. These effects of pituitary GR deletion only happen developmentally, because by P30, the HPA dysregulation phenotypes were normalize in $GR^{POMCCre}$ mice (Figure 5.2.) (Table 5-1.), to levels similar to control mice (45). Development of the HPA axis in mice includes a stress hyporesponsive period (SHRP) established from postnatal day 1 (P1) through P12 (46). During this time, neonates have low basal CORT, and high PVN CRH transcript levels. The alterations in $GR^{POMCCre}$ mice during SHRP but not in adulthood, confirm as previously identified that pituitary GR function is indispensable for HPA axis control during the SHRP (46).

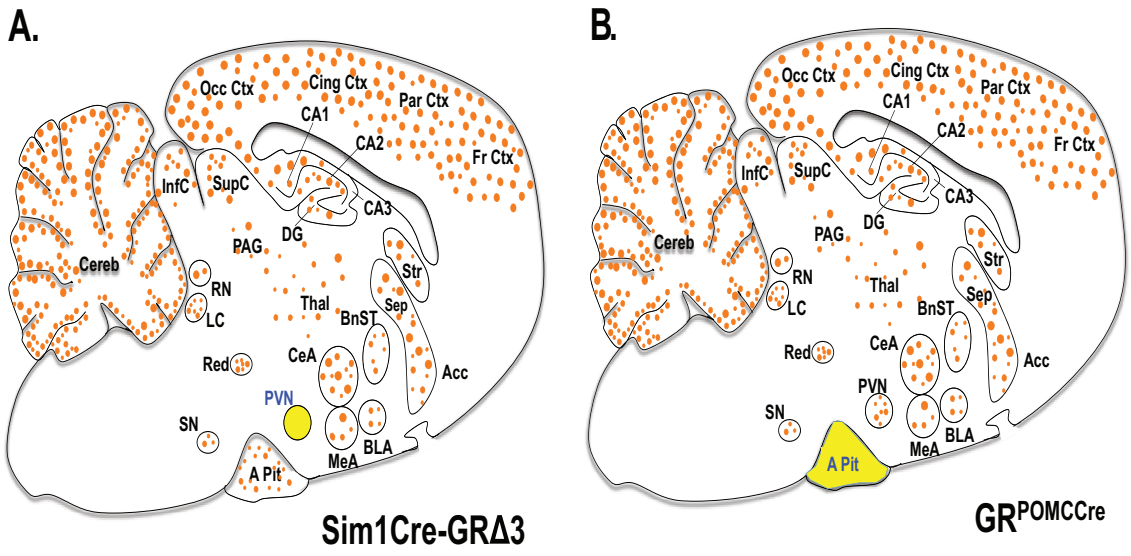

Figure 5.1. Glucocorticoid Receptor Expression in Genetic Mouse Models. Sagittal brain sections depicting areas of GR mRNA expression and deletion in mice with targeted GR deletion. Orange circles (●) represent GR, targeted regions are in yellow highlight and blue font). **A**. Adult Sim1Cre-GRe3Δ mice have GR loss primarily in the PVN. **B**. $GR^{POMCCre}$ mice have GR loss in the anterior pituitary throughout life. ***Abbreviations***: Anterior pituitary, A Pit; Basolateral nucleus of the amygdala, BLA; Bed nucleus of the stria terminalis, BnST; Central nucleus of the amygdala, CeA; Hippocampal areas, CA1, CA2, CA3; Cerebellum, Cereb; Cingulate cortex, Cing Ctx; Dentate gyrus, DG; Frontal cortex, Fr Ctx; Inferior colliculus, InfC; Locus coeruleus, LC; Medial nucleus of the amygdala, MeA; Occipital cortex, Occ Ctx; Periaqueductal gray, PAG; Parietal cortex, Par Ctx; Paraventricular hypothalamic nucleus, PVN; Raphe nucleus, RN; Septum, Sep; Supraoptic nucleus, SN; Superior colliculus, SupC; Thalamus, Thal;

Developmental GC excess in $GR^{POMCCre}$ mice impaired GR-mediated negative feedback in adulthood, as $GR^{POMCCre}$ mice took longer to decrease their CORT levels after restraint stress and in the dexamethasone suppression test (DST) (45). While the initial study of $GR^{POMCCre}$ mice demonstrated no basal anxiety phenotype (45), a subsequent study revealed a more anxious phenotype of $GR^{POMCCre}$ mice in the elevated zero maze test (47). This anxious phenotype was abolished with a chronic social defeat

stress, leading the authors to infer that in the initial study, the mice were inadvertently exposed to a stressor that masks their basal anxiety phenotype. Adult $GR^{POMCCre}$ mice display decreased despair in the forced swim test compared to control mice. The reduced despair behavior was inhibited if $GR^{POMCCre}$ mice were treated with a GR antagonist during the developmental period when CORT levels are high. This finding indicates that transient increases in GR activation early in development have differing effects on anxiety and despair behaviors later in adulthood. This data also suggests that when increased GR activation is persistent, as observed in Sim1Cre-GRe3Δ mice (5), it results in habituation to anxiety and despair behavior under basal circumstances. The data reviewed here is part of a larger body of evidence on the programming effects of early life experience (48–54). The observations described in these studies have strong implications for the role of MR and GR in the predictive adaptive capacity, match/mismatch and cumulative stress exposure theories (55–58).

FBGRKO and Sim1Cre-GRe3Δ both fail to suppress CORT in the DST test (8, 59), and $GR^{POMCCre}$ mice suppress CORT but in a less efficient manner than observed in controls (Table 5-1.). This suggests differential levels of contributions to negative feedback regulation from these regions, with the PVN and forebrain GR contributing more heavily in adulthood and pituitary GR more during early postnatal life. From this data, we conclude that pituitary GR mediates basal HPA activity in early life (young $GR^{POMCCre}$ mice have disrupted PVN CRH, normal plasma CORT and ACTH levels) but not in adulthood (Adult $GR^{POMCCre}$ have normal PVN CRH, normal plasma CORT and ACTH levels). On the other hand, PVN GR is involved in mediating basal HPA activity in adulthood, (adult Sim1Cre-GRe3Δ have disrupted PVN CRH, normal plasma CORT

and ACTH levels).

The data in $GR^{POMCCre}$ and $GR^{CaMKCre}$ (Chapter 1) mice demonstrate the importance of pituitary GR function in early postnatal development. The retention of negative feedback sites in the brain and PVN of $GR^{POMCCre}$ mice prevents the postnatal death that occurs in $GR^{CaMKCre}$ mice (60). This finding suggests that CORT elevations in excess of a certain threshold are lethal. At least one site of negative feedback regulation must be retained for life. This conclusion is supported by the fact that when pituitary GR is the only negative feedback site retained in GR^{NesCre} mice, the mice survive similar to controls. While $GR^{POMCCre}$ mice do show decreases in PVN GR early in life, the level of this GR deletion may not be enough to completely avert negative feedback, and furthermore PVN GR mRNA levels normalize in adulthood while pituitary GR loss is retained (45). Given data in from our group that indicates that 50% loss of PVN GR does not affect HPA axis activity or behavioral phenotypes (5), it seems unlikely that the level of GR loss in $GR^{POMCCre}$ mice is influential in the observed phenotypes. Thus, one question that arises is, when PVN and pituitary GRs are both lost, is GR feedback in the forebrain sufficient to maintain viability?

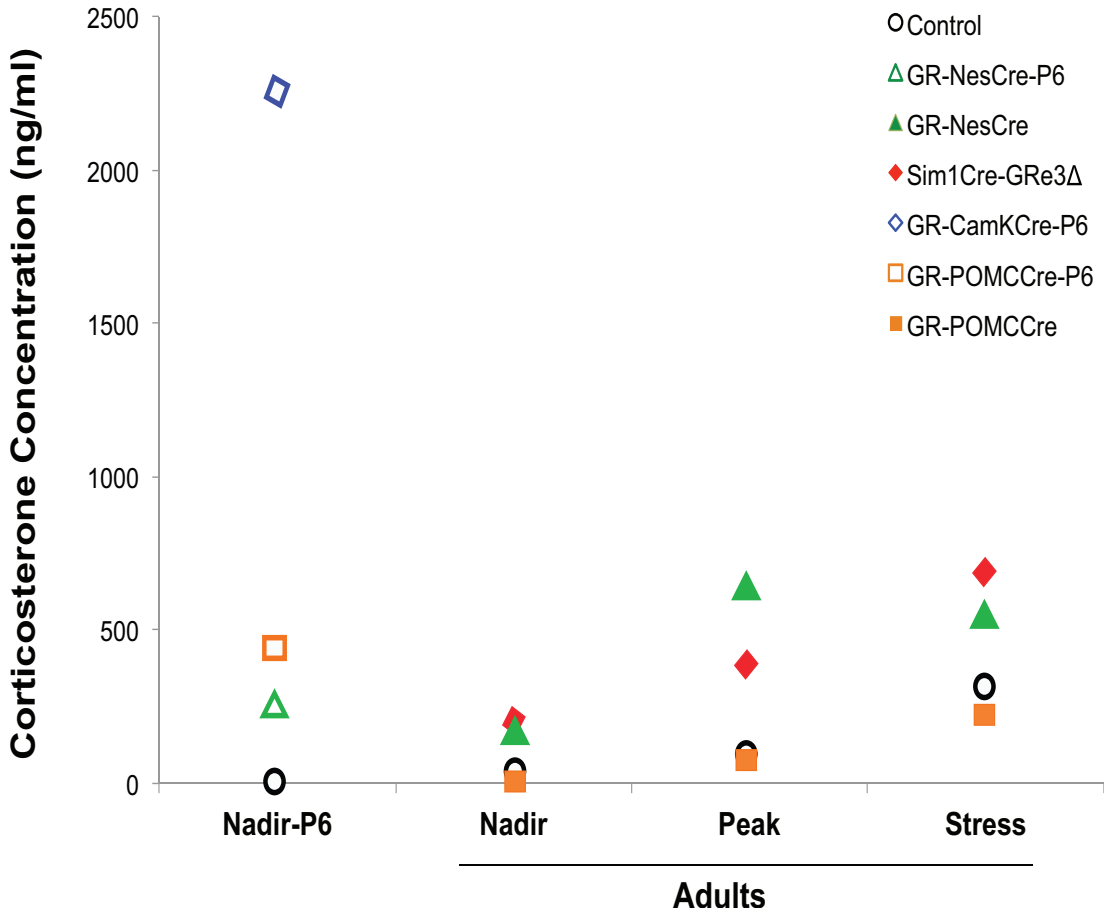

Figure 5.2. Schematic of CORT levels in GR-targeted Mice. This figure depicts the relative levels in plasma CORT depending on the nature of the mutation in GR^{NesCre}, $GR^{CaMKCre}$, $GR^{POMCCre}$, and Sim1Cre-GRe3Δ compared to control mice at postnatal day 6 and in adulthood. Values represent estimates of plasma CORT values provided from respective articles. Controls are averaged values of from individual studies.

After the SHRP, basal plasma CORT levels increase and PVN CRH mRNA levels decrease beginning from P12, while plasma ACTH levels remain constant (61). Moreover, before P9, the stress of a novel environment has no effect on plasma CORT or ACTH levels, but after P12 levels of both hormones increase in response to novelty stress in control mice. The data in $GR^{POMCCre}$ mice indicate that developmentally, pituitary GR is sensitive to stimuli. Generally, GR is known as being responsive to circadian peak or stress-induced but not nadir changes in HPA axis activity (62, 63). However the studies described in $GR^{POMCCre}$ and Sim1Cre-GRe3Δ mice suggest that in early postnatal development, pituitary GR is sensitive to basal HPA axis changes and along development there is a switch to PVN GR becoming sensitive to basal changes in HPA axis activity. A comparison of $GR^{POMCCre}$ and Sim1Cre-GRe3Δ mice also shows that whereas loss of pituitary GR increases anxiety in the EZM and causes stress coping in the FST during adulthood, loss of PVN GR had no effect (Table 5-1.). This outcome is possibly due to lower plasma CORT concentrations early in development of Sim1Cre-GRe3Δ mice than $GR^{POMCCre}$ mice. A closer evaluation of the ontogeny of the HPA axis in Sim1Cre-GRe3Δ mice would better enable this to be resolved.

Table 5-1: Effects of glucocorticoid receptor targeting on HPA axis activity, behavior, and metabolic phenotypes

Phenotype	GR^{NesCre}	FBGRKO Male	FBGRKO Female	$GR^{CaMKCre}$	Sim1Cre-GRe3Δ- Male	Sim1Cre-GRe3Δ-Female	Sim1Cre-GRe2Δ- Male	$GR^{flox+/+}$; $Sim1Cre^{+}$	$GR^{flox+/-}$; $Sim1Cre^{+}$	$GR^{POMCCre}$
GR floxed	Exon 3	Exon 1C-2	Exon 1C-2	Exon 3	Exon 3	Exon 3	Exon 1C-2	Exon 2	Exon 2	Exon 3
Primary region	CNS	Forebrain	Forebrain	CNS + PIT	PVN	PVN	PVN	PVN/Lung	PVN	PIT
GR loss		75-100%	75-100%		87%	87%	43%		70%	90%
Lethality	None	None	None	Postnatal	None	None	None	Embryonic	None	None
POSTNATAL DAY 6										
PVN CRH	↑Prot			↑Prot	↑mRNA	↑mRNA				↓ mRNA
PVN AVP										↑ mRNA
POMC mRNA				↑						↑ N
ACTH				↑N						↑ N
CORT	↑			↑						↑ N
Weight	↓			↓	↓pw	↓pw				↓
Skin pigm.	⇔			↓						
Skin fat	⇔			↓**sc**						
Thymus weight	⇔			↓						⇔
Spleen weight	⇔			↓						
ADOLESCENT										
CORT (P30-50)					↑N/P, ⇔S	⇔				
CORT (P51-73)					↑N/P/S	↑N, ⇔P/S	↑P			
Anxiety - EZM					↑					
ADULT										
PVN CRH	↑Prot	⇔B, ↑S	⇔		↑mRNA		⇔		↑ mRNA	⇔
PVN AVP	⇔	↑	⇔							
ACTH	↑PIT, ↘N	↑P/ S	⇔		↑N/P, ⇔S		⇔ N/P/S		↑B	⇔
CORT	↑N/P	↗N, ↑P/S ↑N, ⇔P	⇔		↑N/P/S		⇔ N/P/S		↑B	⇔
DST		Fail			Fail	Fail	⇔			Impaired
Weight	↓	⇔			↑	↑	⇔		↓	⇔
Fat Mass	↓				↑	↑	⇔			
Fat distribution	Head/neck				Gonad	Gonad	⇔			
Anxiety	↓	↘			⇔ ↑S		⇔			↑
Despair	↓	↑	⇔		⇔		⇔			↓
Stress-coping	↑	↓	⇔		⇔		⇔			↑
Locomotor activity	⇔	↑EZM			↓OF		⇔			⇔
References	(10, 14, 30)	(11, 28, 29)	(12)	(30)	(4)	(4)	(4)	(5)	(5)	(25, 27)

PIT, pituitary; Prot, protein; N, nadir; P, peak; S, stress; B, Basal; sc, subcutaneous; pw, pre-weaning; EZM, elevated zero maze; OF, open field; ↑, increase; ↓ decrease; ↗, trend towards increase; ↘, trend towards decrease; ⇔, similar to controls.

Chronic social defeat stress in $GR^{POMCCre}$ mice not only prevented anxiety phenotypes but also abolished increases in basal CORT that occurred in control stressed mice. There is much evidence of early life stress being protective later in life. The data in $GR^{POMCCre}$ mice supports the notion that the resilience to social defeat stress occurs due to the transient GC excess that mimics the neuroendocrine effects of early life stress (47). It appears though, that while genetic defects that produce a brief period of GC excess postnatally have no effect on HPA axis dysregulation later in adulthood, genetic defects that produce long-term GC excess completely disrupt HPA axis activity.

$GR^{POMCCre}$ mice have stunted growth until during the first two weeks of life (45). Early postnatal growth deficits also occur in GR^{NesCre}, $GR^{CaMKCre}$ and Sim1Cre-GRe3Δ mice, all of which have elevated levels of CORT during that developmental period. In $GR^{POMCCre}$ mice, weight returns to similar levels as that of controls from P28 onwards, and coincides with normalization of HPA axis dysregulation. In contrast to normalized HPA axis in adult $GR^{POMCCre}$ mice, the HPA axis is still hyperactive in GR^{NesCre} and Sim1Cre-GRe3Δ mice in adulthood and leads to a differential impact on growth. These data suggest that GR in the brain and pituitary are required to maintain normal growth processes in mice. Loss of GR early in development leading to GC excess reduces growth, while in adulthood GC influence on growth is region dependent. Pituitary GR, however, does not have a major influence in mediating growth pathways in adulthood. Taken together, these data suggest differential involvement of GR in regulating growth during early development versus during adulthood, as well as differential involvement of GR in specific brain regions in terms of the level of involvement in growth regulation.

Numerous studies in animal models and humans clarify the importance of glucocorticoids and the HPA axis in mediating stress reactivity in terms of endocrine output, behavioral activity, and energy balance. Here in part 1 of the dissertation, we primarily focus on studies in sites of GC negative feedback in the brain and pituitary of genetically targeted mice. In all of the genetic models addressed, diurnal rhythmicity of CORT secretion is maintained indicating that GR in negative feedback sites are not important for circadian regulation of GC synthesis and secretion. During periods of GC excess, increased PVN CRH and/or AVP lead to increased plasma ACTH and CORT. In terms of physical development, it seems that excess GCs during early life cause growth deficits that normalize if GC levels normalize. Early postnatal GC excess impairs growth and may reprogram HPA axis reactivity to stress later in life. Moreover, the effects of this GC excess are dependent on the site where GR is lost. Brief GC elevations during postnatal development, due to pituitary GR loss, slow dexamethasone suppression while increasing anxiety and stress-coping behavior. GC excess due to PVN GR loss results in age and gender-specific differences in impaired negative suppression of CORT and causes a Cushing's phenotype. There is increased anxiety behavior in the EZM during adolescence that is lost in adulthood, perhaps due to habituation of chronically elevated glucocorticoids. There are no basal differences in anxiety or despair behavior in adult Sim1Cre-GRe3Δ mice. However, there is a gene – environment interaction that results in increased anxiety in mice with PVN GR loss after exposure to anxiogenic environment. GR loss in both PVN and pituitary as well as in the brain is incompatible with sustained life, primarily due to the necessity for pituitary GR function early in life.

FUTURE DIRECTIONS

There are a number of lingering questions that can be address with the Sim1Cre-GRe3Δ model. Excess glucocorticoid levels can act on a number of brain regions such as the amygdala, hippocampus, and prefrontal cortex and alter cortical activity (64, 65). Changes in neurocircuitry as a result is increased GC activity have been shown due to GC altering synaptic dendrite morphology (66–68). These changes can lead to dysregulation of excitatory – inhibitory signaling. Our preliminary data demonstrated impaired spatial memory in the Morris water maze test in Sim1Cre-GRe3Δ mice. However, the reductions in speed and locomotor activity and that these mice display confound evaluating the Morris water maze data. Using behavioral tasks that are not influenced by locomotor activity would be beneficial in evaluating learning and memory in Sim1Cre-GRe3Δ mice. Also, functional studies in the brain of Sim1Cre-GRe3Δ mice can reveal the effects of PVN GR disruption role of throughout development.

There remains much to be learned from Sim1Cre-GRe3Δ mice regarding elevated GC levels on metabolic activity. We have already described the Cushingoid phenotype in Sim1Cre-GRe3Δ mice, the growth impairments and altered adipose distribution. We also observed altered stress-induced glucose secretion in Sim1Cre-GRe3Δ mice. The effects of PVN GR on metabolism could prove valuable in uncovering hypothalamic function in metabolic activity. There is also the question of how PVN GR functions depending on gender. Investigations into the influence of gonadal hormones on HPA axis and behavior in Sim1Cre-GRe3Δ mice will prove beneficial in this area. Lastly, the possibility of epigenetic modifications in the GC signaling pathways during development as a result of PVN GR disruption is an attractive area of investigation. Overall, these studies will

provide further insight in understanding HPA axis dysregulation and the molecular basis of psychiatric and metabolic disorders in different genders.

REFERENCES

1. Kalsbeek a, van Heerikhuize JJ, Wortel J, Buijs RM. 1996. A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1 antagonist. J. Neurosci. 16:5555–65.

2. Hermes ML, Coderre EM, Buijs RM, Renaud LP. 1996. GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat. J. Physiol. 496 (Pt 3:749–57.

3. Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, Schibler U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289:2344–7.

4. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang C, Mountjoy K, Kishi T, Elmquist JK, Lowell BB. 2005. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493–505.

5. Laryea G, Schütz G, Muglia LJ. 2013. Disrupting hypothalamic glucocorticoid receptors causes HPA axis hyperactivity and excess adiposity. Mol. Endocrinol. 27:1655–65.

6. Jeanneteau FD, Lambert WM, Ismaili N, Bath KG, Lee FS, Garabedian MJ, Chao M V. 2012. BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. Proc. Natl. Acad. Sci. U. S. A. 109:1305–10.

7. Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, Zink M, Hörtnagl H, Flor H, Henn F a, Schütz G, Gass P. 2005. Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. J. Neurosci. 25:6243–50.

8. Boyle MP, Brewer J a, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ. 2005. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc. Natl. Acad. Sci. U. S. A. 102:473–8.

9. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schütz G. 1998. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93:531–41.

10. Oitzl MS, Reichardt HM, Joëls M, de Kloet ER. 2001. Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proc. Natl. Acad. Sci. U. S. A. 98:12790–5.

11. Webster JC, Cidlowski JA. 1999. Mechanisms of Glucocorticoid-receptor-mediated Repression of Gene Expression. Trends Endocrinol. Metab. 10:396–402.

12. Malkoski SP, Handanos CM, Dorin RI. 1997. Localization of a negative glucocorticoid response element of the human corticotropin releasing hormone gene. Mol. Cell. Endocrinol. 127:189–199.

13. Evans AN, Liu Y, Macgregor R, Huang V, Aguilera G. 2013. Regulation of hypothalamic corticotropin-releasing hormone transcription by elevated glucocorticoids. Mol. Endocrinol. 27:1796–807.

14. Elliott E, Ezra-Nevo G, Regev L, Neufeld-Cohen A, Chen A. 2010. Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. Nat. Neurosci. 13:1351–3.

15. Sharma D, Bhave S, Gregg E, Uht R. 2013. Dexamethasone induces a putative repressor complex and chromatin modifications in the CRH promoter. Mol. Endocrinol. 27:1142–52.

16. Yamamori E, Iwasaki Y, Taguchi T, Nishiyama M, Yoshida M, Asai M, Oiso Y, Itoi K, Kambayashi M, Hashimoto K. 2007. Molecular mechanisms for corticotropin-releasing hormone gene repression by glucocorticoid in BE(2)C neuronal cell line. Mol. Cell. Endocrinol. 264:142–8.

17. Di S, Malcher-Lopes R, Halmos KC, Tasker JG. 2003. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J. Neurosci. 23:4850–7.

18. Malcher-Lopes R, Di S, Marcheselli VS, Weng F-J, Stuart CT, Bazan NG, Tasker JG. 2006. Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release. J. Neurosci. 26:6643–50.

19. Tasker JG. 2006. Rapid glucocorticoid actions in the hypothalamus as a mechanism of homeostatic integration. Obesity (Silver Spring). 14 Suppl 5:259S–265S.

20. Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP. 2010. Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. Endocrinology 151:4811–9.

21. Groeneweg FL, Karst H, de Kloet ER, Joëls M. 2011. Rapid non-genomic effects of corticosteroids and their role in the central stress response. J. Endocrinol. 209:153–67.

22. Groeneweg FL, Karst H, de Kloet ER, Joëls M. 2011. Mineralocorticoid and glucocorticoid receptors at the neuronal membrane, regulators of nongenomic corticosteroid signalling. Mol. Cell. Endocrinol.

23. Dallman MF. 2005. Fast glucocorticoid actions on brain: back to the future. Front. Neuroendocrinol. 26:103–8.

24. Myers B, McKlveen JM, Herman JP. 2012. Neural Regulation of the Stress Response: The Many Faces of Feedback. Cell. Mol. Neurobiol.

25. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schütz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23:99–103.

26. Furay AR, Bruestle AE, Herman JP. 2008. The role of the forebrain glucocorticoid receptor in acute and chronic stress. Endocrinology 149:5482–90.

27. Solomon MB, Furay a R, Jones K, Packard a EB, Packard B a, Wulsin a C, Herman JP. 2012. Deletion of forebrain glucocorticoid receptors impairs neuroendocrine stress responses and induces depression-like behavior in males but not females. Neuroscience 203:135–43.

28. Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

29. Kellendonk C, Eiden S, Kretz O, Schütz G, Schmidt I, Tronche F, Simon E. 2002. Inactivation of the GR in the nervous system affects energy accumulation. Endocrinology 143:2333–40.

30. Drake AJ, Raubenheimer PJ, Kerrigan D, McInnes KJ, Seckl JR, Walker BR. 2010. Prenatal dexamethasone programs expression of genes in liver and adipose tissue and increased hepatic lipid accumulation but not obesity on a high-fat diet. Endocrinology 151:1581–7.

31. Chimin P, Farias T da SM, Torres-Leal FL, Bolsoni-Lopes A, Campaña AB, Andreotti S, Lima FB. 2014. Chronic glucocorticoid treatment enhances lipogenic activity in visceral adipocytes of male Wistar rats. Acta Physiol. (Oxf).

32. Stengel A, Taché Y. 2014. CRF and urocortin peptides as modulators of energy balance and feeding behavior during stress. Front. Neurosci. 8:52.

33. Stengel A, Goebel M, Million M, Stenzel-Poore MP, Kobelt P, Mönnikes H, Taché Y, Wang L. 2009. Corticotropin-releasing factor-overexpressing mice exhibit reduced neuronal activation in the arcuate nucleus and food intake in response to fasting. Endocrinology 150:153–60.

34. Papadimitriou A, Priftis KN. 2009. Regulation of the hypothalamic-pituitary-adrenal axis. Neuroimmunomodulation 16:265–71.

35. Crawford PA, Sadovsky Y, Woodson K, Lee SL, Milbrandt J. 1995. Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. Mol. Cell. Biol. 15:4331–16.

36. Fernandez PM, Brunel F, Jimenez MA, Saez JM, Cereghini S, Zakin MM. 2000. Nuclear receptors Nor1 and NGFI-B/Nur77 play similar, albeit distinct, roles in the hypothalamo-pituitary-adrenal axis. Endocrinology 141:2392–400.

37. Liu Y, Smith LI, Huang V, Poon V, Coello A, Olah M, Spiga F, Lightman SL, Aguilera G. 2013. Transcriptional regulation of episodic glucocorticoid secretion. Mol. Cell. Endocrinol. 371:62–70.

38. Reichardt HM, Schütz G. 1996. Feedback control of glucocorticoid production is established during fetal development. Mol. Med. 2:735–44.

39. Widmaier EP, Dallman MF. 1984. The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perifused pituitaries in vitro: rapid inhibition by glucocorticoids. Endocrinology 115:2368–74.

40. Keller-Wood ME, Dallman MF. 1984. Corticosteroid inhibition of ACTH secretion. Endocr. Rev. 5:1–24.

41. Hinz B, Hirschelmann R. 2000. Rapid non-genomic feedback effects of glucocorticoids on CRF-induced ACTH secretion in rats. Pharm. Res. 17:1273–7.

42. Dayanithi G, Antoni FA. 1989. Rapid as well as delayed inhibitory effects of glucocorticoid hormones on pituitary adrenocorticotropic hormone release are mediated by type II glucocorticoid receptors and require newly synthesized messenger ribonucleic acid as well as protein. Endocrinology 125:308–13.

43. John CD, Christian HC, Morris JF, Flower RJ, Solito E, Buckingham JC. 2004. Annexin 1 and the regulation of endocrine function. Trends Endocrinol. Metab. 15:103–9.

44. Drouin J, Sun YL, Chamberland M, Gauthier Y, De Léan a, Nemer M, Schmidt TJ. 1993. Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. EMBO J. 12:145–56.

45. Schmidt M V, Sterlemann V, Wagner K, Niederleitner B, Ganea K, Liebl C, Deussing JM, Berger S, Schütz G, Holsboer F, Müller MB. 2009. Postnatal glucocorticoid excess due to pituitary glucocorticoid receptor deficiency: differential short- and long-term consequences. Endocrinology 150:2709–16.

46. Schmidt M V, Schmidt M, Levine S, Oitzl MS, van der Mark M, Müller MB, Holsboer F, de Kloet ER. 2005. Glucocorticoid receptor blockade disinhibits pituitary-adrenal activity during the stress hyporesponsive period of the mouse. Endocrinology 146:1458–64.

47. Wagner K V, Wang X-D, Liebl C, Scharf SH, Müller MB, Schmidt M V. 2011. Pituitary glucocorticoid receptor deletion reduces vulnerability to chronic stress. Psychoneuroendocrinology 36:579–87.

48. Francis DD, Champagne FA, Liu D, Meaney MJ. 1999. Maternal care, gene expression, and the development of individual differences in stress reactivity. Ann. N. Y. Acad. Sci. 896:66–84.

49. Liu D. 1997. Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress. Science (80-.). 277:1659–1662.

50. Zhang TY, Chrétien P, Meaney MJ, Gratton A. 2005. Influence of naturally occurring variations in maternal care on prepulse inhibition of acoustic startle and the medial prefrontal cortical dopamine response to stress in adult rats. J. Neurosci. 25:1493–502.

51. Champagne DL, Bagot RC, van Hasselt F, Ramakers G, Meaney MJ, de Kloet ER, Joëls M, Krugers H. 2008. Maternal care and hippocampal plasticity: evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. J. Neurosci. 28:6037–45.

52. Daskalakis NP, Oitzl MS, Schächinger H, Champagne DL, de Kloet ER. 2012. Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity. Physiol. Behav. 106:707–21.

53. Santarelli S, Lesuis SL, Wang X-D, Wagner K V, Hartmann J, Labermaier C, Scharf SH, Müller MB, Holsboer F, Schmidt M V. 2014. Evidence supporting the match/mismatch hypothesis of psychiatric disorders. Eur. Neuropsychopharmacol. 24:907–18.

54. Buwalda B, Stubbendorff C, Zickert N, Koolhaas JM. 2013. Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood: a study on the consequences of social stress in rats. Neuroscience 249:258–70.

55. Champagne DL, de Kloet ER, Joëls M. 2009. Fundamental aspects of the impact of glucocorticoids on the (immature) brain. Semin. Fetal Neonatal Med. 14:136–42.

56. Daskalakis NP, Bagot RC, Parker KJ, Vinkers CH, de Kloet ER. 2013. The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 38:1858–73.

57. Schmidt M V. 2011. Animal models for depression and the mismatch hypothesis of disease. Psychoneuroendocrinology 36:330–8.

58. Nederhof E, Schmidt M V. 2012. Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. Physiol. Behav. 106:691–700.

59. Boyle MP, Kolber BJ, Vogt SK, Wozniak DF, Muglia LJ. 2006. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. J. Neurosci. 26:1971–8.

60. Erdmann G, Schütz G, Berger S. 2008. Loss of glucocorticoid receptor function in the pituitary results in early postnatal lethality. Endocrinology 149:3446–51.

61. Schmidt M V, Enthoven L, van der Mark M, Levine S, de Kloet ER, Oitzl MS. 2003. The postnatal development of the hypothalamic–pituitary–adrenal axis in the mouse. Int. J. Dev. Neurosci. 21:125–132.

62. De Kloet ER, Reul JM. 1987. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12:83–105.

63. De Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M. 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev. 19:269–301.

64. Sandi C. 2011. Glucocorticoids act on glutamatergic pathways to affect memory processes. Trends Neurosci. 34:165–76.

65. Liston C, Gan W-B. 2011. Glucocorticoids are critical regulators of dendritic spine development and plasticity in vivo. Proc. Natl. Acad. Sci. U. S. A. 108:16074–9.

66. Silva-Gómez AB, Aguilar-Salgado Y, Reyes-Hernández DO, Flores G. 2013. Dexamethasone induces different morphological changes in the dorsal and ventral hippocampus of rats. J. Chem. Neuroanat. 47:71–8.

67. Bennett MR, Lagopoulos J. 2014. Stress and trauma: BDNF control of dendritic-spine formation and regression. Prog. Neurobiol. 112:80–99.

68. Liston C, Cichon JM, Jeanneteau F, Jia Z, Chao M V, Gan W-B. 2013. Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance. Nat. Neurosci. 16:698–705.

PART 2

Corticotropin-Releasing Hormone Regulation of Stress Pathways

CHAPTER I

INTRODUCTION TO CORTICOTROPIN-RELEASING HORMONE MEDIATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Gloria Laryea, Melinda G. Arnett and Louis J. Muglia

INTRODUCTION

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide that was first isolated and characterized by Wylie Vale in 1981 (1). It belongs to a family of peptides that has subsequently been found to include Urocortin1, 2, and 3 (Ucn1, 2, 3). This family of peptides shares 18% to 43% sequence homology, and its members are expressed in largely non-overlapping regions of the central nervous system and periphery. They function to maintain physiological activities ranging from appetite control to immune system modulation and mediation of the stress response (*2*). To understand the endogenous role of CRH in organisms, several genetic rodent models have been established and used to address a number of questions. These questions deal with understanding the neuroendocrine and behavioral function of CRH and signaling molecules that mediate CRH neurocircuitry. In this chapter, we begin by discussing CRH circuitry and reviewing the associated genetically altered models. Then we present findings regarding human genetic polymorphisms in CRH pathway genes that are associated with stress and psychiatric disorders. We finish by discussing a role for regulators of CRH activity as potential sites for therapeutic intervention aimed at treating maladaptive behaviors associated with stress.

CRH synthesis

CRH is synthesized as a larger inactive preprohormone that undergoes proteolytic cleavage in the Golgi, mainly by prohormone convertase 2 (PC-2), and in some cases PC-1 (3, 4) to generate mature active CRH. The major site of CRH synthesis is the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN). Other brain regions that show high expression of CRH mRNA are the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BnST), and other limbic areas including the hippocampus (Figure 1). In the PVN, CRH is packaged into secretory vesicles and released in a circadian rhythm or in response to stress. The packaged hormone is released from neurons in the PVN into the hypophyseal portal system that links the hypothalamus to the anterior pituitary gland. CRH binds to receptors on corticotroph cells in the anterior pituitary and stimulates the synthesis and secretion of the adrenocorticotropic hormone (ACTH), which ultimately activates adrenal glucocorticoid (GC) synthesis and secretion. These GCs (corticosterone in rodents, cortisol in humans) function to enable adaptation to the stressor and negatively feedback in the hypothalamus and pituitary (to suppress CRH and ACTH production) to establish homeostasis. In extra-hypothalamic parts of the brain, CRH acts in a neuromodulatory capacity on target neurons.

Arginine vasopressin (AVP) is a non-peptide hormone involved in water homeostasis, HPA axis regulation, and behavioral and vascular tone. In HPA axis regulation, AVP is recognized as an important ACTH secretagogue that functions synergistically with CRH. As such, AVP has been shown to compensate for loss of basal, but not stress-activated, CRH-induced secretion of ACTH in CRH KO (5) and CRH-R1 KO mice (6, 7). Detailed AVP involvement in mediating HPA activity and stress-related behavior (see (8, 9) for review) is beyond the scope of this review.

However, it should be considered that in some studies described here in which HPA axis activity remains normal in the absence of CRH or CRH-R1, AVP may contribute to maintaining normal HPA axis activity.

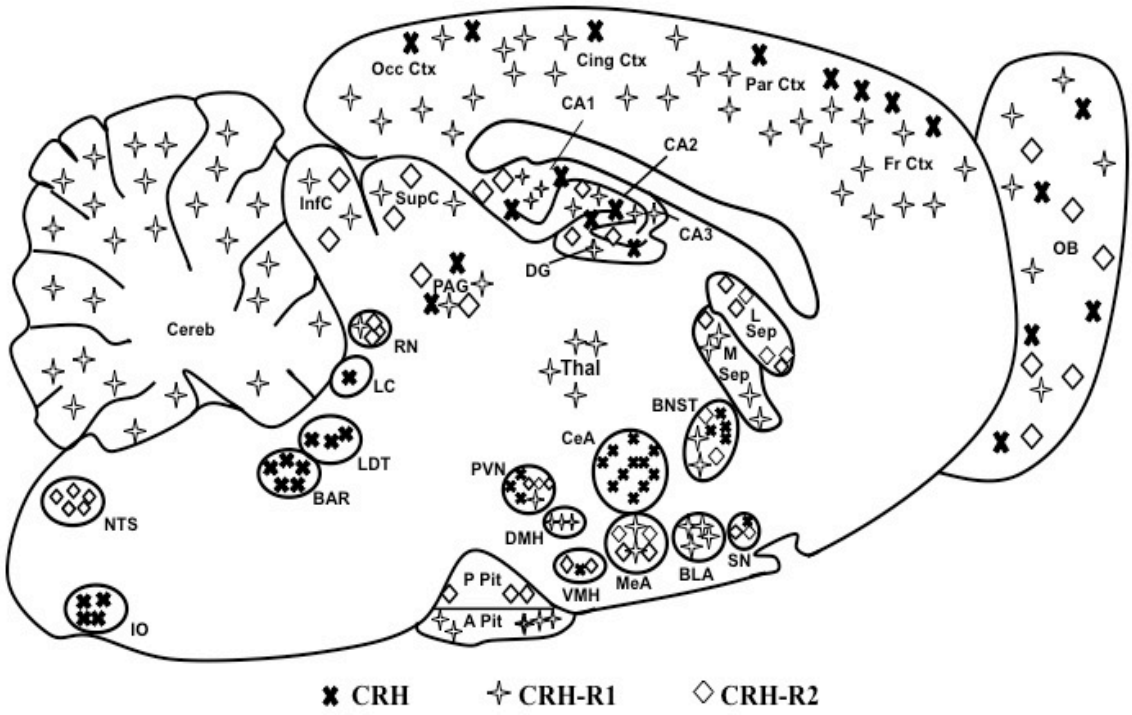

Figure 1. Expression of Corticotropin-releasing hormone (CRH), CRH-R1 and CRH-R2 mRNA in a normal mouse brain. CRH is synthesized in the PVN and shows high expression in the CeA, BnST and the hippocampus. CRH-R1 mRNA is highly expressed in the cortex, cerebellum, A Pit, hippocampus, BLA, MeA and the DMH. CRH-R2 displays high expression in the L Sep, SN, VMH, P Pit and the RN. Abundance of mRNA is shown as the density of representative symbols in an area. Abbreviations: Anterior pituitary, A Pit; Barrington's nucleus, BAR; Basolateral nucleus of the amygdala, BLA; Bed nucleus of the stria terminalis, BnST; Central nucleus of the amygdala, CeA; Hippocampal areas, CA1, CA2, CA3; Cerebellum, Cereb; Cingulate cortex, Cing Ctx; Dentate gyrus, DG; Dorsomedial hypothalamus, DMH; Frontal cortex, Fr Ctx; Inferior colliculus, InfC; Inferior Olive, IO; Locus coeruleus, LC; Lateral dorsal tegmental nucleus, LDT; Medial nucleus of the amygdala, MeA; Medial septum, M Sep; Nucleus tractus solitarii, NTS; Occipital cortex, Occ Ctx; Olfactory bulb, OB; Posterior pituitary, P Pit; Periaqueductal gray, PAG; Parietal cortex, Par Ctx; Paraventricular hypothalamic nucleus, PVN; Raphe nucleus, RN; Supraoptic nucleus, SN; Superior colliculus, SupC; Thalamus, Thal; Ventromedial hypothalamus, VMH.

CRH receptors

CRH has two receptors, CRH-R1 and CRH-R2. These receptors share almost 71% amino acid sequence similarity (10), but are distinctly different in localization and affinities for CRH. In rodents, CRH-R1 displays widespread expression in the cortex and cerebellum, with higher expression in the anterior pituitary, hippocampus, basolateral and medial amygdala, dorsomedial hypothalamus, and parts of the pons/medulla and mesencephalon (Figure 1) (10, 11). CRH-R2, in contrast, is found in more discrete locations in the brain but displays abundant expression throughout the periphery. It is highly expressed in the lateral septum, supraoptic nucleus, ventromedial hypothalamus, cortical amygdala nucleus, raphe nucleus, choroid plexus, and the nucleus tractus solitarii (Figure 1). Regions of overlap between CRH-R1 and CRH-R2 include the olfactory bulb, hippocampus, superior and inferior colliculus, BnST, periaqueductal gray, and medial septum (10, 11). CRH-R1 has a much higher affinity for CRH than CRH-R2 and is the principle receptor involved in the hypothalamic-pituitary adrenal (HPA) axis mediated stress response. Ucn2 and 3 bind selectively to CRH-R2 (2, 12) while Ucn1 binds both receptors with high affinities (13).

In monkey brain, CRH-R1 immunoreactivity is most abundant in the pituitary, cerebellum, and brain stem, with moderate expression in the cerebral cortex, basal forebrain, basal ganglia and thalamus and weak expression in the prefrontal cortex and limbic areas (14). CRH-R2 is present in the neocortex, pituitary, amygdala and hippocampus of monkey brain (15). This is in sharp contrast to expression in the rat brain where only CRH-R1 is found in the pituitary and neocortex.

Finally, in human brain, *CRHR1* is expressed in the hypothalamus, cerebellar cortex, pituitary, amygdala and nucleus accumbens (16) while *CRHR2* is expressed in the pituitary, amygdala, thalamus and hippocampus (17).

The first splice variants of CRH-R1 were identified as CRH-R1α and CRH-R1β (18). CRH-R1α lacks 29 amino acids of exon 6 and is the highest affinity CRH receptor variant. CRH-R1β contains all 14 exons of the CRH-R1 gene, including exon 6, which decreases the affinity of CRH-R1β for CRH. Other CRH-R1 splice variants, including CRH-R1c-h, have been identified, all lacking exon 6 as well as other exons (19). All the aforementioned CRH-R1 splice variants have been identified in humans, while only variants analogous to CRH-R1α, CRH-R1c, CRH-R1e, and CRH-R1f exist in mice. CRH-R2α and CRH-R2β splice variants have been identified in rodents with CRH-R2α expressed mainly in neuronal regions while CRH-R2β is found predominantly in peripheral regions such as the heart, blood vessels and duodenum (13, 20, 21). In humans, both CRH-R2α and CRH-R2β are expressed in peripheral tissues and the brain. Both CRH-R1 and CRH-R2 receptors are G-protein coupled receptors (GPCR), composed of seven-transmembrane domains. Binding of CRH to either receptor results in a conformational change that produces activation of $G\alpha_s$ stimulated adenylate cyclase and activated cyclic AMP and protein kinase A (PKA) signaling pathways (18, 21). The expression of different splice variants of CRH-R1 or CRH-R2 therefore mediate functions associated with their locations through activation of cAMP-mediated pathways.

The specific type of neurons that express the CRH receptors will also factor into their function. In that regard, a recent article by Refojo and colleagues identified the expression of mouse CRH-R1 in forebrain glutamatergic and GABAergic neurons, as well as midbrain dopaminergic neurons and to a lesser extent serotonergic

neurons (*22*). The functional effects of CRH within each of these neuron populations will be discussed in the next chapter.

CRH binding protein

The CRH binding protein (CRH-BP) is another source of regulation for CRH activity at the protein level. During the third trimester of pregnancy in humans, there is a significant increase in the release of placental CRH that predicts the relative timing for parturition (23). Despite this dramatic release of CRH into circulation, there is no increase in ACTH secretion from the pituitary (24). The most reasonable explanation for this came through the identification of the 37 kDa CRH-BP that sequesters CRH and prevents binding to its receptors (25). In primates, CRH-BP is expressed in the brain, pituitary, liver, placenta and plasma (26) and has a higher affinity for CRH than CRH receptors do (27). In rodents, CRH-BP is widely expressed in the cerebral cortex, lateral septum, olfactory bulb and limbic areas such as the CeA and the BnST as well as in pituitary corticotroph cells, with sparse expression in the PVN (27, 28). CRH-BP expression overlaps with CRH receptor expression and is therefore properly positioned to sequester CRH and inhibit its activity. Conversely, low levels of Ucns are also capable of binding CRH-BP and displacing CRH to increase levels of free CRH and thus, CRH activity (29). Whether there are internal or external signals that allow for displacement of CRH by Ucns is unclear, but further investigation may provide insights into regulation.

Synaptic CRH activity

A role for CRH in modulating CNS synaptic transmission outside the HPA axis has become evident. Extrahypothalamic CRH may act as a modulator of neurotransmitters, affecting their excitatory and inhibitory activity. CRH-R1 and CRH-R2 are metabotropic receptors and thus, function slowly in activating signaling cascades, unlike the fast activation of classical neurotransmitters, such as glutamate and GABA, on ionotropic receptors. Synaptic CRH would function to prime post-synaptic neurons for neurotransmitter or neuromodulator activity (30). This activity of CRH is both receptor- and site-specific. For instance, CRH application depresses glutamate-mediated excitatory postsynaptic currents (EPSCs) in the CeA while facilitating these EPSCs in the lateral septum (31). Application of CRH receptor antagonists has indicated that CRH-R1 is responsible for the changes in EPSCs in the CeA and lateral septum, while CRH-R2 facilitates EPSCs in the CeA and depresses them in the lateral septum, although Ucn1 mediates the latter. Further evidence in studies of mice lacking CRH-R1 in forebrain glutamatergic neurons shows that CRH increases excitatory field potentials in the BLA and facilitates action potential firing between hippocampal regions (22). CRH modulation of synaptic activity is observed in other brain regions and provides evidence for the mechanism by which CRH mediates behavior. Evidence supporting this mechanism was demonstrated in a study where acute stress and CRH application facilitated long-term potentiation in the hippocampus and was responsible for enhanced contextual fear conditioning in mice (32). Alterations in CRH expression and signaling are associated with a number of psychiatric disorders that are discussed later in this review. The interplay between extrahypothalamic CRH, its receptors and modulators may regulate synaptic activity that leads to the behavioral changes associated with these disorders.

CRH AGONISTS AND ANTAGONISTS

A subpopulation of patients with major depression and post-traumatic stress disorder (PTSD) have elevated levels of CRH in their cerebrospinal fluid (33, 34) Moreover, HPA axis hyperactivity is the most commonly observed neuroendocrine change in major depressive disorder (MDD). Substantial evidence suggests that normalization of the HPA axis might be a requirement for successful treatment of some individuals with MDD (35). It should be noted that there is biological variation that occurs within these disorders and while some patients do exhibit CRH abnormalities, others do not (36, 37). Furthermore, the presence of CRH abnormalities may relate to the severity of the illness, or to the presence of melancholic features in patients with MDD (35). The hyperactive CRH system and HPA axis abnormalities that occur in some patients suggests a potential role for CRH in modulating the neurocircuitry underlying mood and anxiety disorders. Animal studies have expanded the understanding of CRH activity in neuroendocrine and behavioral changes associated with stress. Important findings from these studies are discussed below.

The use of intracerebroventricular (ICV) CRH has provided further insight into CRH-mediated stress-induced behaviors. ICV injections of CRH in rats results in stimulation of hypothalamic and limbic brain regions that are activated in the stress response (38). In animal models, several behavioral paradigms have been utilized that explore various aspects of anxiety, despair, fear, avoidance, and other stress-related behaviors. Paradigms such as the open field (OF), elevated plus maze (EPM), and light/dark preference (LD) tests are used as indices of anxiety. The forced swim (FST) and tail suspension (TST) tests are used to measure levels of despair, and fear conditioning is utilized as a measure of stress-induced learning and memory. These

tests have been extensively validated and are used frequently in rodent studies. Some studies have demonstrated that ICV CRH increases locomotion, anxiety, fear, despair, and emotionality, decreases food intake, sexual activity, exploration (39) and social interaction and activates the HPA axis and the autonomic nervous system (40–44). Furthermore, these changes can be reversed with CRH antagonists (45–47). CRH-R1 antagonists and antisense oligonucleotides are anxiolytic in rats while CRH-R2 antagonists and antisense oligonucleotides increase despair-like behaviors and alter appetitive behaviors (48, 49).

In vitro studies using rat amygdala and hypothalamic slices suggest that CRH and Ucn1 act through CRH-R1 to increase GABA release (50, 51). Moreover, prolonged daily infusion of Ucn1 into the BLA results in the development of anxiety-like behavior in the EPM and social interaction tests (52). The study determined that this anxiety-like phenotype was inhibited with the administration of NMDA receptor antagonists.

Ucn2 and Ucn3, which are primarily CRH-R2 ligands, have been demonstrated to suppress locomotor activity when injected into the intracerebral ventricles of rats (53, 54). The opposite effect occurs when ovine CRH is administered, it increases locomotor activity. Moreover, while CRH was anxiogenic in the EPM, Ucn3 was anxiolytic and Ucn2 had a delayed anxiolytic effect in the EPM test. These data are supported by evidence in CRH-R2 null mice (discussed in the next chapter) and reinforce the idea that CRH-R2 has a stress-coping function opposite to the stress-activating function of CRH-R1. In contrast to this data, Ucn2 administration in mice is anxiogenic in the EPM, an effect reversed by CRH-R2 specific antagonist (55). Although much data supports an anxiolytic role for CRH-R2, there is still strong data supporting an anxiogenic role for the receptor. Despite the

lack of consistency in data, CRH-R2 does have a role in mediating behavioral responses to stress. The degree of interaction with CRH-R1 in mediating these effects is yet to be determined. These studies and others also indicate the infusion of CRH or Ucn1, -2, or -3 in to the cerebral ventricle reduces feeding behavior in rodents (56).

Pharmacological manipulations have provided evidence in understanding central CRH system effects and continue to be important for investigating the effects of potential therapeutic drugs. In general, however, they are limited by the fact that their effects are short-lived and it is difficult to measure the degree of receptor activation mediating the observed effects. The generation of genetically altered animal models, discussed in the next chapter, has allowed for a closer examination of the endogenous role for CRH.

GENETICALLY ALTERED RODENT MODELS

CRH Mutants

With the generation of CRH deficient mice (CRH KO) (57), the hypothetical role of CRH as an essential mediator of physiological responses to stress could be further tested. CRH KO mice from heterozygous matings are phenotypically normal compared to control mice (58). Their only defining feature is a marked atrophy of the zona fasciculata of the adrenal gland and profound glucocorticoid deficiency. CRH KO mice generated from homozygous parents do not survive longer than 24 hours, due to a lack of glucocorticoids from the mother's placenta to aid in proper lung formation. The aggregate studies in CRH KO mice have demonstrated that CRH KO mice display behavioral responses to stressors remarkably similar to those of wild-type

(WT) animals (59, 60). These mice have low levels of basal plasma concentrations of corticosterone (CORT) that are not compensated for by other ACTH secretagogues such as vasopressin (58) and that do not increase with a foot shock stressor. Stress-induced activation of the HPA axis is absent in these mice whereas stress-induced behavioral responses thought to be mediated by CRH in the brain remain unaffected. It is of note that the use of two distinct CRH-R1 specific antagonists in WT and KO mice attenuated stress-induced behaviors. This indicates the existence of another CRH-like ligand, possibly one of the urocortins, acting through CRH-R1 (59).

CRH Overexpressing (CRH-OE) Mice

General CRH-OE

To study the role of CRH hyperactivity, transgenic mice were generated that constitutively overexpress CRH (Table 1-1.). Stenzel-Poore and colleagues (61) utilized the mouse metallothionein-I (MMT-1) promoter, expressed in the brain and peripheral areas such as the adrenal gland, heart, and testis, to drive CRH overexpression in these areas (CRH-Tg). Inserting rat CRH cDNA into Thy-1 regulatory genes created another model of CRH overexpression (62). The Thy-1 gene drives neuronal expression postnatally through adulthood ($CRH\text{-}OE_{2122}$). In addition, Lu *et al*. inserted a targeting allele consisting of a floxed stop codon, a CRH gene, and an IRES LacZ gene into the Rosa 26 allele ($R26^{+/flop\ CRH}$) and mated them to Cre-lines to overexpress CRH (63). The $R26^{+/flop\ CRH}$ mice, when crossed with Deleter-Cre mice, overexpress CRH ubiquitously throughout the body ($CRH\text{-}COE^{Del}$) (64). These three general transgenic lines display elevated plasma CORT and, in some cases, elevated ACTH levels, altered HPA axis responses and Cushingoid phenotypes. Cushing's syndrome is characterized by hypercortisolemia, truncal obesity, muscle wasting,

thinning of the skin and hair loss (61). These phenotypes are observed in all three transgenic lines described, although the CRH-OE_{2122} mice do not show a Cushing's phenotype until six months of age (61, 64, 65).

Behavioral studies in CRH overexpressing mice show reduced locomotor activity in a novel environment that is further exacerbated by social defeat stress and increased anxiety identified by less time spent in the open arm of the EPM (64, 66), and increased latency to enter the light compartment of a LD preference box (64, 67) and black/white transition test (68). ICV injection of α-helical CRH 9-41, a CRH antagonist, abolished this anxiogenic response (66). Despite heightened anxiety, these mice displayed decreased despair, demonstrated by reduced immobility in the FST (64, 67). CRH-OE_{2122} mice have decreased acoustic startle reactivity, inability to habituate to a startle response, and a deficit in pre-pulse inhibition, all indicative of an impaired ability to process sensory information (62). Moreover they have altered heart rate and increased food and water consumption (69). The CRH-Tg mice from Stenzel-Poore and colleagues also show reduced alcohol preference (70). This finding is surprising given that increased CRH-R1 activity is known to increase alcohol use in stressed rats and humans (71–73). The data from these mice support a role for CRH in stress-mediated coping and sensory processing.

Table 1-1. CRH deletion and overexpression mutants

CRH Deletion Mutant

Line	Manipulation	Main Phenotypes	References
CRH- KO	Constitutive deletion of CRH by insertion of a phosphoglycerate kinase neomycin-resistant cassette	Adrenal insufficiency ↓ Stress CORT No behavioral changes	(57–60)

CRH Overexpression (OE) Mutants

Line	Manipulation	Main Phenotypes	References
CRH-Tg	Mouse metallothionein-1 (MT-1) promoter driven CRH OE in brain, adrenal glands, heart, and testes.	Adrenal Hypertrophy Cushingoid phenotype Attentional Impairment ↑ Basal CORT and ACTH ↓ Locomotion ↑ Anxiety in OF, EPM, LD, and black/white transition test ↑ Active coping in FST ↓ Despair in FST ↓ Sexual receptivity in females ↓ Alcohol preference Gene expression changes	(61,66,67, 70,79,80)
CRH-OE_{2122}	Thy-1 promoter driven CRH OE in neurons postnatally through adulthood.	Adrenal Hypertrophy Cushingoid phenotype at 6 months of age ↑ Basal CORT Dexamethasone non-suppression ↓ Acoustic startle reactivity ↓ Habituation to a startle response Deficit in pre-pulse inhibition ↑ Food and water consumption, and altered heart rate	(62,65,69)
CRH-COE^{Del}	Rosa26 (R26) promoter driven CRH OE in the whole body.	Cushingoid phenotype at 3-weeks of age ↑ Adrenal weight, ↓ thymus weight ↑ Basal CORT ↑ Anxiety in OF, EPM, LD, and black/white transition test ↓ Despair in FST	(64)
CRH-COE^{APit}	R26 and POMC promoter driven CRH OE in the anterior and intermediate lobes of the pituitary	Mild Cushingoid phenotype at 5-6 months of age ↑ Basal CORT	(64)

Table 1-1. Con't

CRH Overexpression (OE) Mutants

Line	Manipulation	Main Phenotypes	References
CRH-COE-Nes	R26 and Nestin promoter driven CRH OE in neurons and glia from embryonic day 10.5	↑ Stress-induced CORT and ACTH in male mice ↓ Despair in FST and TST tests, reversible with CRH-R1 antagonist treatment ↑ REM sleep	(63,75,76)
CRH-COE-Cam	R26 and CamK2 promoter driven CRH OE in forebrain glutamatergic neurons from postnatal day 15	Normal HPA axis activity ↑ REM sleep ↑ Deficit in spatial performance in the MWM and Y-maze tests.	
CRH-COE-Dlx	R26 and Dlx promoter driven CRH OE in GABAergic interneurons from embryonic day 10.5	Normal HPA axis activity and behavior	
FBCRHOElife	CamK2 promoter driven forebrain CRH OE from embryonic day 0 through life	Cushingoid phenotype by 8 weeks of age ↑ Nadir CORT and ACTH	(77)
Crh-COE^{CamCreERT2}	R26 and Camk2a-CreERT2 promoter driven CRH OE in forebrain glutamatergic neurons (OE induced by tamoxifen at postnatal week 8)	↑ Anxiety in LD and EPM tests	(22)
FBCRHOEdev	CamK2 promoter driven forebrain CRH OE from embryonic day 15 to postnatal day 21	↑Basal CORT only during CRH- OE. ↑ Despair in FST and TST test (↓ despair in FST with antidepressants treatment) ↑ Anxiety in OF, EPM, and LD tests ↑ CRH-R1 mRNA in the cingulate cortex, dentate gyrus and CA1 region of the hippocampus	(77)
CRF-OE	CamK2 promoter driven forebrain CRH OE from 8 to 11 weeks of age	↑ Nadir CORT ↓ Thymus weight in females ↓ Locomotion in familiar environment ↓ Despair in FST Trend towards anxiety in LD	(78)

Abbreviations: Acoustic startle response test, ASR; Adrenocorticotropic Hormone, ACTH; Arginine Vasopressin, AVP; Basolateral nucleus of the amygdala, BLA; Bed nucleus of the stria terminalis, BnST; Central nucleus of the amygdala, CeA; Corticosterone, CORT; cAMP response element-binding, CREB; Corticotropin-Releasing Hormone, CRH; Dentate gyrus, DG; Dopamine, DA; Dorsal raphe nucleus, DRN; Elevated Plus Maze, EPM; Forced Swim Test, FST; Lateral Septum, LS; Light/Dark Preference test, LD; Lipopolysaccharide, LPS; Long-term potentiation, LTP; Morris Water Maze, MWM; Open field test, OF; Open Reading Frame, ORF; Overexpression, OE; Paraventricular nucleus of the hypothalamus, PVN; Prefrontal Cortex, PFC; Proopiomelanocotin, POMC; Rapid Eye Movement, REM; Supraoptic nucleus, SON; Tail suspension test, TST; Urocortin, Ucn).

These studies demonstrate that elevated CRH alters emotional regulation. However, analysis of the data may be confounded by the Cushing's phenotype of these mice, including muscle wasting, which may alter their behavioral output. A recent article investigated the effects on GABA and glutamatergic transmission in CRH-OE_{2122} (74). The study showed that constitutive overexpression of CRH reduced sensitivity to the anxiolytic effects of CRH-R1 antagonists, and $GABA_A$ and glutamate receptor agonists in response to stress-induced hypothermia. Additionally, mRNA levels of distinct subunits of the $GABA_A$ receptors and $mGluR_{2/3}$ receptor were differentially altered in amygdala *versus* the hypothalamus (74). The data supports the notion that an imbalance in GABAergic and glutamatergic transmission may underlie the genesis of stress-related maladaptive behaviors.

Spatially Restricted CRH-OE

The general overexpression of CRH in the aforesaid studies makes it difficult to identify which regions are involved in mediating the different phenotypes observed. This section focuses on transgenic mouse models that spatially restrict CRH overexpression to particular brain regions (Table 1-1.). The Rosa 26 allele was inserted with a targeting allele consisting of a floxed stop codon, a CRH gene, and an IRES LacZ gene ($R26^{+/flop\ CRH}$) (63). These mice were bred to Nestin-Cre mice to overexpress CRH in neurons and glia from embryonic day 10.5 through adulthood (CRH-COE-Nes); Cam-Cre to overexpress CRH in forebrain glutamatergic neurons from postnatal day 15 (CRH-COE-Cam); and Dlx-Cre to overexpress CRH in GABA-ergic interneurons from embryonic day 10.5 into adulthood (CRH-COE-Dlx). None of these mutants display Cushingoid phenotypes and all exhibit normal basal CORT and ACTH levels. Stress-induced CORT and ACTH are however, higher in

CRH-COE-Nes males compared to controls, an effect not observed in females. Behaviorally, CRH-COE-Nes mice show decreased despair behavior in both the FST and TST tests that is reversible with CRH-R1 antagonist treatment. The authors postulate that this is an adaptation to actively cope with stress (63). The fact that these behaviors are only observed in the CRH-COE-Nes mice indicates that hindbrain regions are important for mediating the active coping responses observed in CRH-COE-Nes mice. Behavior in the CRH-COE-Cam mice indicates deficits in spatial memory in the Y-maze test and deficits in spatial learning in the Morris Water Maze (MWM) test (75). These spatial learning and memory deficits recapitulate what is observed in mice that have undergone early life stress and do not occur in the absence of forebrain CRH-R1. This indicates that CRH-R1 signaling is important in mediating stress-induced alterations in hippocampus-dependent learning and memory. Further proof of this will be discussed in the CRH-R1 mutants' section (Section 3.3.1.). A separate study also shows that overexpressing CRH in forebrain glutamatergic neurons (Crh-COE^{CamCreERT2}) of mice increases anxiety in the EPM and LD tests, while anxiolysis is observed in the absence of forebrain CRH-R1 (22).

Sleep impairments are observed in a number of psychiatric disorders and are targets of therapy when treating patients with depression, PTSD, and anxiety disorders. CRH-COE-Nes and CRH-COE-Cam mice display increased REM (rapid eye movement) sleep, indicating a role for CRH dysregulation of sleep (76).

The R26$^{+/flop\ CRH}$ mice have also been crossed to Pomc-Cre mice to overexpress CRH in the anterior and intermediate lobes of the pituitary (CRH-COE^{APit}) (64). CRH-COE^{APit} display a Cushingoid-like phenotype at 5–6 months old, decreased body weight but increased adrenal gland weight, increased basal CORT secretion that is arrhythmic, and no differences in the stress-induced CORT secretion,

compared to controls, in males but a blunted effect in females. Behaviorally, these mice spent more time in the inner zone of the OF and less time immobile in the FST, indicative of decreased anxiety and despair-like behaviors, respectively (64).

Spatially and Temporally Restricted CRH-OE

We have generated transgenic mice that overexpress CRH in the forebrain under the control of the CamKII promoter (77). These mice utilize an inducible tetracycline system that enables CRH overexpression to be turned off in the presence of dietary doxycycline (FBCRHOE). CRH mRNA expression was found to be elevated in all forebrain regions excluding the thalamus and PVN. Lifelong forebrain CRH overexpression ($FBCRHOE^{life}$) resulted in a Cushingoid-like phenotype and elevated nadir CORT and ACTH levels. This inducible tetracycline system was further used to study the importance of forebrain CRH overexpression during development in the first three weeks of life, E15 to P21 ($FBCRHOE^{dev}$). Although $FBCRHOE^{dev}$ mice have elevated basal CORT at P15 and P20, these levels normalize in adulthood. Behaviorally, $FBCRHOE^{dev}$ mice display increased despair and anxiety-like behaviors as well as increased CRH-R1 mRNA that are reversed with antidepressant treatment (77). A similar mouse model overexpressing forebrain CRH transiently from 8 to 11 weeks of age, shows increased cortex and hippocampal CRH and increased basal CORT levels with a trend towards mild anxiety in the LD test and active coping in the FST (78). These studies demonstrate how disruption of CRH activity during critical developmental periods can affect behavioral outcomes. These studies have been invaluable in understanding the role of CRH in neuroendocrine and behavioral outcomes associated with stress.

CRH-R1 and CRH-R2 Mutants

CRH-R1 and CRH-R2 null mutant mice have been generated to elucidate the role for each receptor subtype in mediating the observed behaviors. The findings from these studies will be discussed in details below.

CRH-R1 Mutants

CRH-R1 null mutant mice were generated by deleting exons 5–8 of the CRH-R1 gene encoding the last 12 amino acids of the first extracellular domain through the fourth transmembrane domain and replacing them with a PGK neomycin-resistant gene cassette (79). In behavior models used to measure anxiety, these mice showed reduced anxiogenic-like responses compared to the littermate controls (80). A separate study generated CRH-R1 null mutant mice lacking the coding sequences of transmembrane regions V, VI, and VII, including the G-coupling protein domain and the intracellular cytoplasmic tail, resulting in a dysfunctional CRH-R1 unable to transmit any ligand-induced signals (81). These mice displayed similar reductions in anxiety-related behaviors in response to ethanol withdrawal. To determine if CRH-R1 mediates anxiety-like behavior independently of the HPA axis function, Müller and colleagues generated conditional knockout mice with CaMKIIa driving Cre-mediated inactivation of CRH-R1 (Cam-CRHR1) in behaviorally relevant neuronal circuitries of the anterior forebrain and limbic system including the cortex, hippocampus and amygdala (82). Similar to the conventional mutants, these conditional knockouts displayed significantly reduced anxiety-related behaviors indicating that selective disruption of the CRH-R1 signaling pathway reduces anxiety. Furthermore, diminished neuronal activity in regions such as the medial amygdala and the prelimbic cortex of these forebrian CRH-R1 KO mice likely mediates the anxiety

phenotype observed (83). Chronic social defeat stress or early life stress in WT mice causes cognitive impairments reflected in the Y-maze and MWM tests (75, 84). In Cam-CRHR1 mice that undergo the aforementioned stressors, loss of CRH-R1 in forebrain neurons protects against impairments in spatial performance. Moreover, these mice are protected from atrophy in the dendritic spines of CA1 and CA3 neurons and impaired hippocampal LTP. Interestingly, these CRH-R1 deficient mice appear to compensate for stress-induced impairments in synaptic transmission by increasing dendritic spines density and enhancing high-frequency stimulation LTPs in the hippocampus (75). CRH activity through CRH-R1 receptors, therefore, not only mediates anxiety behaviors but functions in hippocampus-dependent cognitive performance. In contrast to forebrain CRH-R1 deletion, deletion of CRH-R1 in all brain neurons, using the Nestin promoter, decreases forced swim stress-induced alcohol consumption in adults (85) and increases basal and stress-induce plasma CORT in neonates (86). Loss of CRH-R1 in all these transgenic lines results in differential HPA axis alterations that implicate divergent roles of forebrain and hindbrain regions in stress neuroendocrinology (Table 1-2) (6, 79, 81, 86–88).

A recent study has also implicated divergent roles of CRH-R1 in different neurotransmitter systems. CRH-R1 was deleted specifically in forebrain glutamatergic neurons ($Crhr1^{Glu\text{-}CKO}$) by crossing floxed CRH-R1 mice to Nex-Cre mice, Nex is a transcription factor that is expressed in mature glutamatergic neurons (22). $Crhr1^{Glu\text{-}CKO}$ mice display reduced anxiety in four distinct test, including EPM and LD preference tests. Conversely, mice with CRH-R1 deleted in midbrain dopamine neurons ($Crhr1^{DA\text{-}CKO}$) display increased anxiety. These data indicate that different neurotransmitter system mediates opposite function of CRH-R1. While glutamatergic neurons mediate the anxiogenic properties of CRH-R1 activity,

dopamanergic neurons mediate the anxiolytic functions. Further analysis of the $Crhr1^{DA\text{-}CKO}$ mice showed a reduced prefrontal cortex response to stress-induced dopamine release. This study also demonstrated that CRH-R1 deletion in gabaergic and serotonergic neurons had no effect on anxiety-related behaviors. This data in general indicates that CRH may function in different neurotransmitter systems to balance CRH-R1 responses to stress (22).

CRH-R2 Mutants

In contrast to the clear reduced-anxiety phenotype in both conventional and conditional CRH-R1 KO mice, significant differences in the behavioral phenotypes are reported amongst three independently generated CRH-R2 KO mouse lines. Subsequently, the physiological role of CRH-R2 in mediating anxiety is still unclear. CRH-R2 null mice have been demonstrated to increase HPA axis reactivity to stress (89). Bale and colleagues generated a CRH-R2 null mutant mouse by constructing a targeting vector in which the portion of CRH-R2 encoding one-half of the fifth transmembrane domain through the end of the seventh transmembrane domain was deleted and replaced with a neomycin-resistant gene cassette (90). In contrast to CRH-R1 mutants, behavioral studies revealed that these mice displayed increased anxiety-like behaviors. Similar increased anxiety and despair-related behaviors have been found in separate studies of CRH-R2 null mice (91, 92). CRH-R2 null mutants have also been produced by homologous recombination with a targeting construct containing CRH-R2 and a neomycin cassette (91). Behavioral studies revealed no difference in anxiety-like behaviors in these mutants. Compared to control mice, these CRH-R2 null mutant mice were also tested for despair-like behavior using the FST and showed longer immobility time indicating increased despair-like behaviors (93). When reared

in an isolated environment after weaning, CRH-R2 deficient mice displayed increased locomotor behavior to a greater extent than what is observed in WT mice (94).

Table 1-2. CRH receptor mutants

CRH Receptor Mutants			
Line	**Manipulation**	**Main Phenotypes**	**References**
CRH-R1$^{-/-}$	Constitutive deletion (exons 5-8) of the CRH-R1	Adrenal gland atrophy ↑ PVN CRH ↓ Basal CORT ↓ Stress–induced CORT and ACTH in males ↓ Anxiety in LD and EPM tests	(79,80)
CRH-R1$^{-/-}$	Constitutive deletion of CRH-R1 (transmembrane regions V, VI, and VII)	↓ CRH-induced cAMP ↓ Basal ACTH in pituitary cultures ↓ Basal CORT in females ↑ PVN CRH only in neonates ↑ Plasma and PVN vasopressin ↓ Stress-induced CORT and ACTH ↓ ACTH-induced CORT ↓ Anxiety in LD test basally and during alcohol withdrawal ↓ Neuronal activity (cFOS)	(6,81,83,87,88)
Crhr1$^{loxP/loxP}$Camk2a-cre Or Cam-CRHR1 Or CRHR1^{Camk2aCre} Or CRF_1-CKO	Camk2a promoter driven deletion CRH-R1 in forebrain	↑ Stress-induced CORT and ACTH in adults and neonates ↑ CRH mRNA in PVN in adults and neonates ↓ Anxiety in LD and EPM test in adults Hyperactive in OF test in adults ↓ Neuronal activity ↓ Deficit in spatial memory in Y-maze tests after chronic social defeat stress No deficit in spatial learning and memory in MWM or Y-maze tests after early life stress No chronic social defeat stress-induced deficits in the novel object recognition test No stress-induced atrophy of apical dendrites in CA3 neurons compared to control mice No stress-induced reduction of GR mRNA in CA1 and CA3 neurons compared to control mice ↑ Weight gain after chronic stress No deficit in hippocampal LTP after early life stress ↑ High frequency stimulation-induced LTP with early life stress	(75,82,84,86)

Table 1-2. Cont'd

CRH Receptor Mutants			
Line	**Manipulation**	**Main Phenotypes**	**Refer**
$Crhr1^{loxP/loxP}$Nes-cre Nes-CRHR1	Nes-Cre promoter driven deletion CRH-R1 in all neurons	↓ Basal CORT in neonates ↑ Stress-induced CORT and ACTH ↓ Alcohol intake after FSS	(85,86)
$Crhr1^{Glu\text{-}CKO}$	Nes-Cre promoter driven CRH-R1 deletion in mature glutamatergic neurons	↓ Anxiety in LD, EPM, novel object exploration, and modified hole board tests No effect on despair behavior in FST No effect auditory fear conditioning ↑ Locomotion in LD test No effect on basal or stress-induced CORT secretion ↓ Excitatory field potentials on glutamatergic neurons in the BLA ↓ Facilitation of action potential firing between hippocampal DG-CA3-CA1 network	(22)
$Crhr1^{DA\text{-}CKO}$	Dat-CreERT2 promoter driven CRH-R1 deletion in midbrain dopaminergic neurons	↑ Anxiety in LD, EPM, novel object exploration, and modified hole board tests No effect on despair behavior in FST No effect auditory fear conditioning No effect on basal or stress-induced CORT secretion ↓ Response to stress-induced DA release in PFC	
$Crhr1^{GABA\text{-}CKO}$	DLX5/6 promoter driven CRH-R1 deletion in forebrain GABAergic neurons	No effect on anxiety behaviors No effect on despair behavior in FST No effect auditory fear conditioning No effect on basal or stress-induced CORT secretion	(22)
$Crhr1^{5HT\text{-}CKO}$	ePet-Cre promoter driven CRH-R1 deletion in brainstem seratonergic neurons		
$Crhr1^{CNS\text{-}CKO}$	Nes-Cre promoter driven CRH-R1 deletion in all neurons	No effect on anxiety behaviors	
$CRH\text{-}R2^{-/-}$	Constitutive deletion by replacing 5th-7th transmembrane domains with a neomycin-resistant cassette	↑ Stress-induced CORT and ACTH ↓ Food intake after stress of food deprivation ↑Anxiety in EPM and OF tests ↑ Despair in FST ↑ CRH mRNA in the CeA ↑ Ucn1 mRNA in Edinger-Westphal (EW) nucleus	(90,93)

Table 1-2. Cont'd

CRH Receptor Mutants			
Line	**Manipulation**	**Main Phenotypes**	**Refer**
CRH-R2$^{-/-}$	Constitutive deletion by replacing exons of the 3rd intracellular loop with neomycin-resistant cassette	↑ Anxiety in EPM and LD tests in males ↑ Locomotion in OF test in males ↑ Stress-induced anxiety in the OF test in males ↓ Neuronal activation measured by levels of phosphorylated CREB ↑ Despair behavior in the FST and TST tests that is prevented when MEK/ERK pathway in the hippocampus is inhibited	(91,92)
CRH-R2$^{-/-}$	Constitutive deletion by replacing the 3rd and 4th transmembrane domains with a neomycin-resistant cassette	↓ cAMP activity in cultured cardiomyocytes ↑ Ucn1 mRNA in Edinger-Westphal (EW) nucleus ↑ Stress-induced CORT and ACTH ↓ Cardiac function with Ucn administration Altered feeding with Ucn administration	(89)
CRH-R1$^{-/-}$/ CRH-R2$^{-/-}$	CRH-R1$^{-/-}$ (79) were crossed to CRH-R2$^{-/-}$ (90) to generate these double knockout mice	↑ PVN CRH ↓ Basal CORT and ACTH ↓ HPA axis reactivity to stress	(7)
CRH-R1$^{-/-}$/ CRH-R2$^{-/-}$	CRH-R1$^{-/-}$(81) were crossed to CRH-R2$^{-/-}$(89) to generate these double knockout mice	↓ Basal CORT and ACTH ↓ Anxiety in EPM and OF tests in females ↑ PVN CRH and AVP	(95)

Abbreviations: Acoustic startle response test, ASR; Adrenocorticotropic Hormone, ACTH; Arginine Vasopressin, AVP; Basolateral nucleus of the amygdala, BLA; Bed nucleus of the stria terminalis, BnST; Central nucleus of the amygdala, CeA; Corticosterone, CORT; cAMP response element-binding, CREB; Corticotropin-Releasing Hormone, CRH; Dentate gyrus, DG; Dopamine, DA; Dorsal raphe nucleus, DRN; Elevated Plus Maze, EPM; Forced Swim Test, FST; Lateral Septum, LS; Light/Dark Preference test, LD; Lipopolysaccharide, LPS; Long-term potentiation, LTP; Morris Water Maze, MWM; Open field test, OF; Open Reading Frame, ORF; Overexpression, OE; Paraventricular nucleus of the hypothalamus, PVN; Prefrontal Cortex, PFC; Proopiomelanocortin, POMC; Rapid Eye Movement, REM; Supraoptic nucleus, SON; Tail suspension test, TST; Urocortin, Ucn).

Combined CRH-R1 and CRH-R2 Mutants

Mice null for both CRH-R1 and CRH-R2 display adrenal cortex atrophy, increased PVN CRH, decreased basal CORT and ACTH levels and diminished HPA axis reactivity in response to stress (90). A similar phenotype is observed in CRH-R1

deficiency alone, indicating that CRH-R2 cannot overcome the loss of CRH-R1 (7). A separate study observed that CORT and ACTH levels were significantly different between CRH-R1/CRH-R2 double KO compared to CRH-R1 KO but both were significantly diminished compared to controls. Further examination indicated that, CRH-R1 KO male mice born from dams who were either CRH-R2 het or CRH-R2 KO, show increased anxiety in OF and EPM, implying that there are influences of genetic background on behavior. Female mice showed decreased anxiety in the EPM, also indicating gender dimorphism and perhaps the influence of gender-specific hormones in mediating behavior (95).

Urocortin Mutants

Urocortins (Ucn) have a high affinity for CRH receptors. While Ucn1 and CRH have high affinities for CRH-R1, Ucn2 and 3 are considered to be the major endogenous ligands for CRH-R2 (96). CRH deficient mice do not differ from WT mice in their freezing response to a foot shock stressor, but CRH-R1 antagonists block freezing behavior in CRH-KO mice (59). This brought about the idea that another CRH-related peptide was compensating for loss of CRH. The most likely candidate was Ucn1 since it also has a high affinity for CRH-R1. Ucn1 null mice however, have normal HPA axis activity and anxiety phenotypes, but display impairments in acoustic startle response, not due to hearing impairments (Table 1-3.) (97). Studying the response of mice deficient for both CRH and Ucn1 to foot shock may better elucidate whether Ucn1 acts on CRH-R1 in the absence of CRH. A different Ucn1 null mouse line generated by a different group shows that Ucn1 deficiency increases anxiety in the OF and EPM (98). However, more data on this line of mice is needed as the hearing impairment and reduction in CRH-R2 mRNA in the

lateral septum in these mice may account for the increased anxiety. These $Ucn^{-/-}$ mice also have reduced ethanol preference (99), implicating a role for Ucn1 in alcohol behavior.

In contrast to Ucn1 null mice, Ucn2 null mice have altered HPA axis regulation of CORT and ACTH (100). Ucn2 KO female mice show higher nocturnal CORT and ACTH levels, not observed if the mice were ovariectomized, implicating estrogen mediation of this effect. The female Ucn2 KO mice also have an increase in hypothalamic AVP mRNA that may account for the rise in ACTH and CORT. Behaviorally, Ucn2 KO mice show no anxiety phenotypes but the females do display less despair in the FST and TST tests (100). This implicates that Ucn2 may be involved in mediating gender differences in relation to HPA axis function and depressive phenotype.

When both Ucn1 and 2 are deleted (Ucn1/Ucn2 dKO), there is a stress-induced elevation in CORT (males only) as well as elevated CRH mRNA in the PVN (101). Ucn1/Ucn2 dKO mice display reduced basal anxiety as well as reduced stress-induced anxiety in the OF and LD tests (Table 1-3.). Since Ucn1 null mice do not show changes in HPA axis regulation, it is plausible that an interaction between Ucn1 and 2 activities is necessary to influence HPA axis response.

Ucn3 deficient mice ($Ucn3^{tZ/tZ}$) only display behavioral alterations in the social discrimination task (102). Whereas controls show no discrimination of a familiar versus unfamiliar subject, $Ucn3^{tZ/tZ}$ are able to discriminate. This was also the case for CRH-R2 KO mice but not $Ucn2^{tZ/tZ}$, indicating that this behavior is specific to Ucn3 action on CRH-R2 receptors. $Ucn3^{tZ/tZ}$ demonstrate no alterations in HPA axis activity or in tests of anxiety, despair, novel object recognition, and conditioned fear.

Table 1-3. Urocortin mutants

Ucn mutants

Line	Manipulation	Main Phenotypes	REF
$Ucn^{-/-}$	Constitutive deletion of Ucn1 by replacement of the coding exon of Ucn with an EGFP-LacZ fusion reporter and a *PGKneo* selection cassette	Normal HPA axis activity No change anxiety in the EPM, OF, and LD tests Impaired acoustic startle response	(97)
$Ucn^{-/-}$	Constitutive deletion of Ucn1 by replacement of region encoding the mature peptide with a neomycin-resistant gene cassette	Normal HPA axis activity and feeding behavior ↑ Anxiety in EPM and OF tests ↓ CRH-R2 mRNA in the LS ↓ Length of hair cell in organ of Corti leading to hearing impairments	(98,99)
Ucn2 KO	Constitutive deletion of Ucn2 by insertion of a neomycin-resistant gene cassette	↑ Nocturnal CORT and ACTH in females ↑ AVP mRNA in the PVN and SON of females - altered drinking habits ↓ Despair in the FST and TST in females No changes in anxiety in EPM and LD tests or in conditioned fear tests ↑ CRH mRNA in the BnST and CeA ↓ Ucn3 mRNA in the median preoptic nucleus and perifornical area ↑ CRH-R2 mRNA in the BnST, LS and DR	(100)
Ucn1/Ucn2 dKO	Cross breeding Ucn1 (98) and Ucn2 (100) single KOs to generate these double knockout mice.	↑ Stress-induced plasma CORT in males ↑ PVN CRH mRNA Hypertrophy of the zona fasciculate ↓ Anxiety in EPM and OF ↓ Behavioral response to acute stress in females ↓ CRH-R2 mRNA in the LS ↑ Amygdala CRH mRNA	(101)
$Ucn3^{tZ/tZ}$	Ucn3 gene was disrupted by homologous recombination and the ORF was replaced by a tau-lacZ reporter gene	Normal basal and stress-induced HPA axis responses No changes in anxiety-related behaviors in EPM, social interaction, and modified hole board tests compared to WT No difference in despair behavior in the FST compared to WT No genotype effect in the ASR test ↑ Cocial discrimination memory	(102)
Ucn tKO	Cross breeding Ucn1, 2, and 3 single KOs from (98,100,102)	↓ Basal exploration in OF 24 hours post-stress ↑ Anxiety in OF, LD and ASR tests 24 hours after an acute stressor ↑ Freezing in cued fear conditioning and ASR tests ↓ Spatial learning in MWM ↑ CRH-R2 mRNA in the LS and DRN ↑ CRH-R1 mRNA in amygdala compared to controls 24 hours post- stress Lack of stress-induced amygdala gene modification compared to controls	(103)

When all three Ucn are deleted, the triple knock out (tKO) exhibit increased anxiety in the OF and LD tests 24 hours after stress but are no different from WT mice in unstressed conditions or immediately after stress (103). The tKO mice display an increase in the acoustic startle response 24 hours post-stress. They also increase freezing in cued fear conditioning but not the contextual test, and have stress-induced deficits in spatial learning in the MWM test. Amygdala gene expression profiles demonstrate that Ucn tKO mice do not have typical changes in stress-induced genes to aid in coping with stress. Thus urocortins appear to be essential for recovery after stress, by modifying expression of stress-related genes in the amygdala. This tKO line may be useful in studying the proposed function of CRH-R2 in reducing sensitivity to stress (21).

Due to the discrepancy in two lines of Ucn1 null mice, the function of Ucn1 in anxiety remains unclear. However, it is clear that Ucn1 is not important for mediating HPA axis activity, a role more attributable to Ucn2 function. The data discussed here also point to a role for Ucn2 activity in mediating despair behavior and stimulating gender differences. The specificity of $Ucn3^{tZ/tZ}$ mice in affecting only social discrimination indicates a role for Ucn3 in processing social cues. This role of Ucn3 is fitting as it is expressed in regions associated with the accessory olfactory system.

CRH-BP Mutants

Mice overexpressing CRH-BP were created by inserting a fragment of the rat CRH-BP, between the mouse metallothionein-I promoter and a 2.1 kb noncoding fragment of the human growth hormone, which contains a polyadenylation sequence. These mice overexpress CRH-BP in the brain and pituitary as well as peripheral sites such as the placenta, plasma, and amniotic fluid (27). Male transgenic mice showed

increased weight gain, to a greater extent than females, and no HPA activity changes (104). Overexpressing CRH-BP only in the anterior pituitary, using the pituitary glycoprotein hormone promoter, similarly produced normal circadian and stress-induced levels of ACTH and CORT (105). Further investigation demonstrated compensatory increases in both PVN CRH and vasopressin (AVP) as the culprits for lack of HPA axis alterations. The behavioral phenotype of these mice includes increased locomotion and a trend towards decreased anxiety in the OF (105).

Interestingly, CRH-BP deficient mice likewise display normal circadian and stress-induced levels of ACTH and CORT. However, anxiety phenotypes are much more evident in these mice in the EPM, OF and defensive withdraw tests, with females exhibiting more anxiety (106). CRH-BP deficient male mice demonstrate reduced food intake and diminished weight gain, reflecting the defined role for CRH in anorexiogenic behavior. The fact that all these mutant CRH-BP mice display normal HPA axis activity (Table 1-4.) underscores the importance of maintaining homeostasis in this pathway, conceivably, through compensatory changes in PVN CRH and AVP transcript levels.

Table 1-4. CRH binding protein mutants

CRH-BP mutants			
Line	**Manipulation**	**Main Phenotypes**	**REF**
CRH-BP (transgenic)	Mouse metallothionein-1 (MT-1) promoter driven CRH-BP OE in the brain and pituitary as well as sites such as the placenta, plasma, and amniotic fluid	Normal CORT and ACTH ↑ Weight gain (gender specific) Blunted ACTH response to LPS injection	(104)
CRH-BP (transgenic)	Pituitary glycoprotein hormone a-subunit (a-GSU) promoter driven CRH-BP OE in the pituitary	Normal CORT and ACTH ↑ PVN CRH and vasopressin ↑ Locomotion Trend towards decreased anxiety in the OF	(105)
CRH-BP$^{-/-}$	Constitutive deletion by replacing exons1-5 with a phosphoglycerate kinase neomycin-resistant cassette	Normal CORT and ACTH ↑ Anxiety in EPM, OF and defensive withdraw (gender specific) ↓ Food intake and weight gain in males	(106)

THE USE OF VIRAL VECTORS TO MODULATE CRH ACTIVITY

In the bulk of studies thus far described, the relatively broad brain regions investigated constrain our ability to understand the role of CRH action in restricted brain nuclei in mediating behaviors. The use of lentiviral vectors has succeeded at overcoming this limitation by allowing for precise spatial regulation of CRH overexpression (Table 1-5.). Regev *et al*. (2010) injected a lentiviral vector, containing rat CRH cDNA, into the CeA of 7-week old male mice. The authors presented that chronic (4-month) overexpression of CRH in the CeA of male mice under basal conditions had minor effects on anxiety in the OF and LD preference tests (107). However, 30 minutes of restraint stress significantly attenuated anxiety-related behaviors, implying possible habituation to a stressor. In a separate study, short-term inducible CeA CRH overexpression increased anxiety after stress (108). Since CeA CRH is known to be anxiogenic, perhaps the 4-month long CeA CRH OE results in compensatory changes that cause the observed habituation response. In male rats, extended elevated CeA CRH (10 weeks) during adulthood differentially dysregulated HPA axis function and increased CRH in the PVN (109). Observed behaviors included decreased locomotion in the OF test, increased anxiety in EPM, and increased time withdrawing and under protection in the defensive withdrawal test. A similar study elevating CeA CRH levels in female rats demonstrated stress pathology changes including increased anxiety and despair-like behaviors, and impaired negative feedback of the HPA axis (110). These female rats show augmented basal anxiety in the acoustic startle response. It is notable that the lentiviral construct used in this latter study was generated to infect neurons that endogenously produce CRH. This may limit confounds of expressing CRH in neurons that do not normally express CRH, and subsequent effects of these neurons on their targets. It therefore appears

that short term CeA CRH OE results in anxiogenic phenotypes while longer term CeA CRH OE causes a blunting of anxiety behaviors. We can however not exclude the effects of gender and/or species differences in mediating the discrepancies in these studies.

Table 1-5. Viral Vectors in Rodent Models.

Line (viral vector)	Manipulation	Main Phenotypes	REF
CeA CRF OE (pCSC-SP-PW-rCRF-IRES/GFP)	Long-term lentiviral CRH OE in the CeA of adult male mice. Behavioral testing began 4 months after lentiviral injections	↓ Basal and stress-induced anxiety in OF and LD tests ↓ Response to acoustic startle Habituation to startle after stress ↑ CRH-R1 mRNA in CeA	(107)
CeA-CRF-OE (rtTA-IRES/GFP + TRE-mCRF-IRES/RFP)	Short-term lentiviral CRH OE in the CeA of adult male mice when Dox is administered. Behavioral testing began 3 days after Dox administration	↑ Stress-induced anxiety in LD test No effects on despair in FST or TST No effects on fear conditioning	(108)
Lenti-CMV-CRF OE (LVCRFp3.CRF)	Lentivirus-induced CRH OE in the CeA of female rats. Behavioral testing began 2 weeks after lentiviral injections	Impaired negative feedback of the HPA axis Disrupted reproductive and sexual function. ↑ Despair in FST ↑ Anxiety in acoustic startle	(110)
CeA CRF OE LVCRFp3.CRF	Lentivirus-induced CRH OE in the CeA of male rats. Behavioral testing began 4 weeks after lentiviral injections	↑ CRH and vasopressin mRNA in the PVN ↑Basal ACTH Dexamethasone non-suppression ↑ Anxiety in EPM and defensive withdrawal tests.	(109)
BnST CRF OE (pCSC-SP-PW-rCRF-IRES/GFP)	Long-term lentiviral CRH OE in the BnST of male mice. Behavioral testing began 4 months after lentiviral injections	↑ Despair in FST ↓ CRH-R1 mRNA in BnST	(107)
BnST CRF OE (LVCRFp3.CRF)	CRH OE in the BnST of adult male rats before or after fear conditioning in ASR tests	No changes in anxiety measures in EPM or DW tests No HPA axis alterations ↓ CRH-R1 binding density in the BnST ↓ CRH-R2 binding density in the DRN	(111)

Table 1-5. Cont'd

Line (viral vector)	Manipulation	Main Phenotypes	REF
BnST CRF OE (LVCRFp3.CRF)	Behavioral testing began ~2 weeks after lentiviral injections	CRH OE induced before conditioning to fearful stimulus: No differences in baseline ASR ↓ Startle sensitization and shock reactivity in ASR ↓ FPS, impaired acquisition of associative fear memory CRH OE induced after conditioning to fearful stimulus: ↑ FPS, enhanced fear memory expression	(111)
BLA CRFR1 KD (Lenti-shCRFR1)	CRH-R1 KD in the BLA of adult male mice. Behavioral testing began ~2 weeks after lentiviral injections	↓ Anxiety in the LD and OF tests	(112)
CeA CRF-KD (Lenti-shCRF)	CRH KD in the CeA of adult male mice Behavioral testing began ~2 weeks after lentiviral	↓ Basal anxiety in the EPM test and stress-induced anxiety in LD test No effects on despair in FST or TST No effects on fear conditioning ↑ Basal plasma CORT levels ↓ Ucn3 mRNA in BnST	(108)
GPe CRFR1 KD (Lenti-shCRFR1)	CRH-R1 KD in the GPe of adult male mice. Behavioral testing began ~2 weeks after lentiviral injection	↑ Anxiety in the LD, OF and EPM tests No changes in locomotion ↓ Enkephalin protein in GPe, possible mechanism for increased anxiety	(113)
rPFA-Ucn3 OE (Lenti-rtTA)	Transgenic mice with Ucn3 under the control of a TRE were injected in the rPFA with lentivirus containing rtTA. Ucn3 OE occurs when Dox is administered	↑ Anxiety in the LD and OF tests ↑ Metabolic rate, but has no effect on food intake ↓ Insulin sensitivity	(114)

Abbreviations: Adrenocorticotropic Hormone, ACTH; Acoustic Startle response test, ASR; Bed nucleus of the stria terminalis, BnST; Basolateral nucleus of the amygdala, BLA; Central nucleus of the amygdala, CeA; Corticosterone, CORT; Corticotropin-Releasing Hormone, CRH; Corticotropin-Releasing Factor, CRF; Dorsal Raphe Nucleus, DRN; Doxycycline, Dox; Elevated Plus Maze, EPM; Fear Potentiated Startle, FPS; Forced Swim Test, FST; Globus Pallidus (external), GPe; Hypothalamic-Pituitary-Adrenal, HPA; Knock-down, KD; Dark/Light Preference test, LD; Overexpression, OE; Open field test, OF; Perifornical Area (rostral), rPFA; Tail suspension test, TST; Tetracycline Response Element, TRE; Urocortin, Ucn.

Like the CeA, the BnST is a part of the extended amygdala involved in anxiety and fear mediation. Two studies of CRH-OE in the BnST in rodents, using distinct lentiviral constructs, show no effect on anxiety behavior in the EPM and defensive withdrawal tests (107, 111). Both studies also demonstrate no HPA axis alterations and compensatory reductions in CRH-R1 mRNA in the BnST. Fear potentiated-startle (FPS) is a behavioral paradigm in which acoustic noise (unconditioned stimulus, US) is paired with a foot-shock (conditioned stimulus, CS). Measures of startle response and shock reactivity in response to only the US provide information on acquisition, retention, and expression of fear learning. CRH-OE in the BnST of male rats prior to fear conditioning impairs acquisition of fear, as these rats have attenuated startle and shock reactivity amplitudes (111). However if CRH is overexpressed after fear conditioning, the BnST CRH-OE mice have potentiate fear responses compared to controls, indicating enhanced retention or expression of fear memory. This abnormal enhancement of FPS is observed in individuals with PTSD, and points a potential mechanism in which elevated BnST CRH activity strengthens association of neutral cued with fear memories.

Environmental enrichment (EE), which is known to reduce anxiety, significantly decreases CRH-R1 mRNA in the BLA (112). Lentiviruses containing small interference RNA (siRNA) were generated to knock down CRH-R1 in the BLA to further investigate its role in anxiety. Behavioral data indicates that siRNA knockdown of BLA CRH-R1 decreased anxiety in the OF and LD and a trend in EPM. The authors suggest the BLA as a potential target of therapeutics that mimics the anxiolytic effects of EE and benzodiazepines (112). Comparatively, when this approach was used to knockdown CeA CRH, mice show decreased anxiety in the EPM (108). Acute restraint stress, which was anxiogenic for control mice, had no

effect on CeA CRH-KD mice. Since restraint stress is known to increase CeA CRH and cause anxiety, knockdown of CeA CRH by the viral vector may counteract the effects of stress. This data indicates that CRH-R1 in both the BLA and CeA functions to mediate anxiety responses.

Limbic regions are not the only modulators of CRH activity in anxiety behavior. This is demonstrated in a study that used lentiviral-based siRNA to knock down 60% of CRH-R1 receptors in the external part of the globus pallidus of the striatum (GPe) (113). This increased anxiety in LD, OF and cause a trend towards increased anxiety in the EPM. CRH-R1 antagonist infused into the GPe also increases anxiety in OF and marble burying tests. These data imply that, in contrast to anxiogenic effects of CRH-R1 activation in the amygdala, activation in the GPe has anxiolytic properties. In this study, restraint stress reduced CRH-R1 mRNA in GPe, further verifying the anxiolytic properties of GPe (113).

Lentiviral vectors have also been used to overexpress Ucn3 in the rostral perifornical area (rPFA) of the hypothalamus that projects to neurons in the lateral septum and ventromedial hypothalamus (114). Mice overexpressing Ucn3 in the rPFA display increased anxiety, which is most likely through rPFA Ucn3 acting on CRH-R2 neurons in the lateral septum.

The use of lentiviral vectors also produced the added advantages of infection neurons and activating CRH receptors on neurons that they project to, unlike pharmacological methods that only act locally. Although the use of viral vectors is still ectopic, it provides more physiological relevance that pharmaceutical methods and more spatial relevance than genetically altered rodents alone.

HUMAN GENE POLYMORPHISMS

CRH hyperactivity is present in several psychiatric disorders. For example, CRH levels are elevated in the CSF of some post-traumatic stress disorder (PTSD) and depressed patients (33, 34). Furthermore, this HPA axis dysregulation, which occurs in a subpopulation of individuals who suffer from a psychiatric disorder, is characterized by elevated secretion of ACTH and CORT that occurs in response to CRH hyperactivity. In the remainder of this review, we will discuss studies of previously identified human polymorphisms in the CRH pathway that are associated with the occurrence of psychiatric disorders (Table 1-6.).

CRH

Smoller and colleagues performed one of the first genetic association studies of *CRH* by studying children from families in which the parents were diagnosed with panic disorders (115). They specifically focused on behavioral inhibition in children as a strong predictor of later development of anxiety disorders. They identified an allele linked to the *CRH* locus (173 bp in the dinucleotide repeat marker CRH-PCR1) that negatively associated with behavioral inhibition, particularly in children whose parents had panic disorder. A subsequent study identified 3 single nucleotide polymorphisms (SNPs) in the *CRH* gene, one of which is in the coding region, and a haplotype comprising SNPs that also associated with behavioral inhibition (Table 1-6.) (116). Although the sample sizes in these studies are relatively small and there exist potential for Type I or Type II errors, they demonstrate an important role for the *CRH* gene in mediating anxiety-like behaviors in humans. Thus suggesting that children with these SNPs in the *CRH* gene are less likely to display behavioral

inhibition and thus, are at a decreased risk for developing anxiety disorders later in life.

CRHR1

The most studied SNPs in the *CRHR1*gene are a T rare allele in rs7209436, an A rare allele in rs110402, and a T allele in rs242924, which also form the *CRHR1* T-A-T haplotype. Adults with a history of childhood abuse that were homozygous for either of the rare alleles had the lowest Beck Depression Inventory (BDI) scores for depression, while those homozygous for the common allele had the highest BDI scores, with the heterozygotes having BDI scores in between the two (117). Moreover, the *CRHR1* T-A-T haplotypes were overrepresented in women with a history of childhood abuse that did not have MDD compared to women with no childhood abuse/no major MDD and women with childhood abuse and MDD. There was an additive effect of the rare alleles in this study (117, 118). Analysis of HPA axis activity in individuals with childhood abuse and the common allele SNPs of *CRHR1* showed increased plasma CORT in response to the Dexamethasone/CRH test (119). The potential protective role for the aforementioned SNPs has also been confirmed in males (120, 121). Further examination by Grabe and colleagues however, showed an increased risk rather than protective effects of this T-A-T haplotype (122) (Table 1-6.). Taken together, the majority of data indicate that the rare allele SNPs and haplotypes in *CRHR1* provide protection against the development of depressive symptoms when an individual has experienced early life stress. Thus, it is possible that the identified SNPs are involved in decreasing the activity of *CRHR1* to reduce CRH hyperactivity-driven maladaptive changes. An additional study presented evidence suggestive of a protective role for the T-A-T

haplotype of the *CRHR1 gene.* The authors propose that the T-A-T haplotype may impair memory consolidation of the emotional effects of childhood abuse and thereby reduce risk of depressive symptoms later in life for women (121). This is a likely inference as studies in rodents have demonstrated CRH involvement in memory formation and consolidation (32, 123, 124). Further investigation of the rs110402 SNP has shown that T common allele carriers have a younger age-of-onset for their first depressive episode and experience more seasonal-related episodes of depression (125). Functional magnetic resonance imaging (fMRI) data has demonstrated the MDD patients with the G allele in the rs110402 SNP have alterations in brain activity consistent with increased vulnerability to depression (16). The use of fMRI in investigating the functional effects of CRHR1 polymorphisms provides for greater avenues of understanding how the CRH signaling pathways affect the generation of psychiatric disorders.

In a depressed population of Mexican Americans, a G-A-G haplotype from SNPs rs1876828, rs242939, and rs242941 in the *CRHR1* gene increases treatment response to antidepressants, fluoxetine and desipramine (126). Two studies have shown that in a group of Chinese patients, a G-G-T haplotype for the same SNPs increases the risk of developing major depression (127, 128). These studies demonstrate that genetic risk for depression depends on factors such as ethnicity and possibly geographic location. This substantiates the need to evaluate the interaction of genes and environment in studies of human diseases.

Stress is associated with an increased risk of substance abuse. A sample of adolescents that had experienced a number of stressful life events and had common variants in *CRHR1* (CC rs187831 or A rs242938) demonstrated increased binge drinking (72) and increased risk of alcohol drinking behavior (129). A subsequent

independent analysis demonstrated that the more stressful life events experienced in adolescents homozygous for CC rs187831, the lower the age of onset for drinking (130). Furthermore, adolescent stress with either genotype (CC rs187831 or A carrier rs242938) increased the risk for young adult alcohol drinking. In addition, CRH gene variations that differ in corticosteroid sensitivity were found to influence the risk for alcohol use and dependence in rhesus macaques (131). Briefly, animals were genotyped for a single-nucleotide polymorphism disrupting a glucocorticoid response element, rh*CRH* - 2232 C > G. The effects of this allele on CSF levels of CRH, behavior and ethanol consumption were measured. Macaques carrying the G allele had lower CSF levels of CRH. Infant macaques carrying the G allele were more exploratory and bold, and in adolescent and adult male macaques, the G allele was associated with increased exploratory/bold behavior when responding to an unfamiliar male. Adults with the C/G genotype also exhibited increased alcohol consumption.

These studies indicate that there exists a gene x environment interaction for risk of alcohol abuse, as studies that examined the effect of the SNPs alone found no association (132, 133). Analysis of a different CRHR1 SNP (rs110402) in schizophrenia patients shows that the T allele interacts with a G allele in a CRHBP SNP (rs3811939) to double the risk of comorbid alcoholism in these patients. Individuals with these SNPs also show a higher CRH-R1/CRH-BP mRNA ratio, indicating an increase in CRH reactivity as the plausible cause (134). Rodent studies have demonstrated that in alcohol preferring (msP) rats, there is an increase in CRH-R1 transcripts in the brain and an increased risk to reinstate alcohol-seeking after a foot-shock stressor (71). These studies indicate that the human SNPs described may function to increase CRH activity mediated through CRH-R1, to increase stress-

induced alcohol consumption. There is also evidence that low stress exposure increases suicidality in individuals with a rs4792887 (TT) genotype in the *CRHR1* gene (135). Studying the interaction of stress and polymorphisms in CRH pathway genes will enable better insight into the ability of stress to precipitate psychiatric diseases.

CRHR2

In depressed individuals, carriers of the G allele of the rs2270007 SNP in *CRHR2* were slower to respond to antidepressant (citalopram) treatment at 4 weeks post-treatment (125) indicating a potential role for this SNP in altering HPA axis homeostasis and as a predictor of antidepressant treatment response.

A separate study provided evidence for an association between *CRHR2* SNPs and increased suicidal behavior in people with bipolar disorder (136).

CRHBP

The CRH binding protein sequesters CRH thereby preventing it from binding its receptors. Thus, reduced CRH-BP activity may cause CRH hyperactivity. The polymorphisms discussed below point to a decrease in expression or activity of CRHBP as the reason for the associated disorders. Patients with MDD that have a rs10473984 (TT) genotype in *CRHBP* show increase HPA axis activity, decreased response to antidepressant treatment, and an increased chance of relapse (35). SNPs in *CRHBP* have also been associated with vulnerability for unipolar depression (137) and stress-induced alcohol craving (Table 1-6.) (138). Interactions between CRHBP and CRHR1 polymorphism have been observed to increase suicide attempts (139) and alcoholism (134) in schizophrenic patients (Table 1-6.). Hyperactivation of CRH

signaling pathways is certainly capable of exerting these effects based on the data reviewed in animal and human studies.

Table 1-6. Human gene variants in CRH pathways associated with psychiatric disorders

GENE	Polymorphism	Associated Disorder	Effect	REF.
CRH	173 bp in the dinucleotide repeat marker CRH-PCR1 rs6999100 (CC) rs6159 (GG) rs1870393 (CC)	Behavioral inhibition	Risk promoting	(115,116)
CRHR1	rs7209436 (TT) rs110402 ((AA) rs242924 (TT) T-A-T haplotype	Depression with adverse early life experiences	Protective	(117–121)
CRHR1	T-A-T haplotype rs17689882 rs16940674 rs16940665	Depression with childhood physical neglect	Risk promoting	(122)
CRHR1	rs110402 (TT)	Depression onset and seasonal episodes	Risk promoting	(125)
CRHR1	rs110402 (GG)	Depression vulnerability	Risk promoting	(16)
CRHR1	G-G-T haplotype rs1876828 (GG) rs242939 (GG) rs242941 (TT)	Genetic susceptibility to major depression and response to antidepressant treatment	Risk promoting	(126–128)
CRHR1	rs1876831 (CC) rs242938 (A)	Alcohol consumption with life stress	Risk promoting	(72,129,130)
CRHR1	rs4792887 (TT)	Suicidality after low stress exposure	Risk promoting	(135)
CRHR2	rs2270007 (GG)	Decreased response to antidepressant treatment in depressed patients	Risk promoting	(125)
CRHR2	5-2-3 haplotype (allele 3 in GT)	Suicidal behavior in bipolar disorder	Risk promoting	(136)
CRHBP	rs10473984 (TT)	Remission and decreased depressive symptoms with citalopram treatment	Risk promoting	(35)
CRHBP	rs1875999 (TT)	Unipolar depression	Risk promoting	(137)
CRHBP	rs10055255 (TT)	Stress-induced alcohol craving and negative mood	Risk promoting	(138)
CRHBP *CRHR1*	rs3811939 (GG) rs110402 (TT)	Comorbid alcoholism in Schizophrenic patients	Risk promoting	(134)
CRHBP *CRHR1*	rs1875999 rs169400665	Suicidal behavior in Schizophrenia	Risk promoting	(139)

The Potential of CRH-Pathways Genetic Studies

CRH plays a critical role in adaptation to stress (140). Finely regulated CRH expression and sensitivity is required for proper function and homeostasis of the HPA axis (141). Chronic stress and early-life trauma can result in permanent disruptions in the CRH system and may lead to psychopathology in adulthood. The data summarized in Section 4 begins to lend support to the notion that genetic variation in CRH-related pathways contributes to psychiatric disorders in humans, and could serve as a potential target for therapeutic intervention. As each of these psychiatric diagnoses is likely to reflect divergent pathogenic mechanisms, the ability to personalize therapies based upon established risk-altering genotypes would be particularly valuable.

TARGETING THE CRH PATHWAY FOR THERAPY

The studies reviewed here demonstrate that abnormal CRH function may dysregulate HPA axis activity and produce maladaptive behavioral responses. Animal studies with CRH antagonists have shown both anxiolytic and antidepressant effects. Additionally, antidepressant treatment in depressed patients that normalizes HPA activity decreases the likelihood of relapse. In humans, CRH antagonists have been used in clinical trials to evaluate efficacy in treatment of MDD. A clinical trial in 20 patients with MDD demonstrated that the CRH-R1 antagonist, R121919, is able to decrease anxiety and despair in multiple inventories (142). When the patients ceased taking R121919, their symptoms returned. Furthermore, this drug was safely tolerated by the patients, improved sleep (143) and did not impair normal brain activity, heart conductance, or HPA axis activity (144). High doses of R121919 in control

individuals elevated liver enzymes and resulted in discontinued production. Currently, only one other CRH receptor antagonist is undergoing clinical trials to evaluate efficacy in treating anxiety disorders ([clinicaltrials.gov;NCT01018992]). It should be noted that other placebo-controlled trials with CRHR1 antagonists have failed to show efficacy in the treatment of generalized anxiety disorder (145) or major depression (146). The CRB-BP also served as a potential target for therapy, but much more research in needed to evaluate how it can be effectively targeted to reduce or elevate CRH activity depending on the disorder.

CONCLUSIONS

The summarized data presented in this chapter provide important insights into a role for CRH in mediating the maladaptive behaviors associated with major mood disorders and demonstrate the complexity of the underlying neurocircuitries involved in mediating CRH activity. In addition, these studies implicate components of the CRH system as potential therapeutic targets for interventions aimed at treating the negative behavior sequelae of mood disorders.

The CRH-CRH receptor-signaling pathway provides a consistent and robust series of translational studies linking basic neuroscience investigation in model systems to human psychiatric diseases. Initial behavioral and physiological experiments in rodents have reliably predicted that human variation in the CRH-CRH receptor and CORT pathways would influence the risk of neuropsychiatric disorders. In the future, these variants may serve as potential predictors of risk for the development of psychiatric illnesses and forecast individual responses to particular drug based treatments.

REFERENCES

1. Vale W, Spiess J, Rivier C, Rivier J. 1981. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394–7.

2. Lewis K, Li C, Perrin MH, Blount a, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW. 2001. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc. Natl. Acad. Sci. U. S. A. 98:7570–5.

3. Brar B, Sanderson T, Wang N, Lowry PJ. 1997. Post-translational processing of human procorticotrophin-releasing factor in transfected mouse neuroblastoma and Chinese hamster ovary cell lines. J. Endocrinol. 154:431–40.

4. Grigoriadis DE, Lovenberg TW, Chalmers DT, Liaw C, De Souze EB. 1996. Characterization of corticotropin-releasing factor receptor subtypes. Ann. N. Y. Acad. Sci. 780:60–80.

5. Muglia LJ, Jacobson L, Luedke C, Vogt SK, Schaefer ML, Dikkes P, Fukuda S, Sakai Y, Suda T, Majzoub JA. 2000. Corticotropin-releasing hormone links pituitary adrenocorticotropin gene expression and release during adrenal insufficiency. J. Clin. Invest. 105:1269–77.

6. Muller MB. 2000. Selective Activation of the Hypothalamic Vasopressinergic System in Mice Deficient for the Corticotropin-Releasing Hormone Receptor 1 Is Dependent on Glucocorticoids. Endocrinology 141:4262–4269.

7. Preil J, Müller MB, Gesing a, Reul JM, Sillaber I, van Gaalen MM, Landgrebe J, Holsboer F, Stenzel-Poore M, Wurst W. 2001. Regulation of the hypothalamic-pituitary-adrenocortical system in mice deficient for CRH receptors 1 and 2. Endocrinology 142:4946–55.

8. Surget A, Belzung C. 2008. Involvement of vasopressin in affective disorders. Eur. J. Pharmacol. 583:340–9.

9. Bao A-M, Swaab DF. 2010. Corticotropin-releasing hormone and arginine vasopressin in depression focus on the human postmortem hypothalamus. Vitam. Horm. 82:339–65.

10. Chalmers DT, Lovenberg TW, De Souza EB. 1995. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J. Neurosci. 15:6340–50.

11. Reul J. 2002. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr. Opin. Pharmacol. 2:23–33.

12. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias C a, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE. 2001. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc. Natl. Acad. Sci. U. S. A. 98:2843–8.

13. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull A V, Lovejoy D, Rivier C. 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378:287–92.

14. Kostich WA, Grzanna R, Lu NZ, Largent BL. 2004. Immunohistochemical visualization of corticotropin-releasing factor type 1 (CRF1) receptors in monkey brain. J. Comp. Neurol. 478:111–25.

15. Sánchez MM, Young LJ, Plotsky PM, Insel TR. 1999. Autoradiographic and in situ hybridization localization of corticotropin-releasing factor 1 and 2 receptors in nonhuman primate brain. J. Comp. Neurol. 408:365–77.

16. Hsu DT, Mickey BJ, Langenecker SA, Heitzeg MM, Love TM, Wang H, Kennedy SE, Peciña M, Shafir T, Hodgkinson CA, Enoch M-A, Goldman D, Zubieta J-K. 2012. Variation in the Corticotropin-Releasing Hormone Receptor 1 (CRHR1) Gene Influences fMRI Signal Responses during Emotional Stimulus Processing. J. Neurosci. 32:3253–60.

17. Hiroi N, Wong ML, Licinio J, Park C, Young M, Gold PW, Chrousos GP, Bornstein SR. 2001. Expression of corticotropin releasing hormone receptors type I and type II mRNA in suicide victims and controls. Mol. Psychiatry 6:540–6.

18. Chen R, Lewis K a, Perrin MH, Vale WW. 1993. Expression cloning of a human corticotropin-releasing-factor receptor. Proc. Natl. Acad. Sci. U. S. A. 90:8967–71.

19. Pisarchik A, Slominski AT. 2001. Alternative splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression. FASEB J. 15:2754–6.

20. Perrin M, Donaldson C, Chen R, Blount a, Berggren T, Bilezikjian L, Sawchenko P, Vale W. 1995. Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. Proc. Natl. Acad. Sci. U. S. A. 92:2969–73.

21. Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, De Souza EB, Oltersdorf T. 1995. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. Proc. Natl. Acad. Sci. U. S. A. 92:836–40.

22. Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, Vogl AM, Dedic N, Schumacher M, von Wolff G, Avrabos C, Touma C, Engblom D,

Schütz G, Nave K-A, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM. 2011. Glutamatergic and Dopaminergic Neurons Mediate Anxiogenic and Anxiolytic Effects of CRHR1. Science 333:1903–1907.

23. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. 1995. A placental clock controlling the length of human pregnancy. Nat. Med. 1:460–463.

24. Behan DP, De Souza EB, Lowry PJ, Potter E, Sawchenko P, Vale WW. 1995. Corticotropin releasing factor (CRF) binding protein: a novel regulator of CRF and related peptides. Front. Neuroendocrinol. 16:362–82.

25. Orth DN, Mount CD. 1987. Specific high-affinity binding protein for human corticotropin-releasing hormone in normal human plasma. Biochem. Biophys. Res. Commun. 143:411–417.

26. Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, Vale WW. 1991. Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. Nature 349:423–6.

27. Seasholtz a F, Burrows HL, Karolyi IJ, Camper S a. 2001. Mouse models of altered CRH-binding protein expression. Peptides 22:743–51.

28. Potter E, Behan DP, Linton EA, Lowry PJ, Sawchenko PE, Vale WW. 1992. The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. Proc. Natl. Acad. Sci. U. S. A. 89:4192–6.

29. Behan DP, Khongsaly O, Ling N, De Souza EB. 1996. Urocortin interaction with corticotropin-releasing factor (CRF) binding protein (CRF-BP): a novel mechanism for elevating "free' CRF levels in human brain. Brain Res. 725:263–7.

30. Gallagher JP, Orozco-Cabal LF, Liu J, Shinnick-Gallagher P. 2008. Synaptic physiology of central CRH system. Eur. J. Pharmacol. 583:215–25.

31. Liu J, Yu B, Neugebauer V, Grigoriadis DE, Rivier J, Vale WW, Shinnick-Gallagher P, Gallagher JP. 2004. Corticotropin-releasing factor and Urocortin I modulate excitatory glutamatergic synaptic transmission. J. Neurosci. 24:4020–9.

32. Blank T, Nijholt I, Eckart K, Spiess J. 2002. Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. J. Neurosci. 22:3788–94.

33. Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W. 1984. Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science (80-.). 226:1342–1344.

34. Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS. 1997. Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154:624–629.

35. Binder EB, Owens MJ, Liu W, Deveau TC, Rush a J, Trivedi MH, Fava M, Bradley B, Ressler KJ, Nemeroff CB. 2010. Association of polymorphisms in genes regulating the corticotropin-releasing factor system with antidepressant treatment response. Arch. Gen. Psychiatry 67:369–79.

36. Mitchell A. 1998. The role of Corticotropin Releasing Factor in Depressive Illness: a Critical Review. Neurosci. Biobehav. Rev. 22:635–651.

37. Bonne O, Gill JM, Luckenbaugh DA, Collins C, Owens MJ, Alesci S, Neumeister A, Yuan P, Kinkead B, Manji HK, Charney DS, Vythilingam M. 2011. Corticotropin-releasing factor, interleukin-6, brain-derived neurotrophic factor, insulin-like growth factor-1, and substance P in the cerebrospinal fluid of civilians with posttraumatic stress disorder before and after treatment with paroxetine. J. Clin. Psychiatry 72:1124–8.

38. Imaki T, Shibasaki T, Hotta M, Demura H. 1993. Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress. Brain Res. 616:114–25.

39. Campbell BM, Morrison JL, Walker EL, Merchant KM. 2004. Differential regulation of behavioral, genomic, and neuroendocrine responses by CRF infusions in rats. Pharmacol. Biochem. Behav. 77:447–55.

40. Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W. 1982. Corticotropin releasing factor produces behavioural activation in rats. Nature 297:331–333.

41. Dunn A. 1990. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res. Rev. 15:71–100.

42. Rivest S, Deshaies Y, Richard D. 1989. Effects of corticotropin-releasing factor on energy balance in rats are sex dependent. Am. J. Physiol. 257:R1417–22.

43. Glowa JR, Barrett JE, Russell J, Gold PW. 1992. Effects of corticotropin releasing hormone on appetitive behaviors. Peptides 13:609–21.

44. Sherman JE, Kalin NH. 1988. ICV-CRH alters stress-induced freezing behavior without affecting pain sensitivity. Pharmacol. Biochem. Behav. 30:801–7.

45. Britton KT, Lee G, Vale W, Rivier J, Koob GF. 1986. Corticotropin releasing factor (CRF) receptor antagonist blocks activating and “anxiogenic” actions of CRF in the rat. Brain Res. 369:303–6.

46. Heinrichs SC, Pich EM, Miczek K a, Britton KT, Koob GF. 1992. Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. Brain Res. 581:190–7.

47. Kalin NH, Sherman JE, Takahashi LK. 1988. Antagonism of endogenous CRH systems attenuates stress-induced freezing behavior in rats. Brain Res. 457:130–5.

48. Heinrichs S., Lapsansky J, Lovenberg T., De Souza E., Chalmers D. 1997. Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. Regul. Pept. 71:15–21.

49. Liebsch G, Landgraf R, Engelmann M, Lörscher P, Holsboer F. 1999. Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. J. Psychiatr. Res. 33:153–163.

50. Bagosi Z, Jászberényi M, Szabó G, Telegdy G. 2008. The effects of CRF and the urocortins on [3H]GABA release from the rat amygdala--an in vitro superfusion study. Brain Res. Bull. 75:15–7.

51. Bagosi Z, Csabafi K, Jászberényi M, Telegdy G. 2012. The effects of corticotropin-releasing factor and the urocortins on hypothalamic gamma-amino butyric acid release--the impacts on the hypothalamic-pituitary-adrenal axis. Neurochem. Int. 60:350–4.

52. Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A. 2004. Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. J. Neurosci. 24:3471–9.

53. Valdez GR, Inoue K, Koob GF, Rivier J, Vale W, Zorrilla EP. 2002. Human urocortin II: mild locomotor suppressive and delayed anxiolytic-like effects of a novel corticotropin-releasing factor related peptide. Brain Res. 943:142–50.

54. Valdez GR, Zorrilla EP, Rivier J, Vale WW, Koob GF. 2003. Locomotor suppressive and anxiolytic-like effects of urocortin 3, a highly selective type 2 corticotropin-releasing factor agonist. Brain Res. 980:206–12.

55. Pelleymounter MA, Joppa M, Ling N, Foster AC. 2004. Behavioral and neuroendocrine effects of the selective CRF2 receptor agonists urocortin II and urocortin III. Peptides 25:659–66.

56. Chen P, Vaughan J, Donaldson C, Vale W, Li C. 2010. Injection of Urocortin 3 into the ventromedial hypothalamus modulates feeding, blood glucose levels, and hypothalamic POMC gene expression but not the HPA axis. Am. J. Physiol. Endocrinol. Metab. 298:E337–45.

57. Muglia L, Jacobsen L, Majzoub JA. 1996. Production of Corticotropin-releasing Hormone-deficient Mice by Targeted Mutation in Embryonic Stem Cells. Ann. N. Y. Acad. Sci. 780:49–59.

58. Muglia L, Jacobson L, Dikkes P, Majzoub JA. 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373:427–32.

59. Weninger SC. 1999. Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. Proc. Natl. Acad. Sci. 96:8283–8288.

60. Dunn AJ, Swiergiel AH. 1999. Behavioral responses to stress are intact in CRF-deficient mice. Brain Res. 845:14–20.

61. Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale W. 1992. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. Endocrinology 130:3378–3386.

62. Dirks A, Groenink L, Schipholt MI, van der Gugten J, Hijzen TH, Geyer M a, Olivier B. 2002. Reduced startle reactivity and plasticity in transgenic mice overexpressing corticotropin-releasing hormone. Biol. Psychiatry 51:583–90.

63. Lu a, Steiner M a, Whittle N, Vogl a M, Walser SM, Ableitner M, Refojo D, Ekker M, Rubenstein JL, Stalla GK, Singewald N, Holsboer F, Wotjak CT, Wurst W, Deussing JM. 2008. Conditional mouse mutants highlight mechanisms of corticotropin-releasing hormone effects on stress-coping behavior. Mol. Psychiatry 13:1028–42.

64. Dedic N, Touma C, Romanowski CP, Schieven M, Kühne C, Ableitner M, Lu A, Holsboer F, Wurst W, Kimura M, Deussing JM. 2011. Assessing Behavioural Effects of Chronic HPA Axis Activation Using Conditional CRH-Overexpressing Mice. Cell. Mol. Neurobiol.

65. Groenink L, Dirks A, Verdouw PM, Schipholt ML, Veening JG, van der Gugten J, Olivier B. 2002. HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. Biol. Psychiatry 51:875–81.

66. Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. 1994. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J. Neurosci. 14:2579–2584.

67. Van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T. 2002. Effects of transgenic overproduction of CRH on anxiety-like behaviour. Eur. J. Neurosci. 15:2007–2015.

68. Heinrichs SC, Min H, Tamraz S, Carmouché M, Boehme SA, Vale WW. 1997. Anti-sexual and anxiogenic behavioral consequences of corticotropin-releasing factor overexpression are centrally mediated. Psychoneuroendocrinology 22:215–224.

69. Dirks A, Groenink L, Bouwknecht JA, Hijzen TH, van der Gugten J, Ronken E, Verbeek JS, Veening JG, Dederen PJWC, Korosi A, Schoolderman LF, Roubos EW, Olivier B. 2002. Overexpression of corticotropin-releasing

hormone in transgenic mice and chronic stress-like autonomic and physiological alterations. Eur. J. Neurosci. 16:1751–1760.

70. Palmer A a, Sharpe AL, Burkhart-Kasch S, McKinnon CS, Coste SC, Stenzel-Poore MP, Phillips TJ. 2004. Corticotropin-releasing factor overexpression decreases ethanol drinking and increases sensitivity to the sedative effects of ethanol. Psychopharmacology (Berl). 176:386–97.

71. Hansson AC, Cippitelli A, Sommer WH, Fedeli A, Björk K, Soverchia L, Terasmaa A, Massi M, Heilig M, Ciccocioppo R. 2006. Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. Proc. Natl. Acad. Sci. U. S. A. 103:15236–41.

72. Treutlein J, Kissling C, Frank J, Wiemann S, Dong L, Depner M, Saam C, Lascorz J, Soyka M, Preuss UW, Rujescu D, Skowronek MH, Rietschel M, Spanagel R, Heinz A, Laucht M, Mann K, Schumann G. 2006. Genetic association of the human corticotropin releasing hormone receptor 1 (CRHR1) with binge drinking and alcohol intake patterns in two independent samples. Mol. Psychiatry 11:594–602.

73. Heilig M, Koob GF. 2007. A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci. 30:399–406.

74. Vinkers CH, Hendriksen H, van Oorschot R, Cook JM, Rallipalli S, Huang S, Millan MJ, Olivier B, Groenink L. 2012. Lifelong CRF overproduction is associated with altered gene expression and sensitivity of discrete GABA(A) and mGlu receptor subtypes. Psychopharmacology (Berl). 219:897–908.

75. Wang X-D, Rammes G, Kraev I, Wolf M, Liebl C, Scharf SH, Rice CJ, Wurst W, Holsboer F, Deussing JM, Baram TZ, Stewart MG, Müller MB, Schmidt M V. 2011. Forebrain CRF_1 modulates early-life stress-programmed cognitive deficits. J. Neurosci. 31:13625–34.

76. Kimura M, Müller-Preuss P, Lu a, Wiesner E, Flachskamm C, Wurst W, Holsboer F, Deussing JM. 2010. Conditional corticotropin-releasing hormone overexpression in the mouse forebrain enhances rapid eye movement sleep. Mol. Psychiatry 15:154–65.

77. Kolber BJ, Boyle MP, Wieczorek L, Kelley CL, Onwuzurike CC, Nettles S a, Vogt SK, Muglia LJ. 2010. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. J. Neurosci. 30:2571–81.

78. Vicentini E, Arban R, Angelici O, Maraia G, Perico M, Mugnaini M, Ugolini A, Large C, Domenici E, Gerrard P, Bortner D, Mansuy IM, Mangiarini L, Merlo-Pich E. 2009. Transient forebrain over-expression of CRF induces plasma corticosterone and mild behavioural changes in adult conditional CRF transgenic mice. Pharmacol. Biochem. Behav. 93:17–24.

79. Smith GW, Aubry JM, Dellu F, Contarino a, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley C a, Sawchenko PE, Koob GF, Vale W, Lee KF. 1998. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 20:1093–102.

80. Contarino A, Dellu F, Koob GF, Smith GW, Lee KF, Vale W, Gold LH. 1999. Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. Brain Res. 835:1–9.

81. Timpl P, Spanagel R, Sillaber I, Kresse a, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W. 1998. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat. Genet. 19:162–6.

82. Müller MB, Zimmermann S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P, Kormann MSD, Droste SK, Kühn R, Reul JMHM, Holsboer F, Wurst W. 2003. Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat. Neurosci. 6:1100–7.

83. Nguyen NK, Keck ME, Hetzenauer A, Thoeringer CK, Wurst W, Deussing JM, Holsboer F, Müller MB, Singewald N. 2006. Conditional CRF receptor 1 knockout mice show altered neuronal activation pattern to mild anxiogenic challenge. Psychopharmacology (Berl). 188:374–85.

84. Wang X-D, Chen Y, Wolf M, Wagner K V, Liebl C, Scharf SH, Harbich D, Mayer B, Wurst W, Holsboer F, Deussing JM, Baram TZ, Müller MB, Schmidt M V. 2011. Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. Neurobiol. Dis. 42:300–10.

85. Molander A, Vengeliene V, Heilig M, Wurst W, Deussing JM, Spanagel R. 2012. Brain-specific inactivation of the Crhr1 gene inhibits post-dependent and stress-induced alcohol intake, but does not affect relapse-like drinking. Neuropsychopharmacology 37:1047–56.

86. Schmidt M V, Deussing JM, Oitzl MS, Ohl F, Levine S, Wurst W, Holsboer F, Müller MB, de Kloet ER. 2006. Differential disinhibition of the neonatal hypothalamic- pituitary-adrenal axis in brain-specific CRH receptor 1-knockout mice. Eur. J. Neurosci. 24:2291–8.

87. Schmidt M V, Oitzl MS, Müller MB, Ohl F, Wurst W, Holsboer F, Levine S, De Kloet ER. 2003. Regulation of the developing hypothalamic-pituitary-adrenal axis in corticotropin releasing hormone receptor 1-deficient mice. Neuroscience 119:589–95.

88. Müller MB, Preil J, Renner U, Zimmermann S, Kresse AE, Stalla GK, Keck ME, Holsboer F, Wurst W. 2001. Expression of CRHR1 and CRHR2 in mouse pituitary and adrenal gland: implications for HPA system regulation. Endocrinology 142:4150–3.

89. Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, Hollis JH, Murray SE, Hill JK, Pantely GA, Hohimer AR, Hatton DC, Phillips TJ, Finn DA, Low MJ, Rittenberg MB, Stenzel P, Stenzel-Poore MP. 2000. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat. Genet. 24:403–9.

90. Bale TL, Contarino a, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF. 2000. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat. Genet. 24:410–4.

91. Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J. 2000. Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat. Genet. 24:415–9.

92. Todorovic C, Sherrin T, Pitts M, Hippel C, Rayner M, Spiess J. 2009. Suppression of the MEK/ERK signaling pathway reverses depression-like behaviors of CRF2-deficient mice. Neuropsychopharmacology 34:1416–26.

93. Bale TL, Vale WW. 2003. Increased depression-like behaviors in corticotropin-releasing factor receptor-2-deficient mice: sexually dichotomous responses. J. Neurosci. 23:5295–301.

94. Gresack J, Powell S, Geyer M, Poore M-S, Coste S, Risbrough V. 2010. CRF2 null mutation increases sensitivity to isolation rearing effects on locomotor activity in mice. Neuropeptides 44:349–53.

95. Bale TL, Picetti R, Contarino A, Koob GF, Vale WW, Lee K-F. 2002. Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior. J. Neurosci. 22:193–9.

96. Gysling K, Forray MI, Haeger P, Daza C, Rojas R. 2004. Corticotropin-releasing hormone and urocortin: redundant or distinctive functions? Brain Res. Brain Res. Rev. 47:116–25.

97. Wang X, Su H, Copenhagen LD, Vaishnav S, Pieri F, Shope C Do, Brownell WE, De Biasi M, Paylor R, Bradley A. 2002. Urocortin-deficient mice display normal stress-induced anxiety behavior and autonomic control but an impaired acoustic startle response. Mol. Cell. Biol. 22:6605–10.

98. Vetter DE, Li C, Zhao L, Contarino A, Liberman MC, Smith GW, Marchuk Y, Koob GF, Heinemann SF, Vale W, Lee K-F. 2002. Urocortin-deficient mice show hearing impairment and increased anxiety-like behavior. Nat. Genet. 31:363–9.

99. Giardino WJ, Cocking DL, Kaur S, Cunningham CL, Ryabinin AE. 2011. Urocortin-1 within the centrally-projecting Edinger-Westphal nucleus is critical for ethanol preference. PLoS One 6:e26997.

100. Chen A, Zorrilla E, Smith S, Rousso D, Levy C, Vaughan J, Donaldson C, Roberts A, Lee K-F, Vale W. 2006. Urocortin 2-deficient mice exhibit gender-specific alterations in circadian hypothalamus-pituitary-adrenal axis and depressive-like behavior. J. Neurosci. 26:5500–10.

101. Neufeld-Cohen A, Evans AK, Getselter D, Spyroglou A, Hill A, Gil S, Tsoory M, Beuschlein F, Lowry CA, Vale W, Chen A. 2010. Urocortin-1 and -2 double-deficient mice show robust anxiolytic phenotype and modified serotonergic activity in anxiety circuits. Mol. Psychiatry 15:426–41, 339.

102. Deussing JM, Breu J, Kühne C, Kallnik M, Bunck M, Glasl L, Yen Y-C, Schmidt M V, Zurmühlen R, Vogl AM, Gailus-Durner V, Fuchs H, Hölter SM, Wotjak CT, Landgraf R, de Angelis MH, Holsboer F, Wurst W. 2010. Urocortin 3 modulates social discrimination abilities via corticotropin-releasing hormone receptor type 2. J. Neurosci. 30:9103–16.

103. Neufeld-cohen A, Tsoory MM, Evans AK, Getselter D, Gil S. 2010. A triple urocortin knockout mouse model reveals an essential role for urocortins in stress recovery. PNAS.

104. Lovejoy D a, Aubry JM, Turnbull a, Sutton S, Potter E, Yehling J, Rivier C, Vale WW. 1998. Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain. J. Neuroendocrinol. 10:483–91.

105. Burrows HL, Nakajima M, Lesh JS, Goosens KA, Samuelson LC, Inui A, Camper SA, Seasholtz AF. 1998. Excess corticotropin releasing hormone-binding protein in the hypothalamic-pituitary-adrenal axis in transgenic mice. J. Clin. Invest. 101:1439–47.

106. Karolyi IJ, Burrows HL, Ramesh TM, Nakajima M, Lesh JS, Seong E, Camper SA, Seasholtz AF. 1999. Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 96:11595–600.

107. Regev L, Neufeld-Cohen A, Tsoory M, Kuperman Y, Getselter D, Gil S, Chen A. 2010. Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation. Mol. Psychiatry 16:714–728.

108. Regev L, Tsoory M, Gil S, Chen A. 2012. Site-specific genetic manipulation of amygdala corticotropin-releasing factor reveals its imperative role in mediating behavioral response to challenge. Biol. Psychiatry 71:317–26.

109. Flandreau EI, Ressler KJ, Owens MJ, Nemeroff CB. 2012. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. Psychoneuroendocrinology 37:27–38.

110. Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME. 2009. Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol. Psychiatry 14:37–50.

111. Sink KS, Walker DL, Freeman SM, Flandreau EI, Ressler KJ, Davis M. 2012. Effects of continuously enhanced corticotropin releasing factor expression within the bed nucleus of the stria terminalis on conditioned and unconditioned anxiety. Mol. Psychiatry.

112. Sztainberg Y, Kuperman Y, Tsoory M, Lebow M, Chen A. 2010. The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1. Mol. Psychiatry 15:905–17.

113. Sztainberg Y, Kuperman Y, Justice N, Chen A. 2011. An anxiolytic role for CRF receptor type 1 in the globus pallidus. J. Neurosci. 31:17416–24.

114. Kuperman Y, Issler O, Regev L, Musseri I, Navon I, Neufeld-Cohen A, Gil S, Chen A. 2010. Perifornical Urocortin-3 mediates the link between stress-induced anxiety and energy homeostasis. Proc. Natl. Acad. Sci. U. S. A. 107:8393–8.

115. Smoller JW, Rosenbaum JF, Biederman J, Kennedy J, Dai D, Racette SR, Laird NM, Kagan J, Snidman N, Hirshfeld-Becker D. 2003. Association of a genetic marker at the corticotropin-releasing hormone locus with behavioral inhibition. Biol. Psychiatry 54:1376–1381.

116. Smoller JW, Yamaki LH, Fagerness J a, Biederman J, Racette S, Laird NM, Kagan J, Snidman N, Faraone S V, Hirshfeld-Becker D, Tsuang MT, Slaugenhaupt S a, Rosenbaum JF, Sklar PB. 2005. The corticotropin-releasing hormone gene and behavioral inhibition in children at risk for panic disorder. Biol. Psychiatry 57:1485–92.

117. Bradley RG, Binder EB, Epstein MP, Tang Y, Nair HP, Liu W, Gillespie CF, Berg T, Evces M, Newport DJ, Stowe ZN, Heim CM, Nemeroff CB, Schwartz A, Cubells JF, Ressler KJ. 2008. Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. Arch. Gen. Psychiatry 65:190–200.

118. Kranzler HR, Feinn R, Nelson EC, Covault J, Anton RF, Farrer L, Gelernter J. 2011. A CRHR1 haplotype moderates the effect of adverse childhood experiences on lifetime risk of major depressive episode in African-American women. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 156B:960–8.

119. Tyrka AR, Price LH, Gelernter J, Schepker C, Anderson GM, Carpenter LL. 2009. Interaction of childhood maltreatment with the corticotropin-releasing hormone receptor gene: effects on hypothalamic-pituitary-adrenal axis reactivity. Biol. Psychiatry 66:681–5.

120. Heim C, Bradley B, Mletzko TC, Deveau TC, Musselman DL, Nemeroff CB, Ressler KJ, Binder EB. 2009. Effect of Childhood Trauma on Adult Depression and Neuroendocrine Function: Sex-Specific Moderation by CRH Receptor 1 Gene. Front. Behav. Neurosci. 3:41.

121. Polanczyk G, Caspi A, Williams B, Price TS, Danese A, Sugden K, Uher R, Poulton R, Moffitt TE. 2009. Protective effect of CRHR1 gene variants on the development of adult depression following childhood maltreatment: replication and extension. Arch. Gen. Psychiatry 66:978–85.

122. Grabe HJ, Schwahn C, Appel K, Mahler J, Schulz A, Spitzer C, Fenske K, Barnow S, Lucht M, Freyberger HJ, John U, Teumer A, Wallaschofski H, Nauck M, Völzke H. 2010. Childhood maltreatment, the corticotropin-releasing hormone receptor gene and adult depression in the general population. Am. J. Med. Genet. B. Neuropsychiatr. Genet. 153B:1483–93.

123. Pitts MW, Todorovic C, Blank T, Takahashi LK. 2009. The central nucleus of the amygdala and corticotropin-releasing factor: insights into contextual fear memory. J. Neurosci. 29:7379–88.

124. Roozendaal B, Schelling G, McGaugh JL. 2008. Corticotropin-releasing factor in the basolateral amygdala enhances memory consolidation via an interaction with the beta-adrenoceptor-cAMP pathway: dependence on glucocorticoid receptor activation. J. Neurosci. 28:6642–51.

125. Papiol S, Arias B, Gastó C, Gutiérrez B, Catalán R, Fañanás L. 2007. Genetic variability at HPA axis in major depression and clinical response to antidepressant treatment. J. Affect. Disord. 104:83–90.

126. Licinio J, O'Kirwan F, Irizarry K, Merriman B, Thakur S, Jepson R, Lake S, Tantisira KG, Weiss ST, Wong M-L. 2004. Association of a corticotropin-releasing hormone receptor 1 haplotype and antidepressant treatment response in Mexican-Americans. Mol. Psychiatry 9:1075–82.

127. Liu Z, Zhu F, Wang G, Xiao Z, Tang J, Liu W, Wang H, Liu H, Wang X, Wu Y, Cao Z, Li W. 2007. Association study of corticotropin-releasing hormone receptor1 gene polymorphisms and antidepressant response in major depressive disorders. Neurosci. Lett. 414:155–8.

128. Xiao Z, Liu W, Gao K, Wan Q, Yang C, Wang H, Wang X, Wang G, Liu Z. 2011. Interaction between CRHR1 and BDNF genes increases the risk of recurrent major depressive disorder in Chinese population. PLoS One 6:e28733.

129. Blomeyer D, Treutlein J, Esser G, Schmidt MH, Schumann G, Laucht M. 2008. Interaction between CRHR1 gene and stressful life events predicts adolescent heavy alcohol use. Biol. Psychiatry 63:146–51.

130. Schmid B, Blomeyer D, Treutlein J, Zimmermann US, Buchmann AF, Schmidt MH, Esser G, Rietschel M, Banaschewski T, Schumann G, Laucht

M. 2010. Interacting effects of CRHR1 gene and stressful life events on drinking initiation and progression among 19-year-olds. Int. J. Neuropsychopharmacol. 13:703–14.

131. Barr CS, Dvoskin RL, Yuan Q, Lipsky RH, Gupte M, Hu X, Zhou Z, Schwandt ML, Lindell SG, McKee M, Becker ML, Kling MA, Gold PW, Higley D, Heilig M, Suomi SJ, Goldman D. 2008. CRH haplotype as a factor influencing cerebrospinal fluid levels of corticotropin-releasing hormone, hypothalamic-pituitary-adrenal axis activity, temperament, and alcohol consumption in rhesus macaques. Arch. Gen. Psychiatry 65:934–44.

132. Dahl JP, Doyle GA, Oslin DW, Buono RJ, Ferraro TN, Lohoff FW, Berrettini WH. 2005. Lack of association between single nucleotide polymorphisms in the corticotropin releasing hormone receptor 1 (CRHR1) gene and alcohol dependence. J. Psychiatr. Res. 39:475–9.

133. Soyka M, Preuss UW, Koller G, Zill P, Hesselbrock V, Bondy B. 2004. No association of CRH1 receptor polymorphism haplotypes, harm avoidance and other personality dimensions in alcohol dependence: results from the Munich gene bank project for alcoholism. Addict. Biol. 9:73–9.

134. Ribbe K, Ackermann V, Schwitulla J, Begemann M, Papiol S, Grube S, Sperling S, Friedrichs H, Jahn O, Sillaber I, Gefeller O, Krampe H, Ehrenreich H. 2011. Prediction of the Risk of Comorbid Alcoholism in Schizophrenia by Interaction of Common Genetic Variants in the Corticotropin-Releasing Factor System. Arch. Gen. Psychiatry 68:1247–56.

135. Wasserman D, Sokolowski M, Rozanov V, Wasserman J. 2008. The CRHR1 gene: a marker for suicidality in depressed males exposed to low stress. Genes. Brain. Behav. 7:14–9.

136. De Luca V, Tharmalingam S, Kennedy JL. 2007. Association study between the corticotropin-releasing hormone receptor 2 gene and suicidality in bipolar disorder. Eur. Psychiatry 22:282–7.

137. Claes S, Villafuerte S, Forsgren T, Sluijs S, Del-Favero J, Adolfsson R, Van Broeckhoven C. 2003. The corticotropin-releasing hormone binding protein is associated with major depression in a population from Northern Sweden. Biol. Psychiatry 54:867–872.

138. Ray LA. 2011. Stress-induced and cue-induced craving for alcohol in heavy drinkers: Preliminary evidence of genetic moderation by the OPRM1 and CRH-BP genes. Alcohol. Clin. Exp. Res. 35:166–74.

139. De Luca V, Tharmalingam S, Zai C, Potapova N, Strauss J, Vincent J, Kennedy JL. 2010. Association of HPA axis genes with suicidal behaviour in schizophrenia. J. Psychopharmacol. 24:677–82.

140. Claes SJ. 2004. Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. Ann. Med. 36:50–61.

141. Austin MC, Janosky JE, Murphy HA. 2003. Increased corticotropin-releasing hormone immunoreactivity in monoamine-containing pontine nuclei of depressed suicide men. Mol. Psychiatry 8:324–32.

142. Zobel A. 2000. Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J. Psychiatr. Res. 34:171–181.

143. Held K, Künzel H, Ising M, Schmid D a., Zobel a., Murck H, Holsboer F, Steiger a. 2004. Treatment with the CRH1-receptor-antagonist R121919 improves sleep-EEG in patients with depression. J. Psychiatr. Res. 38:129–136.

144. Künzel HE, Zobel AW, Nickel T, Ackl N, Uhr M, Sonntag A, Ising M, Holsboer F. 2003. Treatment of depression with the CRH-1-receptor antagonist R121919: endocrine changes and side effects. J. Psychiatr. Res. 37:525–533.

145. Coric V, Feldman HH, Oren D a, Shekhar A, Pultz J, Dockens RC, Wu X, Gentile K a, Huang S-P, Emison E, Delmonte T, D'Souza BB, Zimbroff DL, Grebb J a, Goddard AW, Stock EG. 2010. Multicenter, randomized, double-blind, active comparator and placebo-controlled trial of a corticotropin-releasing factor receptor-1 antagonist in generalized anxiety disorder. Depress. Anxiety 27:417–25.

146. Binneman B, Feltner D, Kolluri S, Shi Y, Qiu R, Stiger T. 2008. A 6-week randomized, placebo-controlled trial of CP-316,311 (a selective CRH1 antagonist) in the treatment of major depression. Am. J. Psychiatry 165:617–20.

CHAPTER II

SITE-SPECIFIC MODULATION OF BRAIN GLUCOCORTICOID RECEPTOR AND CORTICOTROPIN-RELEASING HORMONE EXPRESSION USING LENTIVIRAL VECTORS

Gloria Laryea, Melinda G. Arnett, Lindsay Wieczorek, and Louis J. Muglia

INTRODUCTION

Defining the genes and gene networks involved in mediating the stress response will enhance understanding of the underlying pathophysiology of mental health disorders. The **hypothalamic-pituitary-adrenal (HPA)** axis is a major part of the neuroendocrine system that responds to stress. Regulation of the HPA axis is critical for adaptation to environmental changes. The principal regulator of the HPA axis is corticotrophin-releasing hormone (CRH), which is made in the paraventricular nucleus (PVN) of the hypothalamus and is an important target for negative feedback by glucocorticoids (GCs) – the key effector of the HPA axis. They exert their effects through ubiquitous nuclear hormone receptor superfamily members, in particular GR (NR3C1) (1).

Disruption of normal HPA axis activity is a major risk factor for neuropsychiatric disorders where decreased expression of GR and altered CRH activation has been documented (2, 3). The use of transgenic and gene knockout mice has helped to define roles for particular genes in mediating altered stress responsiveness and neuroendocrine function (4–6). However, these data are limited due to their inability to regulate spatial and temporal disruption of gene expression. Viral vectors have become the most commonly used gene delivery vehicles due to their high transduction efficiencies (7). In particular, lentiviral (LV) vectors have

become powerful tools in basic and translational research representing one of the most effective gene delivery vehicles allowing for stable long-term transgene expression in both dividing and non-dividing cells (8, 9).

Our lab has generated two LV vectors in an effort to investigate the role of CRH in specific brain nuclei. In this paper, we will discuss their design, production and possible future application in mice.

MATERIALS and METHODS

Animals

All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Vanderbilt University (Nashville, TN) and Cincinnati Children's Medical Center (Cincinnati, OH). Mice were housed on a 12 h/12 h (Vanderbilt) or 14 h/10 h (Cincinnati Children's Medical Center) light/dark cycle with *ad libitum* access to rodent chow and water. For use with the tetracycline-inducible system, some mice were given *ad libitum* access to doxycycline (doxy) chow (0.0625% doxy; Test Diets) to control transgene expression

Generation of Tetop-CRH mice

These mice were generated as previously described (10). Briefly, the CRH gene was placed under the control of a tetracycline-responsive cytomegalovirus minimal promoter (tetop) and inserted into a pUHC13-3 backbone vector. The resulting tetop-CRH plasmid was injected into inbred C57Bl/6 oocytes.

Generation of CaMKIIα-tTA mice

These mice were generated as previously described (11). These mice express the tetracycline transactivator under the control of the CaMKIIα promoter (Jackson Laboratories).

Lentiviral Vectors

For each LV vector, individual plasmids were designed as described below and sent to the Hope Center Viral Vector Core (Washington University, St. Louis, MO) for packaging. Briefly, plasmids were packaged using three helper plasmids in 293-T cells into replication deficient virions. Viral vector titers were determined by transduction of HT1080 cells and assayed for reporter expression using flow cytometry or real time PCR. We were able to reliably obtain a titer of 10^7-10^9 infectious units/mL for each LV.

LV-rtTA Vector

To generate the pLV-rtTA plasmid, we used a LV plasmid that is under the control of a modified moloney murine leukemia virus long terminal repeat (MNDU3; pMNDU3 plasmid generously given by M. Sands, Washington University in St. Louis) (12). We directly inserted the reverse tetracycline transactivator (rtTA) from Clontech's rtTA-advanced plasmid (PT3899-5) into the LV backbone with EcoRI and BamHI cuts.

LV-tetop-CRH Vector

We inserted tetop-CRH (10), the CRH gene under the control of the tetop promoter, into the XhoI and EcoRV sites of pLenti-III-EF1α (Applied Biological Materials, Richmond, BC., Canada).

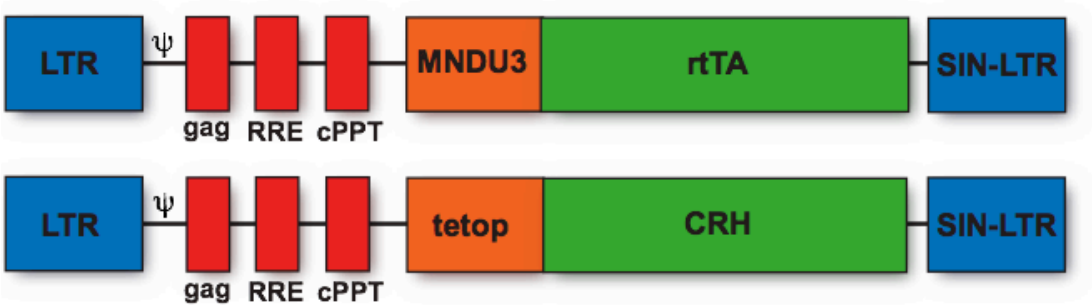

Figure 2.1. Constructs of lentiviral vectors packaged into replication deficient virions. Each LV vector contains an HIV-based system including 3' and 5' long terminal repeats (LTR), a 5' self-inactivating (SIN) element to prevent replication and a woodchuck post-transcriptional regulatory element to enhance protein expression (A) LV-rtTA (B) LV-tetopCRH. Ψ = packaging signal; RRE = Rev response element; cPPT = central polyurine tract; gag = group-specific antigen; EF1α, MNDU3, and tetop = promoters; nls = nuclear localization signal; rtTA = reverse tetracycline transactivator; CRH = corticotropin-releasing hormone.

Cell Culture

The LV-GR was transiently infected into Chinese hamster ovarian (CHO)-K1 cells (ATCC, CCL-61). Cells were seeded in a 9-well plate at 3 x 10^5 cells/ml and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), and 1% pen strep (Gibco, New York, NY). Cells were cultivated at 37°C in a humidified atmosphere containing 5% CO2. CHO-K1 cells were infected with LV-GR (7 µl, 7.4 x 10^8 TU/ml) or LV-GFP in triplicate while control wells contained CHO-K1 cells only. Whole cell extracts were prepared after a 72-hour incubation using total protein extraction buffer (PE buffer [10mM Hepes, 60mM KCl, 1mM EDTA], 250mM dithiothreitol, 100% NP-40 and protease inhibitor mixture [0.5

μg/ml aprotinin, 1 μg/ml pepstatin and leupeptin). Analysis of the integrated proviral genome was determined by Western blotting.

Western Blotting

60 μg of whole cell extracts were resolved through sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4–12% gradient Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad CA), transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare Life sciences, Pittsburg, PA) using a semidry transfer system (Bio Rad, Hercules, CA), and blocked with 5% dry milk in PBS and 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO). Blots were probed with anti-GR antisera (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1/2,000 dilution overnight at 4°C. Binding of horseradish peroxidase labeled goat anti-rabbit antibody (sc-2004) was determined using SuperSignal West Chemiluminescent substrate (Pierce, Rockford, IL). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with an anti-β-actin antibody (Sigma Aldrich, St. Louis, MO) as an internal control to ensure equal loading of protein.

Luciferase Assay

We transfected CHO cells with a pTetop-luciferase (pTetop-luc) plasmid in conjunction with pLV-rtTA. After administering doxy at 0, 10, and 100 ng/mL concentrations, we used a luminometer to measure relative light units (RLU) as an indicator of luciferase expression.

CRH Immunohistochemistry

Mice were anesthetized with 2.5% Avertin, and transcardially perfused with 4% paraformaldehyde. The brains were immersed in 20% DEPC sucrose solution and sectioned at 30 um into PBS with 0.25% Triton X-100 (PBS-T); sections were stored at 4ºC until use. 0.3% hydrogen peroxide in PBS was used to quench endogenous peroxides, and sections were blocked in 10% normal goat serum (NSG) in PBS-T to prevent non-specific binding. Sections were incubated in primary antibody, 1:1000 rabbit anti-CRF (Bachem, San Carlos, CA) at 4ºC overnight. Then, sections were labeled with biotinylated goat anti-rabbit IgG secondary (1:200, Vector Laboratories, Burlingame, CA) at room temperature for an hour, followed by an hour in avidin/biotin complex solution (Vector Laboratories, Burlingame, CA), and detected with diaminobenzidine (DAB) staining. The sections were air-dried on slides overnight, dehydrated with ethanol and xylene, and cover-slipped with Permount (Fisher Scientific, Pittsburg, PA). Stained slides were visualized under a light microscope (Zeiss Axio Imager) and images were prepared using Adobe Photoshop.

Hematoxylin and Eosin (H & E) Staining

Sections adjacent to those used for CRH immunohistochemistry were placed on slides and air-dried overnight. Slides were processed as follows: 2 min wash in 70% ethanol; 5 min wash in distilled water; 10 min staining in hematoxylin (Polysciences Inc, Warrington, PA); 3 washes in distilled water; 1 min in saturated lithium chloride; 3 washes in distilled water; 2 min in 70% ethanol; 2 min in 80% ethanol; 90 sec staining in eosin (Polysciences Inc, Warrington, PA); 2 min in 70% ethanol; 2 min in 80% ethanol; three 2 min washes in 95% ethanol; three 2 min washes in 100% ethanol; three 2 min washes in xylene; coverslipped with Permount (Fisher Scientific,

Pittsburg, PA).

Stereotaxic Injection

We gave mice a 10 mg/kg dose of ketoprofen (Fort Dodge, IA) and an hour later, anesthetized them with a 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) solution and mounted them on a stereotaxic frame (Kopf Instruments, Tujunga, CA). We drilled small holes at the coordinates provided below and injected the LV at either a rate of 0.2µl/min into the central nucleus of the amygdala (CeA) or 0.5µl/min into the striatum. The mice were monitored for recovery and given a 10mg/kg dose of ketoprofen each day for 3 days post-surgery. Fourteen days after the injection, brains were harvested and processed for immunohistochemistry. Coordinates: CeA (1.25mm bregma, ±2.75 lateral, 4.75 ventral); striatum (0mm bregma, 2.5 lateral, 3.8 ventral).

RESULTS AND DISCUSSION

CRH modulation in the brain of animal models has provided extensive evidence for their involvement in neuroendocrine, behavioral and autonomic responses to stress. An important question that arises is whether CRH activity differs with respect to disease state and whether CRH activity in distinct brain regions contributes in different proportions to anxiety versus depression, or any other affective disorder state for that matter. Elevated forebrain CRH produced different neuroendocrine and behavior responses depending on the length of time of CRH overexpression and the specific developmental period in which this overexpression occurred (10, 13). Some of the forebrain regions with altered CRH included the

basolateral and central amygdalar nuclei, hippocampus, striatum, PVN of the hypothalamus, and nucleus accumbens. The ability to define how these regions contribute to the observed phenotypes will help discern which regions are important targets for specific psychiatric disorders. We can evaluate this using LV vectors, directly delivered to brain nuclei of interest, in mice with specific transgenes to alter expression of CRH.

CRH modulation

We used the tetracycline inducible method to enable modulation of CRH expression along developmental periods. We generated transgenic mice with the tetop promoter driving CRH gene expression (tetop-CRH).

To enable inducible expression of CRH in a site-specific manner, we developed an HIV-1-derived replication-defective LV vector that possesses rtTA (LV-rtTA) (Figure 2.1. A). The rtTA forms a Tet-on system and when bound to a tetop promoter can activate expression of any downstream gene of interest. This expression is inducible: turned on in the presence of a tetracycline derivative, doxy, or off in its absence. The rtTA has high inducibility (within hours of doxy administration) and low background (14, 15).

To determine the *in vitro* efficacy of the virus, we transfected CHO cells with a combination of pTetop-luc and pLV-rtTA (pMNDU-rtTA). We administered doxy at various concentrations and detected the luciferase expression as measured by the relative light units (RLU) (Figure 2.2.). In the presence of both pTetop-luc and pLV-rtTA, doxy significantly increases the RLU, which was minimal with no doxy present, indicating that doxy allowed rtTA to bind to tetop and drive luciferase expression.

To determine the *in vivo* efficacy of our virus, we performed unilateral stereotaxic injections into the striatum of tetop-CRH mice that were either on or off doxy with 3µl of LV-rtTA (7.36 x 10^9 infectious units/mL). Two weeks after viral infection, we harvested the brains and performed immunohistochemistry to evaluate CRH protein expression. The data indicate that CRH is expressed only in the presence of both LV-rtTA and doxy in mice with the tetop-CRH transgene (Figure 2.3. A). To evaluate the tissue in the injected region, we performed H & E staining. The H & E staining in Figure 2.3. B illustrates mononuclear cell infiltration and cell toxicity in the LV-rtTA injected region. This data raised concerns of a possible host-inflammatory response induced by the rtTA protein.

To eliminate this confound, we decided to use mice that express tTA during development and thus are inherently tolerant to it (CamKIIα-tTA mice). Consequently, we generated an LV delivery system, LV-tetop CRH (Figure 2.1. B), complementary to the CamKIIα-tTA transgenic mice, by inserting the tetop-CRH gene into an LV vector, pLenti-III-EF1α (Applied Biological Materials Inc., Richmond, BC. Canada).

To test *in vivo* efficacy, we unilaterally injected the striatum of CamKIIα-tTA mice that were either on or off doxy with 3µl of LV-tetopCRH (titer 8.62x10^7 infectious units/ml). We chose striatum because it has no endogenous CRH expression and thus it is easier to visualize CRH introduced by the virus over background staining. We evaluated CRH protein expression two weeks after viral infection. In comparison to the Tet-on system – LV-rtTA in the presence of doxy turns on CRH gene expression – in this system (Tet-off), LV-tetopCRH binds to the tTA in the mice with the CamKIIα-tTA transgene and drives CRH expression only in the absence of doxy. Thus, placing the mice on a doxy diet would turn off CRH

expression. This allows us to retain the ability to spatially and temporally restrict CRH expression.

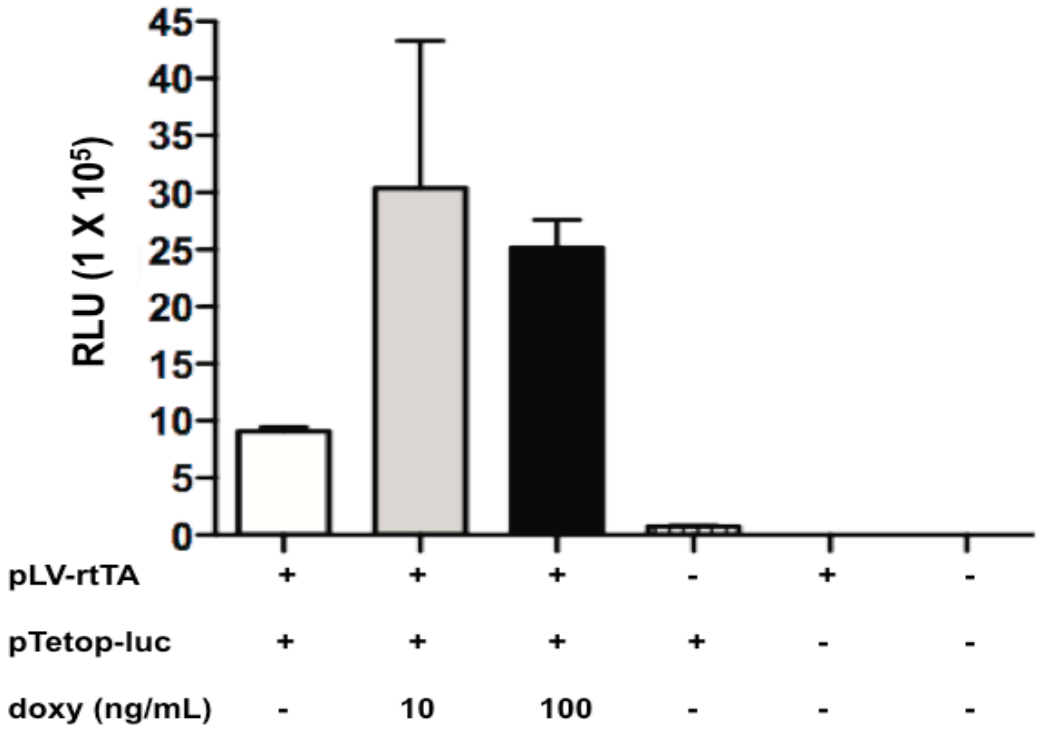

Figure 2.2. ***In vitro*** **analysis of pLV-rtTA.** CHO cells were co-transfected with pTetop-luc and pLV-rtTA in the presence and absence of doxy to test the efficiency of the pLV-rtTA plasmid. Luciferase expression was then measured. pLV-rtTA displays robust expression in the presence of doxy with low levels of expression in the absence of doxy. RLU = relative light units, doxy = doxycycline

The immunohistochemistry data indicate that CRH is expressed only in the presence of LV-tetopCRH (Figure 2.4. A). Mice on a doxy diet or non-injected mice do not show this striatal CRH expression (Figure 2.4. B,C). H & E staining in Figure 2.4. D-F illustrates that the cells in the LV-tetopCRH injected region do not display the same toxic effect observed in the LV-rtTA injected mice. The data support the functionality of this new model.

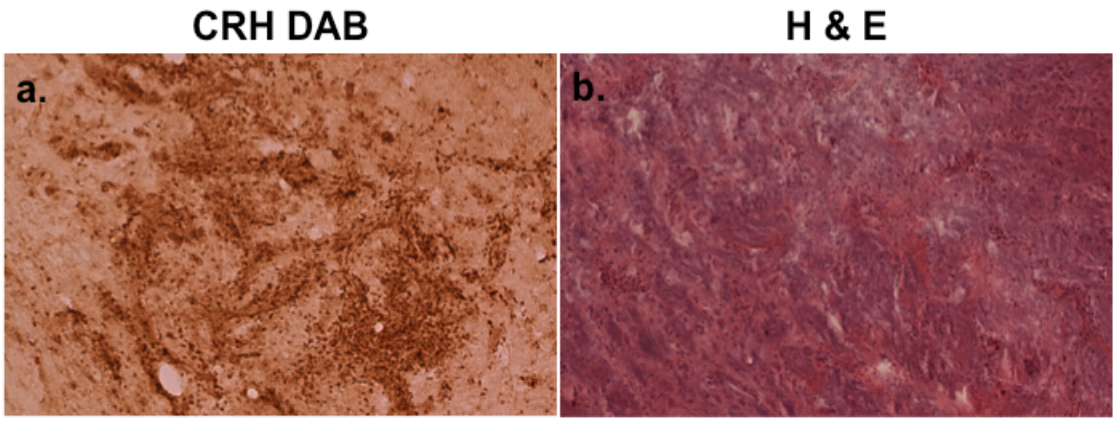

Figure 2.3. CRH immunoreactivity and hemotoxylin & eosin (H&E) staining in tetopCRH mice injected with LV-rtTA. (A) CRH protein (dark brown pigment) is present when mice are on doxy chow. (B) H & E staining displays inflammatory cell infiltration within the injected region.

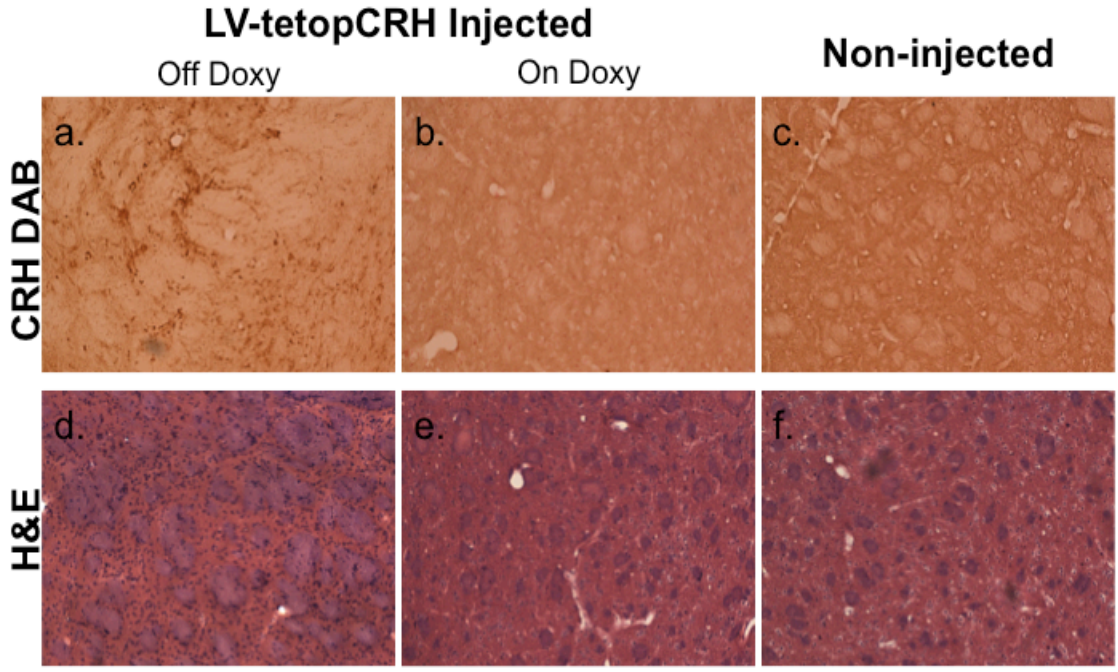

Figure 2.4. CRH immunoreactivity and hemotoxylin & eosin (H & E) staining in CamKIIα-tTA mice injected with LV-tetopCRH. (A) CRH protein (dark brown pigment) is present when mice are off doxy chow. (B ,C) CRH protein is absent when mice are on doxy chow or in non-injected controls. H & E staining shows normal cells in the (D) absence or (E) presence of doxy as well as in (F) non-injected controls.

Previous literature supports the use of LV vectors to define the role of CRH in brain regions such as the CeA and bed nucleus of the stria terminalis (BnST) – two brain regions involved in mediating anxiety and fear behaviors (See (16) for review). CeA CRH elevation in male mice was found to affect anxiety behavior: reduce it with long-term elevation (17) but increase it with short-term elevation (18). On the other hand, BnST CRH overexpression was involved in inducing despair behavior (17). In comparison, CeA CRH elevation in rats demonstrated pronounced maladaptive alteration in measures of anxiety and despair, as well as HPA axis function, similar to that observed in human disorders (19, 20). These studies indicate how different the effects of CRH overexpression are dependent on region, length of over-expression, gender and/or species of organism

The inducibility of our tetracycline system will enable CRH overexpression for short periods of time during development to evaluate how CRH activity at specific time periods can result in different phenotypic outcomes. The strength of our model is the ability to terminate CRH overexpression after a length of time to allow for molecular and circuit changes that may occur and influence future sensitivity to stress. For instance, does brief CRH overexpression in amygdalar nuclei early in development prime and provide resilience to fearful stimuli later in life? Or does it predispose pathological states of anxiety or despair? Perhaps the developmental period during which CRH overexpression occurs will predict whether it is beneficial or destructive when exposed to subsequent stressors.

CONCLUSIONS

One of the limitations in treatment of psychiatric disorders is the limited knowledge of their underlying molecular etiology. We believe that LV constructs in combination with transgenic mouse models provide an avenue for evaluating molecular pathways specific to different affective disorders. The use of this paradigm to study other stress-related molecules and monoamines will possibly prove beneficial in understanding the causation of individual differences in the manifestation and prognosis of mood disorders.

REFERENCES

1. Samarasinghe RA, Witchell SF, DeFranco DB. 2012. Cooperativity and complementarity: synergies in non-classical and classical glucocorticoid signaling. Cell Cycle 11:2819–27.

2. Holsboer F, Ising M. 2010. Stress hormone regulation: biological role and translation into therapy. Annu. Rev. Psychol. 61:81–109, C1–11.

3. Pariante CM. 2009. Risk factors for development of depression and psychosis. Glucocorticoid receptors and pituitary implications for treatment with antidepressant and glucocorticoids. Ann. N. Y. Acad. Sci. 1179:144–52.

4. Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale W. 1992. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. Endocrinology 130:3378–3386.

5. Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schütz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23:99–103.

6. Van Gaalen MM, Stenzel-Poore M, Holsboer F, Steckler T. 2003. Reduced attention in mice overproducing corticotropin-releasing hormone. Behav. Brain Res. 142:69–79.

7. Naldini L, Blömer U, Gage FH, Trono D, Verma IM. 1996. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. U. S. A. 93:11382–8.

8. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–7.

9. Naldini L. 1998. Lentiviruses as gene transfer agents for delivery to non-dividing cells. Curr. Opin. Biotechnol. 9:457–63.

10. Kolber BJ, Boyle MP, Wieczorek L, Kelley CL, Onwuzurike CC, Nettles S a, Vogt SK, Muglia LJ. 2010. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. J. Neurosci. 30:2571–81.

11. Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER. 1996. Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678–83.

12. Haas DL, Lutzko C, Logan AC, Cho GJ, Skelton D, Jin Yu X, Pepper KA, Kohn DB. 2003. The Moloney Murine Leukemia Virus Repressor Binding Site Represses Expression in Murine and Human Hematopoietic Stem Cells. J. Virol. 77:9439–9450.

13. Vicentini E, Arban R, Angelici O, Maraia G, Perico M, Mugnaini M, Ugolini A, Large C, Domenici E, Gerrard P, Bortner D, Mansuy IM, Mangiarini L, Merlo-Pich E. 2009. Transient forebrain over-expression of CRF induces plasma corticosterone and mild behavioural changes in adult conditional CRF transgenic mice. Pharmacol. Biochem. Behav. 93:17–24.

14. Gossen M, Bonin AL, Bujard H. 1993. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. Trends Biochem. Sci. 18:471–5.

15. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc. Natl. Acad. Sci. U. S. A. 97:7963–8.

16. Laryea G, Arnett MG, Muglia LJ. 2012. Behavioral Studies and Genetic Alterations in Corticotropin-Releasing Hormone (CRH) Neurocircuitry: Insights into Human Psychiatric Disorders. Behav. Sci. (Basel). 2:135–171.

17. Regev L, Neufeld-Cohen A, Tsoory M, Kuperman Y, Getselter D, Gil S, Chen A. 2010. Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation. Mol. Psychiatry 16:714–728.

18. Regev L, Tsoory M, Gil S, Chen A. 2011. Site-Specific Genetic Manipulation of Amygdala Corticotropin-Releasing Factor Reveals Its Imperative Role in Mediating Behavioral Response to Challenge. Biol. Psychiatry.

19. Flandreau EI, Ressler KJ, Owens MJ, Nemeroff CB. 2012. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. Psychoneuroendocrinology 37:27–38.

20. Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME. 2009. Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol. Psychiatry 14:37–50.

CHAPTER III

BEHAVIORAL CONSEQUENCES OF CORTICOTROPIN-RELEASING HORMONE OVEREXPRESSION IN THE CENTRAL NUCLEUS OF THE AMYGDALA IN ADOLESCENT MICE

INTRODUCTION

Dysregulation of the HPA axis has been strongly implicated in the genesis of stress-related disorders such as anxiety and depression(1–3). One characteristic these disorders share is an increased emotional response to neutral stimuli(4). Given that the amygdala mediates emotional responses to stress(5, 6), it is an important structure to study in order to further understand predispositions of certain individuals to psychiatric diseases. The amygdala receives input from sensory modalities and integrates this information to activate behavioral and physiological responses(7). Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies(8) in individuals with depression and PTSD show enhanced amygdala activation(9). Furthermore, ablation studies of the amygdala demonstrate decreased fear behavior and more docile demeanor in animals(10), implicating a role for this limbic structure in fear potentiation.

Among the amygdala nuclei, the CeA stands out as the major nuclei of the amygdala for three main reasons. First, the CeA functions as the central integration point for most of the other amygdala nuclei and many other brain regions(11). Specifically, the basolateral amygdala (BLA) receives fear memory information from the hippocampus and sends it to the CeA, causing output to regions involved in the behavioral expression of fear (12, 13). The CeA itself receives direct cortical innervations from the prefrontal cortex (PFC), sensory areas, brainstem and

hypothalamus(11). Information about the salience of danger and cognition associated with it is sent to the CeA directly from the mPFC or indirectly through the BLA(4). This sensation of danger is heightened in individuals with anxiety disorders such that even neutral stimuli can evoke an emotional response(4). Noradrenergic projections from the locus coeruleus to the CeA increases CeA activity and CRH mRNA in the CeA and affects autonomic activation(14). The information sent into the CeA is thus integrated in a process that remains to be elucidated and is expressed in the form of autonomic and behavioral responses in CeA outputs.

Second, the CeA is the major amygdala output structure(11) to regions involved in the stress response. Efferents of the CeA go to bed nucleus of the stria terminalis (BNST), hypothalamus, brainstem and midbrain nuclei, and modulate autonomic and behavioral functions(11). The CeA mainly contains GABAergic output and as such, its projections to the BNST disinhibit BNST inhibition of the PVN(11, 15). Due to limited direct CeA to PVN projections(16), this pathway is believed to be the mechanism by which the CeA activates the HPA axis(6). The CeA activates brainstem nuclei such as the periaqueductal gray to cause freezing and vocalization in the conditioned fear response to shock(11), and hence generates relevant fear responses to aversive stimuli(17). In psychiatric diseases, stimuli can activate the sympathetic nervous system, possibly through CeA activation of the locus coeruleus(18), resulting in increased heart rate and blood pressure(19). Electrical stimulation of the CeA also activates the sympathetic nervous system (11, 19) and is believed to activate the HPA axis and lead to an increase in plasma CORT in rats(20).

Third, the CeA is a site containing large populations of CRH neurons(13, 18) most of which colocalize with GAD65/67, implying their GABAergic inhibitory activity(18). Moreover, electrophysiological data shows that CRH application

increases GABA inhibitory post-synaptic currents (IPSCs) in the CeA(2, 21). Not only does CRH act at receptors in the CeA, but it also activates CeA targets through interneurons disinhibition(2). However, other studies using neuronal tract tracing methods demonstrate that CRH immunoreactive CeA neurons form excitatory synapses with locus coeruleus dendrites(22) to cause excitation of the sympathetic nervous system. This indicates that CeA CRH can function both through excitatory and inhibitory pathways to exert its function. This circuitry of the CeA identifies a role for it in mediating pathways involved in behavioral, autonomic, and endocrine responses to stimuli(23), in part mediated by CRH. The role of CRH in affecting CeA targets may cause changes in behavior that increase risk for psychiatric disease.

Role of CRH in CeA function

The CRH peptide is distributed throughout the brain but found more concentrated in the PVN, CeA, and BnST(13, 24). CRH has two receptors, CRH-R1 and CRH-R2, which have primarily non-overlapping expressions in the brain(25, 26). Restraint stress in rodents significantly increases the levels of CRH and CRH-R1 mRNA in the PVN(27). In the amygdala, CRH-R1 receptors are the predominant type expressed(26). Functionally, inhibition of CRH-R1 receptors prevents the CRH-induced increases in GABAergic IPSCs in the CeA (2) and may also affect autonomic and behavioral functions of the CeA.

Studies in patients with depression and PTSD show distinct elevations in CRH levels in the cerebrospinal fluid(28–33). Treatment of these depressed patients with CRH-R1 antagonist ameliorates the symptoms of anxiety and depression(34) and decreases HPA axis activity(35). Many anxiety and despair symptoms can be recapitulated in rodent models upon intracerebroventricular CRH administration,

through a pathway that is thought to be HPA axis–independent(36). This observation leads to the implication that extrahypothalamic sources of CRH are the main source of CSF CRH(36). Since CeA CRH is anxiogenic(17) and CeA stimulation exacerbates the effects of intracerebroventricular CRH(5), the CeA is a probable extrahypothalamic source of CSF CRH. This hypothesis is supported in rodent models using both in-vivo microdialysis studies that demonstrate elevated CeA CRH following restraint stress(37), as well as chronic CORT administrations. Furthermore, elevated CeA CRH in animal models also demonstrate elevated anxiety(38) suggesting that changes in CeA CRH levels are a potential model of CeA dysfunction in psychiatric disease.

When CeA CRH is reduced with the use of antisense oligonucleotides (13) or by GR deletion in the CeA (CeAGRKO)(39), memory retention in conditioned fear test is impaired. Furthermore, the CeAGRKO mice show rescue of conditioned fear behavior if intracerebroventricular CRH is administered before conditioned fear training(39). It appears therefore that there is a homeostatic level of CRH required in the CeA for normal function and any deviation above or below this level can result in behavioral and neuroendocrine problems. CRH receptor antagonists applied to the CeA reduce elevated plus maze(40) and CRH-mediated anxiety behavior(41). CRH-R1 antagonists diminish anxiety and HPA axis response, and increases exploratory behavior in primates(42). Although the anxiety attenuating effects of CRH R1 antagonist was not observed in a study looking at novelty-suppressed feeding in rat(43), most studies to date do suggest a role for CeA CRH in anxiogenic behaviors(44), fear memory consolidation(39), as well as autonomic responses (15).

Behavioral data also shows that the CeA is involved in the psychological aspect of stress. Studies comparing the effects of the physical stress of treadmill

running, to psychological restraint stress, show that the increase in CeA CRH throughout the restraint stress was not seen to the same extent in treadmill running rats (45). This implies that the CeA is more a mediator of the psychological stress response and this mediation may occur through modulation by CRH (17).

Studies in transgenic mice that overexpress CRH show reduced locomotor activity in a novel environment and increased anxiety behaviors (36). Forebrain CRH overexpression in the first three weeks of life produces anxiety-like behavior and despair-like behavior (46). The use lentiviral vectors to specifically overexpress CRH in the CeA and the BNST of male mice demonstrate that chronic CRH overexpression in the CeA does not affect anxiety under basal conditions (47). However upon a 30 minutes restraint stress, anxiety behavior is attenuated, implying that chronic overexpression can cause habituation to a stressor(47). In contrast lentiviral vectors to overexpress CRH in the CeA of female rats, showed increased anxiety, increased despair, and impaired negative feedback of HPA axis, all changes associated with stress pathology (6). The discrepancy in these two studies may be a result of the length of over-expression time, and gender and/or species differences.

The studies describe above were all performed in adult mice, and do not evaluate the effects of CeA CRH overexpression early in life. Stress in early life is known to increase the risk of psychiatric disorders later in life, suggesting that the developmental time period of stress exposure affects neuroendocrine and behavioral outcomes. Adults who experienced some form of childhood stress show elevated basal cortisol, increases in ACTH responsiveness, and heightened emotional responses to stressful stimuli than control patients with childhood stress (48). It is therefore necessary to evaluate the effects of CeA CRH overexpression early in life to understand its development role in the stress regulation.

In order to test the hypothesis that CeA CRH overexpression in adolescent leads to maladaptive stress responses, we combine the use of a lentiviral vectors with the tetracycline inducible system. Our lab has developed a lentiviral vector described in the previous chapter that will allow for spatial and temporal CRH overexpression (49). Targeting the CeA in adolescent mice will allow the study of specific changes that occur in this brain region upon HPA-axis dysregulation and how this affects diseases such as anxiety and depression.

MATERIAL AND METHODS

Animals

All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Medical Center (Cincinnati, OH). Mice were housed on a 14 h/10 h light/dark cycle with *ad libitum* access to rodent chow and water. *CamtTA mice* (*CaMKIIα-tTA)*: mice express the tetracycline transactivator under the control of the CaMKIIα promoter (Jackson Laboratories).

LV-tetop-CRH Vector

Tetop-CRH (46), the CRH gene under the control of the tetop promoter, was inserted into the XhoI and EcoRV sites of pLenti-III-EF1α (Applied Biological Materials, Richmond, BC, Canada). The resulting plasmid was sent to the Hope Center Viral Vector Core (Washington University, St. Louis, MO) for packaging (described in Chapter 3).

Stereotaxic Injections

We treated mice with 10 mg/kg of ketoprofen (Fort Dodge, IA) one hour before anesthetizing them with a 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) solution. Mice were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA) and drilled small holes for CeA coordinates (1mm bregma, ±3 lateral, 4.3 ventral). We injected control or CamtTA mice at postnatal day 28-31 (P28-31) with 1µl of LV-tetop CRH (titer: 6.8 x 10^8 IU/ml) at a rate of 0.25µl/min into the CeA. We monitored the mice for recovery and gave them a 10mg/kg dose of ketoprofen each day for 3 days post-surgery. Behavioral testing in mice occurred two weeks post-surgery.

CRH *in situ* hybridization

Mice were anesthetized mice with a 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) solution, perfused with DEPC 1x PBS followed by 4% DEPC PFA, and the brains were post-fixed overnight in 4% DEPC PFA. After 48-hour immersion in 20% DEPC sucrose solution at 4ºC, brains were embedded in OCT. 16um sections were obtained from brains at -15ºC in a cryostat and mounted onto Superfrost+ slides (VWR), vacuum-dried overnight, and stored at -80ºC until *in situ* hybridization was performed. ^{33}P UTP was used to label an RNA probe complementary to a 0.32 KB fragment on exon 2 of the CRH mRNA. Sections were hybridized to the labeled probe at 65ºC for 16 hours and washed in 0.1X SSC at 60ºC for 30 min. Slides were exposed to Maximum resonance film (Kodax BioMAx, Rochester N.Y.) for 4 days. We scanned the autoradiographic images at 3200 dots/inch into Photoshop on an Epson Perfection V600 scanner and quantified using NIH Image J software.

Behavioral Testing

All behavioral testing was performed in mice two weeks after lentiviral injections when the mice were between postnatal day 53 – 73 (P53-P73). Behavioral tests were performed between 8:00 a.m. and 12:00 p.m. Mice acclimated in a room adjacent to the testing room for an hour before behavioral testing. Behavioral tests were performed in the Cincinnati Children's Hospital behavior core.

Open field (OF) test: Locomotor exploration/activity was measured in a 41 cm × 41cm chamber (Accuscan Instruments, Columbus, OH). The chamber was equipped with a 16 LED-photocells 2 cm above the floor in x- and y-planes 2.5cm apart. Each trial lasted for a period of 30 minutes, and was analyzed in 5-minute interval. During that time, the PAS Activity program (San Diego Instruments, CA) was used to measure behaviors such as time spent, distance travelled, and number of entries into the inner and outer zones of the chamber.

Light dark (LD) test: The same apparatus used in the OF test was used in this LD test. A rectangular black acrylic (40 cm x 20 cm) insert with an opening was placed into the box and provided a half light, half dark box. Mice were placed a corner of the dark side and allowed to explore for 10 minutes. We recorded latency to enter the light zone, number of entries and time spent in the different zones.

Elevated Zero Maze (EZM) test: This maze (50cm inner diameter, 5cm lane width, 15cm wall height, placed 50 cm off the ground on 4 braced legs) consists of a round maze with opposing open and closed arms. Mice are placed in the center of the open arm and activity is scored for 5 minutes. Time spent in the open and closed areas, as

well as latency and number of entries into the open and closed areas was measured.

Tail suspension test (TST): This test used a rectangular tabletop platform with a hole through which the mouse's tail was suspended. Latency to immobile and time spent immobile were measured manually with the use of a computer software.

Forced swim test (FST): This test utilizes a clear 25 cm x 20 cm acrylic cylinder filled ¾ full with water at room temperature (25°C). Mice are placed in the beaker for 1 minute to acclimate to the chamber, after which we tested the mice for 5 minutes. Latency to immobile and time spent immobile were measured. Mice were placed in a warm cage and toweled dried.

Statistical analysis

The results are presented as mean ± SEM after data were subjected to Student t-test, one- or two-way ANOVA test (Prism 5.0 software; GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as a p value ≤ 0.05 and was analyzed using the Tukey or Bonferroni post hoc test.

RESULTS

Overexpression of CRH in the CeA of adolescent mice

We used the tetracycline-inducible system in combination with lentiviral vectors to specifically overexpress CRH in the CeA (Figure 3.1.). Using a lentivirus containing GFP, we show accurate targeting of the CeA in mice at P28 (Figure 3.2. A). We overexpressed CRH in the CeA in adolescence by injecting LV-tetop CRH into wild-type (Control) or CamtTA (CeA CRH-OE) when mice were between P28-P30. CRH *in situ* hybridization was evaluated 2 weeks after lentiviral injection, and demonstrated a 2-fold increase in CeA CRH-OE compared to control mice (Figure 3.2. B)

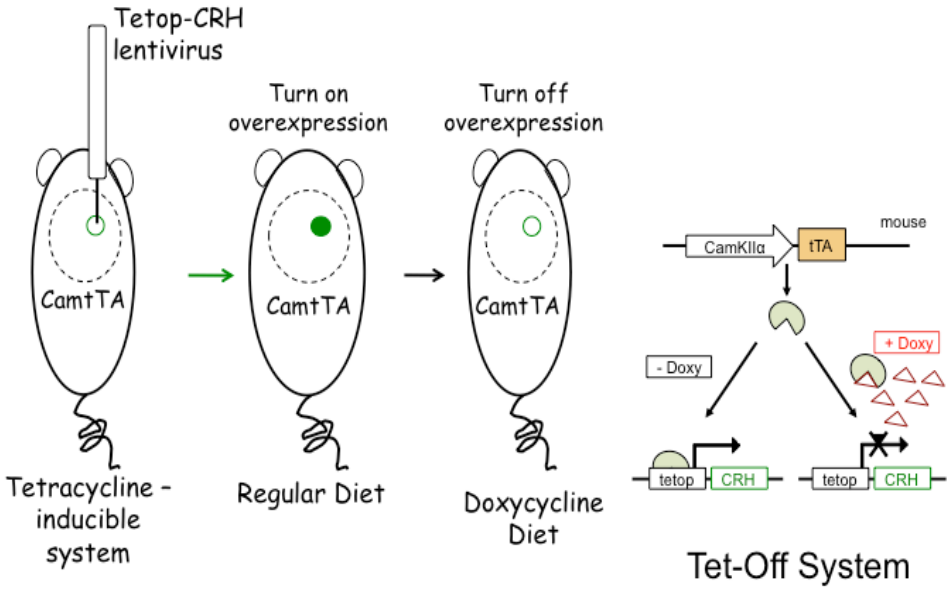

Figure 3.1. CRH overexpression model. Spatial and temporal restriction of CRH overexpression using the tetracycline-inducible system. In the absence of doxycycline, the tetracycline transactivator (tTA) binds to the tetracycline-responsive promoter (tetop) and drives CRH expression. In the presence of doxycycline, CRH overexpression is prevented.

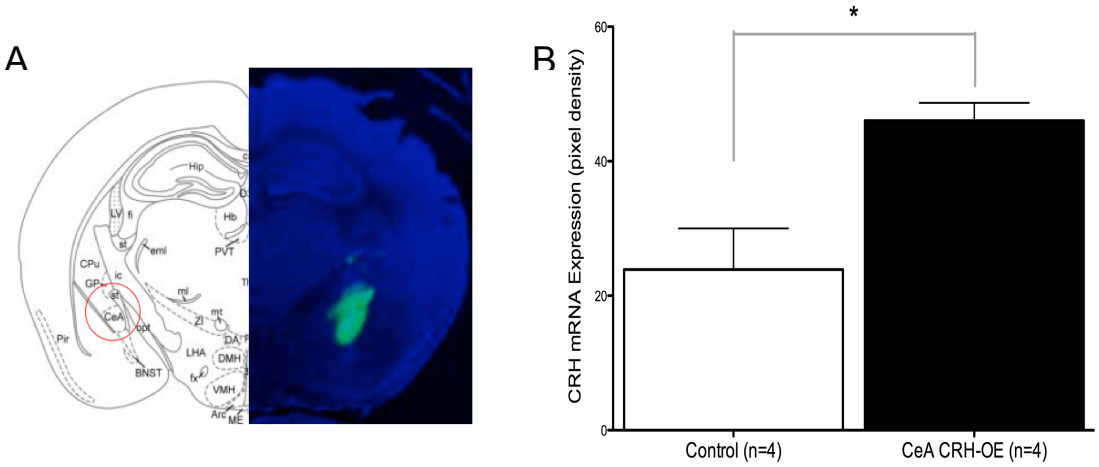

Figure 3.2. CeA targeting and increased CRH mRNA expression. (A) Picture depicting accurate targeting of the CeA through GFP injection. (B) *In situ* hybridization quantification demonstrating a 2-fold increase in CRH mRNA expression in adolescent mice.

Adolescent CeA CRH-OE mice display no anxiety or despair phenotypes

To evaluate the effects of CeA CRH overexpression on behavior in adolescent mice, we tested control and CeA CRH-OE mice in a number of behavioral paradigms during the late adolescent period (P53-P73). We observe normal locomotor behavior in CeA CRH-OE mice in terms of distance traveled in the OF test (Figure 3.3. A). However we do not observe increased anxiety behaviors in the OF (Figure 3.3. B-D). The LD and EZM tests also reveal no differences in anxiety behaviors in CeA CRH-OE mice compared to controls (Figure 3.4. A-F). When we evaluated the effects of amygdala CRH increase on despair-like behaviors in the TST and FST tests, we observed no differences between control and CeA CRH-OE mice (Figure 3.5. A-D).

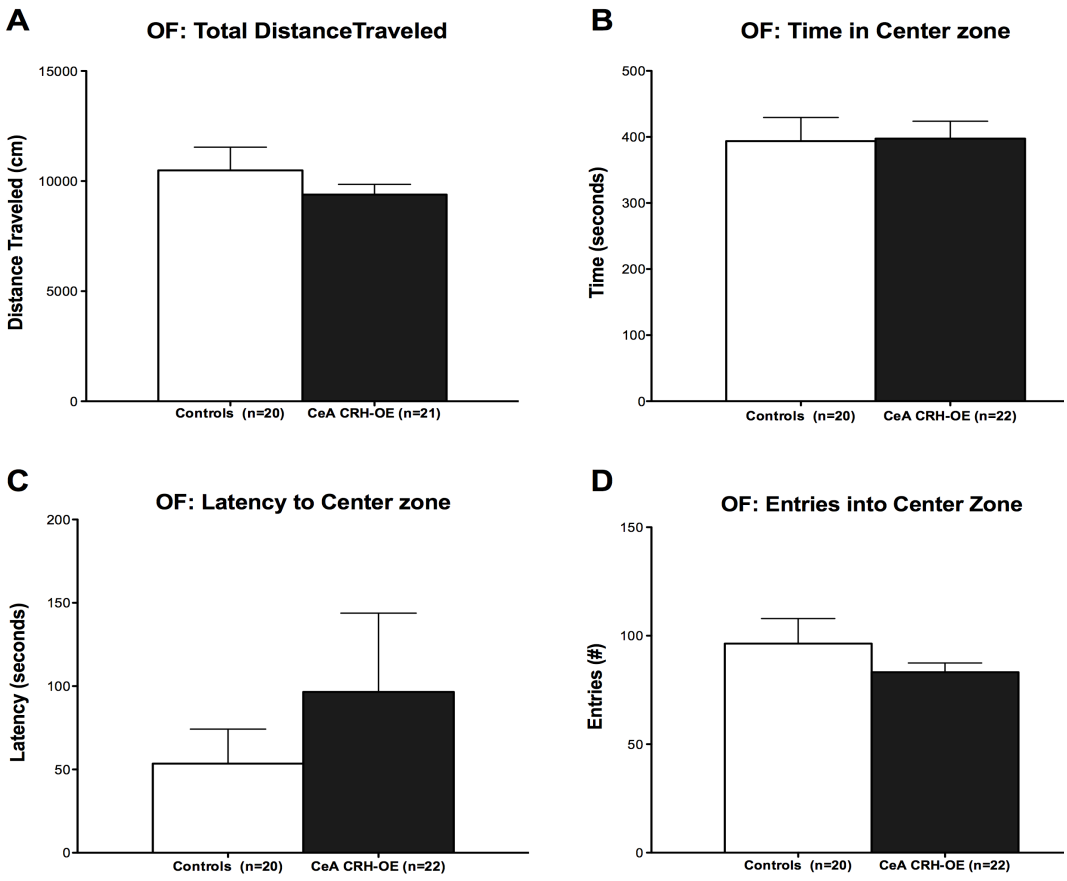

Figure 3.3. Adolescent CeA CRH-OE mice display no locomotor or anxiety phenotypes in the OF test. Adolescent male CeA CRH-OE mice show normal locomotor activity (A) and no anxiety behavior in terms of time spent (B), latency (C) and number of entries (D) into the center zone.

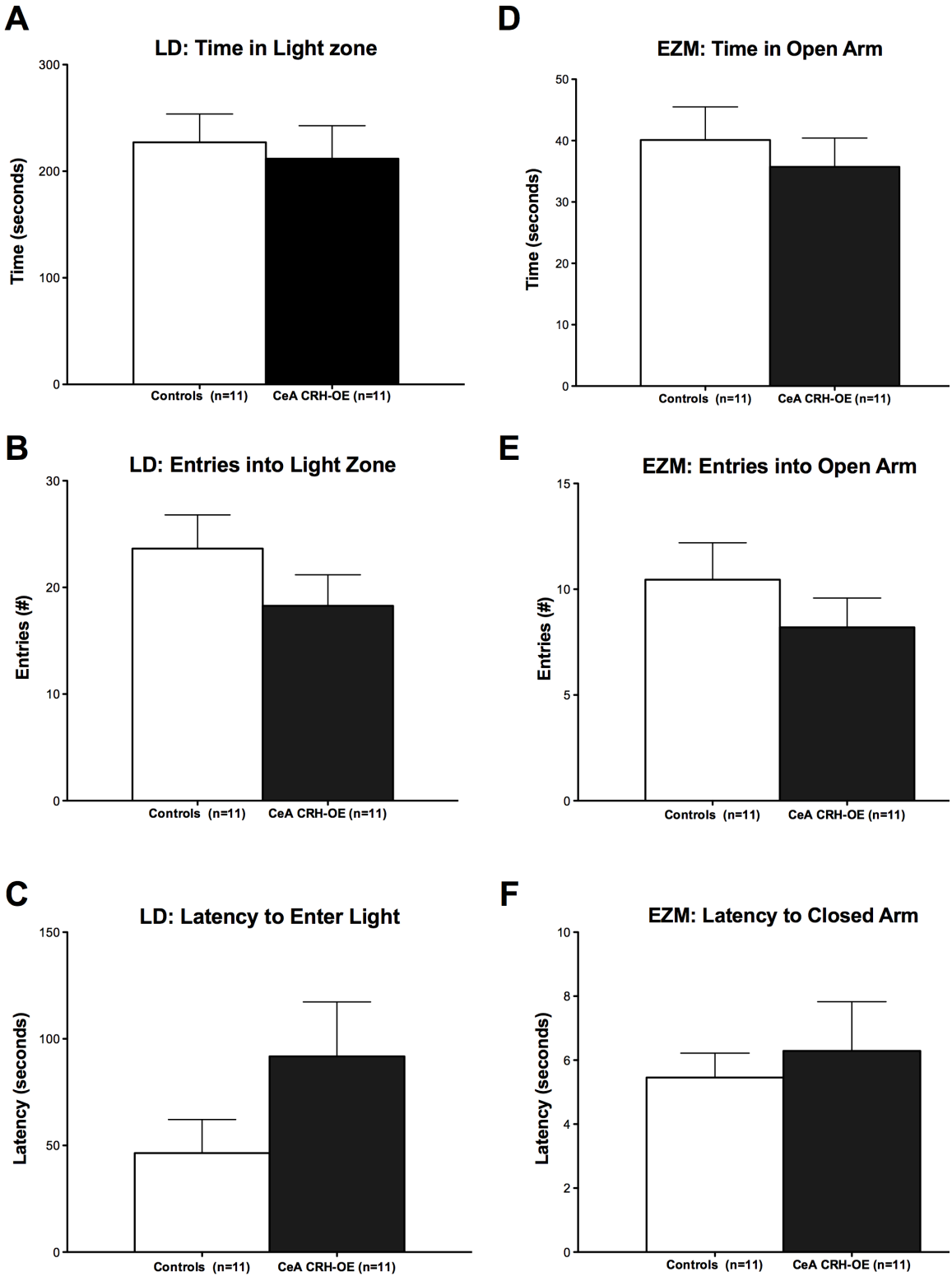

Figure 3.4. Adolescent CeA CRH-OE mice display no anxiety phenotypes in the LD and EZM tests. Adolescent male CeA CRH-OE mice show no anxiety behavior in the LD test in terms of time spent (A), entries (B) and latency (C) into the light zone. No anxiety behavior was observed in the EZM test in terms of time spent (A) and entries (B) into the open arm and no chance in the latency to enter the closed zone (C).

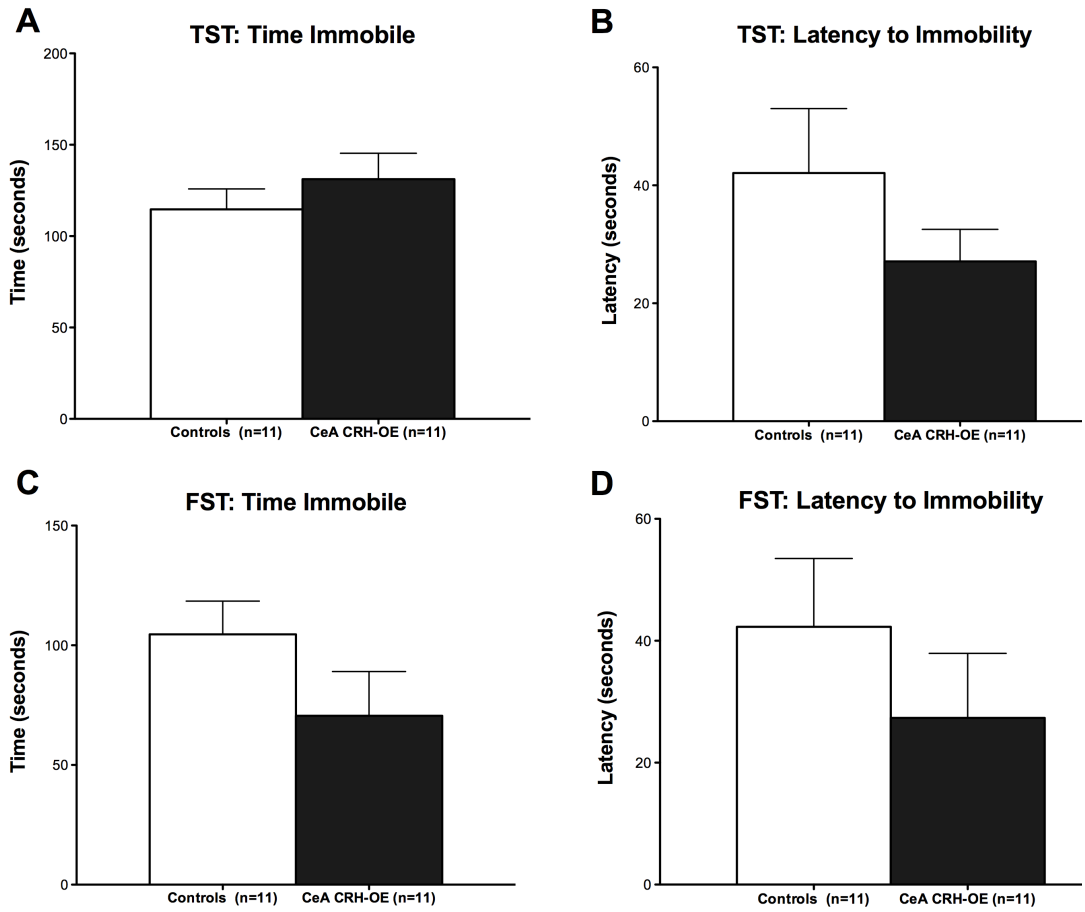

Figure 3.5. Adolescent CeA CRH-OE mice do not display any despair phenotypes in the TST and FST tests. Adolescent male CeA CRH-OE mice show no despair behavior in the TST test in terms of time spent (A) or latency (B) to the immobility. No despair behavior was observed in terms of time spent (C) or latency (D) to the immobility in the FST test.

DISCUSSION

The amygdala plays a key role in generating emotional responses to stress and is coupled with HPA axis hyperactivity. One way the HPA axis responds to stress is through increasing levels of CRH in the CeA. The effects of persistent elevations in CeA CRH in mediating behavioral and neuroendocrine responses to stress remain unclear. Additionally, the effects of transient CeA CRH overexpression during developmentally critical periods, such as adolescence, are unexplored. We hypothesized that transiently elevated CeA CRH during adolescence will lead to anxiety and despair-like behaviors.

The use of tetracycline-inducible transgenic mice models to overexpress CRH during specific periods of development, in combination with stereotaxic injections of viral vectors, allowed for the CeA-specific increases in CRH. Our model for CRH overexpression using CamtTA mice is limited by basal functional changes in CamtTA mice ((50), data not shown). Since we did not observed any behavioral difference in CeA CRH-OE mice compared to controls, it is possible that the elevated CeA CRH may cause adaptations or compensation in CRH signaling through regulation of the availably and activity of CRH receptors. By understanding region-specific functions of CRH in the stress response, the function of the CRH system can be elucidated to determine when a response will either cause a return to homeostasis or a drive towards psychiatric disease.

REFERENCES

1. Boyle MP, Brewer J a, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ. 2005. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc. Natl. Acad. Sci. U. S. A. 102:473–8.

2. Koob GF. 2009. Brain stress systems in the amygdala and addiction. Brain Res. 1293:61–75.

3. Chourbaji S, Gass P. 2008. Glucocorticoid receptor transgenic mice as models for depression. Brain Res. Rev. 57:554–60.

4. De la Mora MP, Gallegos-Cari A, Arizmendi-García Y, Marcellino D, Fuxe K. 2010. Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: Structural and functional analysis. Prog. Neurobiol. 90:198–216.

5. Schulkin J, McEwen BS, Gold PW. 1994. Allostasis, amygdala, and anticipatory angst. Neurosci. Biobehav. Rev. 18:385–96.

6. Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME. 2009. Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. Mol. Psychiatry 14:37–50.

7. De Kloet ER, Joëls M, Holsboer F. 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6:463–75.

8. Krishnan V, Nestler EJ. 2008. The molecular neurobiology of depression. Nature 455:894–902.

9. Van Marle HJF, Hermans EJ, Qin S, Fernández G. 2010. Enhanced resting-state connectivity of amygdala in the immediate aftermath of acute psychological stress☆. Neuroimage 53:348–354.

10. Davis M. 1992. The role of the amygdala in fear and anxiety. Annu. Rev. Neurosci. 15:353–75.

11. Sah P, Faber ESL, Lopez De Armentia M, Power J. 2003. The amygdaloid complex: anatomy and physiology. Physiol. Rev. 83:803–34.

12. Roozendaal B, McEwen BS, Chattarji S. 2009. Stress, memory and the amygdala. Nat. Rev. Neurosci. 10:423–33.

13. Pitts MW, Todorovic C, Blank T, Takahashi LK. 2009. The central nucleus of the amygdala and corticotropin-releasing factor: insights into contextual fear memory. J. Neurosci. 29:7379–88.

14. Koob GF. 1999. Corticotropin-releasing factor, norepinephrine, and stress. Biol. Psychiatry 46:1167–1180.

15. Ulrich-Lai YM, Herman JP. 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. 10:397–409.

16. Weiser MJ, Foradori CD, Handa RJ. 2010. Estrogen receptor beta activation prevents glucocorticoid receptor-dependent effects of the central nucleus of the amygdala on behavior and neuroendocrine function. Brain Res. 1336:78–88.

17. Makino S, Shibasaki T, Yamauchi N, Nishioka T, Mimoto T, Wakabayashi I, Gold PW, Hashimoto K. 1999. Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. Brain Res. 850:136–143.

18. Day HEW, Curran EJ, Watson SJ, Akil H. 1999. Distinct neurochemical populations in the rat central nucleus of the amygdala and bed nucleus of the stria terminalis: Evidence for their selective activation by interleukin-1β. J. Comp. Neurol. 413:113–128.

19. Iwata J, Chida K, LeDoux JE. 1987. Cardiovascular responses elicited by stimulation of neurons in the central amygdaloid nucleus in awake but not anesthetized rats resemble conditioned emotional responses. Brain Res. 418:183–8.

20. Dunn A. 1990. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res. Rev. 15:71–100.

21. Sanders S, Morzorati S, Shekhar A. 1995. Priming of experimental anxiety by repeated subthreshold GABA blockade in the rat amygdala. Brain Res. 699:250–259.

22. Van Bockstaele EJ, Colago EE, Valentino RJ. 1998. Amygdaloid corticotropin-releasing factor targets locus coeruleus dendrites: substrate for the co-ordination of emotional and cognitive limbs of the stress response. J. Neuroendocrinol. 10:743–57.

23. Campbell BM, Morrison JL, Walker EL, Merchant KM. 2004. Differential regulation of behavioral, genomic, and neuroendocrine responses by CRF infusions in rats. Pharmacol. Biochem. Behav. 77:447–55.

24. A W, Bohus B, Koolhaas J. 1993. Corticotropin-releasing hormone microinfusion in the central amygdala diminishes a cardiac parasympathetic outflow under stress-free conditions. Brain Res. 625:219–227.

25. Chalmers DT, Lovenberg TW, De Souza EB. 1995. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific

subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J. Neurosci. 15:6340–50.

26. Perrin MH, Vale WW. 1999. Corticotropin releasing factor receptors and their ligand family. Ann. N. Y. Acad. Sci. 885:312–328.

27. Imaki T. 1996. Corticotropin-releasing factor up-regulates its own receptor mRNA in the paraventricular nucleus of the hypothalamus. Mol. Brain Res. 38:166–170.

28. Van Den Eede F, Van Broeckhoven C, Claes SJ. 2005. Corticotropin-releasing factor-binding protein, stress and major depression. Ageing Res. Rev. 4:213–39.

29. Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS. 1997. Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154:624–629.

30. Kasckow J. 2001. Corticotropin-releasing hormone in depression and post-traumatic stress disorder. Peptides 22:845–851.

31. Banki CM, Karmacsi L, Bissette G, Nemeroff CB. 1992. Cerebrospinal fluid neuropeptides in mood disorder and dementia. J. Affect. Disord. 25:39–45.

32. Banki CM, Karmacsi L, Bissette G, Nemeroff CB. 1992. CSF corticotropin-releasing hormone and somatostatin in major depression: response to antidepressant treatment and relapse. Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol. 2:107–113.

33. Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W. 1984. Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science (80-.). 226:1342–1344.

34. Zobel A. 2000. Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J. Psychiatr. Res. 34:171–181.

35. Keller P a, McCluskey A, Morgan J, O'connor SMJ. 2006. The role of the HPA axis in psychiatric disorders and CRF antagonists as potential treatments. Arch. Pharm. (Weinheim). 339:346–55.

36. Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. 1994. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J. Neurosci. 14:2579–2584.

37. Merali Z, Michaud D, McIntosh J, Kent P, Anisman H. 2003. Differential involvement of amygdaloid CRH system(s) in the salience and valence of the stimuli. Prog. Neuropsychopharmacol. Biol. Psychiatry 27:1201–12.

38. Thompson B. 2004. Corticosterone facilitates retention of contextually conditioned fear and increases CRH mRNA expression in the amygdala. Behav. Brain Res. 149:209–215.

39. Kolber BJ, Roberts MS, Howell MP, Wozniak DF, Sands MS, Muglia LJ. 2008. Central amygdala glucocorticoid receptor action promotes fear-associated CRH activation and conditioning. Proc. Natl. Acad. Sci. U. S. A. 105:12004–9.

40. Rassnick S, Heinrichs S, Britton K, Koob G. 1993. Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res. 605:25–32.

41. Britton KT, Lee G, Vale W, Rivier J, Koob GF. 1986. Corticotropin releasing factor (CRF) receptor antagonist blocks activating and "anxiogenic" actions of CRF in the rat. Brain Res. 369:303–6.

42. Zoumakis E, Rice KC, Gold PW, Chrousos GP. 2006. Potential uses of corticotropin-releasing hormone antagonists. Ann. N. Y. Acad. Sci. 1083:239–51.

43. Merali Z, Khan S, Michaud DS, Shippy S a, Anisman H. 2004. Does amygdaloid corticotropin-releasing hormone (CRH) mediate anxiety-like behaviors? Dissociation of anxiogenic effects and CRH release. Eur. J. Neurosci. 20:229–39.

44. Wiersma a, Baauw a D, Bohus B, Koolhaas JM. 1995. Behavioural activation produced by CRH but not alpha-helical CRH (CRH-receptor antagonist) when microinfused into the central nucleus of the amygdala under stress-free conditions. Psychoneuroendocrinology 20:423–32.

45. Hand GA, Hewitt CB, Fulk LJ, Stock HS, Carson JA, Davis JM, Wilson MA. 2002. Differential release of corticotropin-releasing hormone (CRH) in the amygdala during different types of stressors. Brain Res. 949:122–130.

46. Kolber BJ, Boyle MP, Wieczorek L, Kelley CL, Onwuzurike CC, Nettles S a, Vogt SK, Muglia LJ. 2010. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. J. Neurosci. 30:2571–81.

47. Regev L, Neufeld-Cohen A, Tsoory M, Kuperman Y, Getselter D, Gil S, Chen A. 2010. Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation. Mol. Psychiatry 16:714–728.

48. Carpenter LL, Tyrka AR, McDougle CJ, Malison RT, Owens MJ, Nemeroff CB, Price LH. 2004. Cerebrospinal fluid corticotropin-releasing factor and perceived early-life stress in depressed patients and healthy control subjects. Neuropsychopharmacology 29:777–784.

49. Laryea G, Arnett MG, Muglia LJ. 2012. Behavioral Studies and Genetic Alterations in Corticotropin-Releasing Hormone (CRH) Neurocircuitry: Insights into Human Psychiatric Disorders. Behav. Sci. (Basel). 2:135–171.

50. McKinney BC, Schneider JS, Schafer GL, Lowing JL, Mohan S, Zhao MX, Heng MY, Albin RL, Seasholtz AF, Akil H, Murphy GG. 2008. Decreased locomotor activity in mice expressing tTA under control of the CaMKII alpha promoter. Genes. Brain. Behav. 7:203–13.