Bhave Shreyas, Glucocorticoid Receptor Mediated CRH Gene Repression. Doctor of Philosophy (Biomedical Sciences), September 2015, 140 pages, 27 illustrations, 63 titles.

The hypothalamic pituitary adrenal (HPA) axis is an important neuroendocrine system which mediates the mammalian stress response. Dysregulation of the HPA axis, often due to failed glucocorticoid receptor (GR) mediated negative feedback, is tightly associated with many psychiatric disorders including depression. Glucocorticoids attenuate the activated HPA axis partly by suppressing Corticotropin releasing hormone (CRH) gene (*crh*) expression. Though regulation of *crh* is a critical component of the HPA axis, the molecular mechanisms are poorly understood. In this study, we sought to investigate the molecular mechanism by which GR regulates *crh* expression. The results indicate that Histone deacetylase (HDAC) 1 and methyl CpG binding protein 2 (MeCP2) interact with GR forming a putative complex and that this interaction is GR-ligand, Dexamethasone (Dex), dependent. Furthermore, results indicate that DNA methyltransferase (DnMT) 3b also interacts with GR and this interaction is Dex dependent. Next we tested the role of MeCP2 and DNA methylation in GR mediated *crh* repression. The results suggest that MeCP2 is necessary for maintenance of basal *crh* levels. Decreased levels of MeCP2 are associated with the increased *crh* expression and Dex fails to repress *crh* in the absence of MeCP2. Then we examined the role of DNA methylation in *crh* regulation. The data suggest that Dex increases promoter methylation at specific sites, and that inhibition of methylation at these sites by 5-Aza-2-deoxycytidine (5-AzaDC) is associated with increased expression of *crh*. The results also suggest that inhibition of DNA methylation

abrogates Dex mediated repression of *crh.* In fact, Dex activates *crh* in the face of reduced promoter methylation. While 5-AzaDC and Dex do not alter the protein levels of GR and MeCP2, inhibition of DNA methylation decreases the ability of GR and MeCP2 to occupy the *crh* promoter. Taken together, the data indicate that Dex mediated repression of *crh* is mediated through the formation of a putative complex and requires MeCP2 and site specific promoter methylation. The findings from this study add novel aspects to the molecular mechanism of GR mediated *crh* repression. This will lead to better treatment and management of depression which affects nearly 10% of the US population.

GLUCOCORTICOID RECEPTOR MEDIATED CRH GENE REPRESSION

Dissertation

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

For the Degree of

DOCTOR OF PHILOSOPHY

By

Shreyas Bhave, B. Pharm.

Fort Worth, Texas

September, 2015

ACKNOWLEDGMENT

I would like to express my gratitude and respect to my mentor Dr. Rosalie Uht. I thank her for her guidance, patience, and support. I would like to extend my gratitude to my committee members Drs. Meharvan Singh, Eric Gonzales, Abe Clark, Subhrangsu Mandal and Hassan Alizadeh for their support, valuable time and the guidance they provided throughout my graduation. I am very thankful to Dr. Nathalie Sumien for being an excellent Graduate Advisor. Her support and guidance at each stage was vital for my success.

I thank my colleagues from Uht Lab, Dr. Dharmendra Sharma, Elaine Gregg, Winfred Stacey, Dhwanil Dalwadi and Navita Lopez for their friendship, encouragement and support. I would also like to thank Yang Lab and Clark Lab for their technical help. I am grateful to Dr. Dorota Stankowska from Krishnamoorthy Lab for technical guidance.

I would like to thank my parents for their support, love and encouragement. It was impossible for me to complete this journey without my wife, Amruta. Her understanding and love mean a lot to me. My family is my biggest strength. I am very fortunate to have wonderful friends here in the USA as well as back in India. I am thankful to all of them.

I thank everyone in the Department of Pharmacology & Neuroscience and University of North Texas Health Science Center for helping me achieve my goals. I will cherish this experience for life.

-Shreyas Bhave

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER Page

CHAPTER 1

CHAPTER 2

LIST OF ABBREVIATIONS

5-AzaDC	5-Aza-2-deoxycytidine
ACTH	Adrenocorticotrophic Hormone
AP	Activator Protein
AVP	Arginine-vasopressin
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
CREM	CRE modulator
CREM	Repressor Isoform of CRE Modulator
CRF	Corticotropin Releasing Factor
CRH	Corticotropin Releasing Hormone
CRTC	CREB Protein Regulated Transcription Co-activator
DBD	DNA Binding Domain
Dex	Dexamethasone

GABA	Gamma Amino Butyric Acid
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HDAC	Histone Deacetylase
HPA	Hypothalamic Pituitary Adrenal
HSP	Heat Shock Protein
ICER	Inducible cAMP Early Repressor
LBD	Ligand Binding Domain
MD	Maternal Deprivation
MeCP2	Methyl CpG binding protein 2
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
nGRE	Negative GRE
PKA	Phosphokinase A
POMC	Pro-opiomelanocortin
PVN	Paraventricular Nucleus
SIK	Salt Inducible Kinase
STAT	Signal Transducer and Activator of Transcription
TORC	Transducer of Regulated CREB-Binding Proteins
TSLP	Thymic Stromal Lymphopoietin

CHAPTER 1

Introduction

The mammalian stress response is a culmination of complex neuroendocrine activities. It is a major component of homeostatic mechanisms aimed at maintaining the internal milieu[1]. A response to the external environment is a natural characteristic of all living organisms. The term 'stress response' was first coined by Hans Selye. He defined it as a non-specific response of the body to any noxious stimuli[2,3]. Evolution has shaped the mammalian stress response through the process of natural selection[4]. Formation of a bacterial spore or the more sophisticated "fight and flight" response are two extreme examples of responses to unfavorable environments.

Animals respond to stressful stimuli through various behavioral and physiological changes[5]. There are three major components of a response initiated by an animal during the stress stimulus. This includes a behavioral response, an endocrine response and the autonomic nervous system response[6,7]. All three components are essential for survival. Of these, the endocrine response is mediated through hypothalamic pituitary adrenal (HPA) axis[5].

Dysregulation of the HPA axis is associated with many neuro-pathological conditions such as major depression[8], neuro-degeneration[9], cognitive[10] and other psychiatric disorders[11].

1.a. The hypothalamic pituitary adrenal axis and its regulation

The hypothalamus is a collection of a large number of specialized nuclei, each with its own function. This diencephalic part of the brain is connected to the pituitary gland by the infundibulum on the ventral side[12]. The response to the stress starts with the perception of an unfavorable environment such as pain, danger, inflammation and other. This includes clues from various sensory inputs. The various inputs are integrated and converge at the paraventicular nucleus of the hypothalamus (PVN), which is the starting point of HPA axis[13,14] (Figure 1). The brain centers, that have direct and indirect innervations to the PVN, include the cortex, hippocampus and other parts of the limbic system[15].The PVN is the focal point of Corticotropin releasing hormone (CRH) neurons. Catecholaminergic A2 and C2 neuronal inputs from the nucleus of solitary tract converge onto CRH neurons in the PVN. The PVN also receives direct input from subfornical organ. Many indirect inputs are mediated through GABA-ergic neurons in the peri-PVN area[5,15,16] (Figure 2).

Parvocellular neurons of the PVN synthesize and release CRH and Arginine vasopressin (AVP)[15,17]. Stress stimulates the CRH neurons of the PVN to release CRH into the portal circulation, which carries it to the anterior part of the pituitary. CRH stimulates the pituitary to secrete adrenocorticotrophic hormone (ACTH) into the systemic circulation. ACTH in the systemic blood stimulates the cortex of the adrenal glands to release glucocorticoids (GCs), thus completing HPA axis[5,18]. Released GCs have various actions depending upon the target organ. All these actions are aimed at resisting the imbalance caused to homeostasis due to

stress[19,20]. Along with this, GCs perform a major function of limiting the activity of HPA axis[21]. This is mediated through a negative feedback mechanism by which the end product of a system attenuates its activation.

The action of GCs is mediated through glucocorticoid receptors (GR) and the GR mediated action of GCs is an essential component of HPA axis negative feedback[22]. The GR mediated down regulation of the HPA axis takes place at different levels. GR can down regulate the CRH gene (*crh*) as well as the ACTH gene[22,23]. It also represses the HPA axis at the level of the hippocampus[24]. This GR mediated negative feedback is essential to restrain the HPA axis activity and thus limits the release of excessive GCs[21]. An uncontrolled HPA axis can result in large amounts of GCs secreted into the circulation resulting in deleterious consequences[5]. High levels of GCs are associated with energy imbalance, immune system failure, faulty lipid metabolism, etc.[19]. This dysregulated HPA axis is primarily due to failed GR mediated negative feedback[8]. CRH positive neurons in the PVN of the hypothalamus are a major site for GR mediated negative feedback. In summary, GR mediated down regulation of *crh* plays an important role in limiting the activity of the HPA axis and maintenance of homeostasis[23].

1.b. Corticotropin releasing hormone (CRH)

CRH, or Corticotropin releasing factor (CRF), as it was first termed, was characterized by Vale et al. in 1981[25]. His group was the first to isolate, purify and test the 41-amino acid protein from bovine hypothalamic extracts. They found that this peptide was able to stimulate the release of corticotropin (ACTH) from cultured anterior pituitary cells, thus its name. They went on to synthesize and sequenced the CRH peptide. The rat and human CRH peptide have identical sequence and slightly differ from bovine peptide[25].

The CRH is expressed in major areas of central nervous system (CNS). It is also expressed in peripheral organs such as lymphatic organs and the placenta[26,27]. In the CNS, it is expressed in cortex, hippocampus, amygdala and hypothalamus[17].

1.c. Corticotropin releasing hormone gene regulation

CRH gene (*crh*) expression is a tightly regulated process that is essential to the maintenance of HPA axis homeostasis[23]. Two regions of the brain that express CRH and respond to stress stimuli are the PVN and central nucleus of the amygdala. Earlier studies have shown that these two groups of CRH neurons respond differently to the elevated levels of glucocorticoids[28-30]. Both these nuclei are important in the management of the stress response. CRH positive neurons in the PVN receive inputs from various parts of the brain which are then integrated and converge onto *crh*. In the amygdala CRH plays an important role in fear and anxiety associated with stress response[28-30]. The result of these inputs is altered gene expression.

Molecular mechanism of *crh* stimulation

Many molecular mechanisms of *crh* up-regulation or stimulation are due to the cyclic AMP (cAMP) signaling pathway and are well understood[15] (Figure 3A). Various external stimuli, such as neurotransmitters, lead to increased levels of cAMP in CRH neurons. This in turn activates the proteinkinase A (PKA)-mediated signaling cascade. Activated PKA translocates into the nucleus and phosphorylates the cAMP response element binding (CREB) protein[15]. pCREB then binds to the cAMP response element (CRE) present in the proximal promoter of *crh*. The CRE is a major component of the promoter which mediates transcriptional up-regulation and is located at -224bp upstream of transcription start site (Figure 4A). The CREB protein recruits the CREB binding

protein (CBP), which is a histone acetylase. Histone deacetylation leads to opening of chromatin and activation of the gene. The phosphorylation-mediated activation of the CREB protein is necessary but not sufficient for *crh* activation[31].

Along with CBP, CREB protein activity also depends on the availability of another co-activator. CREB protein regulated transcription co-activator 2 (CRTC2), or transducer of regulated CREB activity (TORC2) as it was known earlier, is a co-activator of CREB protein. Dephosphorylation and eventual translocation of CRTC2 to the nucleus is critical for CREB mediated *crh* activity. A study by Liu et al. suggested that CRTC2 or TORC2 phosphorylation and its presence in the nucleus plays an important role in cAMP dependent stimulation of *crh* activity[32] (Figure 3B).

Regulation of phosphorylation of CRTC2 is mediated by a network of kinases and phosphatases. Liu et al. suggested that the salt inducible kinases (SIK) 1 and 2 are involved in CRCT2 phosphorylation[33]. A study by Jeanneateau et al. showed that the phosphatase calcineurin also plays a role in this process. The Jeanneateau study further suggested that glucocorticoids regulate the phosphorylation of CRTC2 and hence its nuclear-cytoplasmic distribution. In the neurons of mice treated with the synthetic glucocorticoid, dexamethasone (Dex), CRTC2 is hyper-phosphorylated and thus restricted to the cytoplasm[34].

Molecular mechanisms of *crh* repression

The molecular mechanism of positive regulation of *crh* is an extensively studied and well understood process. On the other hand the molecular mechanisms of *crh* down regulation are poorly understood. Though the response to stress in animal models and subsequent negative feedback by glucocorticoids is well known[21], the underlying mechanisms remain elusive. The

crh promoter lacks a consensus glucocorticoid response element (GRE)[35,36]. Earlier studies by Malkoski et al. of the human *crh* promoter have shown that distinct regions are involved in gene activation and repression[35]. A conserved CRE at -224 bp relative to the transcription start site is the main locus of gene activation. On the other hand, the composite negative GRE (nGRE) present at -278 to -249 bp is important in gene repression (Figure 4A)[35,36]. Ligand bound GR (GR holoreceptor) binds to the *crh* promoter at this region. More recent studies in rat hypothalamic immortalized cells have also shown binding of GR in this region of *crh* promoter following Dex treatment [37-39].

There are two possible components of the mechanism of GR mediated *crh* regulation – genomic and non-genomic. Genomic regulation assumes direct or indirect interaction of the GR in the environment of the *crh* promoter followed by chromatin modification, which leads to gene repression. The non-genomic mechanism involves the regulation of kinases and phosphatases, which in turn controls the activation and/or nuclear translocation of transcription factors and co-regulators involved in *crh* regulation.

Role of chromatin modification in *crh* regulation

Here the term "chromatin modification" refers to the covalent changes in histone proteins and DNA (chromatin) which lead to alterations in gene expression[40]. This includes methylation of DNA and modifications to histone proteins. The *crh* promoter has a CpG island which plays an important role in its regulation of gene[41,42] (Figure 4B). A CpG island is a region of DNA where the GC content of DNA is more than 50%. Thus it is a cluster of CpG dinucleotides (Figure 4B). *In vivo* studies in the mouse and rat have shown that exposure to stress is associated with decreased methylation of the *crh* promoter and subsequent increased gene expression[41,42]. Also,

the CRH mRNA level is elevated following treatment with the DNA methyltransferase inhibitor 5-Aza-2-deoxycytidine (5-AzaDC) in mouse hypothalamic N42 cells[42]. The role of DNA methylation in *crh* regulation is also evident from the experiments done in maternal deprivation (MD) model of stress in rats. According to Chen et al., animals exposed to MD after birth have elevated levels of hypothalamic CRH mRNA, following restraint stress in adulthood. These elevated levels correspond to reduced methylation of CpG dinucleotides in the *crh* promoter[41].

Similar to DNA modifications, covalent changes of histone tails also influence *crh* regulation[37]. These changes include acetylation and methylation of specific histone residues. In general, histone acetylation is associated with gene activation, and histone methylation is associated with the gene repression; both modifications appear to be at play in the *crh* promoter. Sharma et al. have shown that, in IVB cells, Dex exposure increases histone 4, and not histone 3, acetylation in the region of the *crh* promoter[37]. This change in acetylation coincides with the recruitment of histone deacetylase (HDAC) 1 to the promoter[37]. Although increased acetylation and gene repression appear to be incongruous, it is possible that the opening of chromatin actually allows entry to the co-repressors which further modify chromatin to repress the gene.

Methylated DNA recruits many proteins that are involved in gene repression. Methyl CpG binding protein 2 (MeCP2) binds to methylated CpG and forms a co-repressive complex with HDACs to repress the gene [43,44]. The role of MeCP2 in *crh* regulation has been demonstrated through the mouse model of Rett syndrome[45]. Truncated MeCP2, as is the case in Rett syndrome, is associated with elevated levels of CRH mRNA in the PVN of transgenic mice. This increase in gene expression is associated with significantly lower promoter enrichment of MeCP2. Interestingly, transgenic mice with truncated MeCP2 show no change in *crh* promoter methylation. Secondary ChIP experiments in wild type mice revealed that in the hypothalamic

region MeCP2 is associated with methylated histone (Dimethyl H3K9), which is a marker for repressed promoter, but not with acetylated histone[45]. Taken together, chromatin modifications play important roles in regulation of *crh.*

Non genomic mechanisms of *crh* regulation

A body of literature supports the existence of non-genomic mechanisms by which negative feedback operates. This school of thought comes from the earlier studies done in $GR^{dim/dim}$ mice. $GR^{dim/dim}$ mice carry a point mutation in GR that prevents GR binding to the DNA[46]. These mice do not show any changes in *crh* expression, but ACTH repression by GCs is aborted. These findings suggested that the GR regulation of ACTH is DNA-binding dependent while *crh* regulation does not require genomic action of GR. Recent studies have also shown that following stress there is no GR enrichment of the *crh* promoter region in hypothalamus[47]. One study also suggests that GR differentially regulates CRH mRNA and heteronuclear RNA suggesting a role of GR at the post-transcription stage[48].

As mentioned earlier, GCs can regulate *crh* through modulation of regulatory proteins[33,34]. A study by Shepard et al. indicates that the GC mediated decrease in *crh* is mediated through the repressor isoform of CRE modulator (CREM) inducible cAMP early repressor (ICER). Those authors suggest that increased GCs following restrain stress in rats, is associated with marked increase in ICER in the PVN region and increased recruitment of CREM to the *crh* promoter. This is in parallel with decreased polymerase II binding to the *crh* promoter[49]. Subsequent studies showed that ICER plays an important role in limiting stimulated *crh* and has no effect on basal levels of *crh*[31,50].

In summary, the *crh* gene is regulated through multiple molecular mechanisms. Some mechanisms are involved in the regulation of basal levels while others are important in case of limiting stimulation. *crh* activation is mediated through CRE, its repression and maintenance of basal levels are mediated through the proximal nGRE and DNA methylation. In this study, we have analyzed molecular mechanisms of *crh* repression and the interplay between DNA methylation and GR recruitment to the promoter. Taken together, the available literature suggests that the regulation of *crh* takes place through multi-modal mechanisms, which allows fine tuning of the stress response and maintenance of homeostasis. Since all stress stimuli converge on to the *crh* neurons in the PVN and it is also a primary target of negative feedback, understanding the GR mediated molecular mechanisms that regulate *crh* is extremely important.

1.d. Glucocorticoid Receptors (GR)

GR is the 1st member of group C of 3rd nuclear receptor subfamily (NR3C1). It is encoded by NR3C1 gene present on the 5th chromosome in humans and on the 18th chromosome in mouse and rat. The gene has 9 exons and 8 introns. It has a DNA binding domain (DBD) consisting of two zinc fingers and ligand binding domain (LBD) (Figure 5)[51-53]. The DBD is present at the center of the protein while LBD is at the C-terminal of the protein. The prototypic GR-DNA interaction involves GR dimerization and subsequent zinc finger interaction with a major groove of a DNA helix. GR is expressed throughout the body and it acts as a transcription factor that regulates a plethora of genes[54]. It is widely expressed in the cortex, mid brain region and limbic system[55,56]. There is a dominant negative form, GRβ, which arises from variation in splicing[57]. This study, however focuses on the GRα isoform or simply GR.

1.e GR mediated gene regulation

GR is a major ligand induced transcription factor. The endogenous ligands for GR are corticosteroids released by the adrenal glands. In the absence of ligand, GR is sequestered in the cytoplasm by heat shock proteins (HSPs). The binding of ligand leads to conformational changes and dissociation from HSPs which leads to translocation of GR to the nucleus[52,58]. In the nucleus, the GR homodimerizes and binds DNA elements known as Glucocorticoid Response Elements (GREs)[59,60]. This is followed by recruitment of co-regulators, which then alter gene expression[58].

GR has a broad range of target genes. These include inflammatory genes, genes related to metabolism and genes related to HPA axis[61,62]. There are many mechanisms which have been proposed to explain the spectrum of GR action. GR mediated activation of a gene results from of GR holoreceptors binding to a palindromic response elements-GREs[63]. This is followed by co-activator recruitment to activate transcription[58] (Figure 6A). Alternatively GR can interact with other transcription factors and activate genes through composite GREs (Figure 6B) or by tethering mechanism where GR does not directly interact with DNA (Figure 6C). For example the Beta –casein gene is induced through the GR interaction with the signal transducer and activator of transcription (STAT)5[51,63]. However GR mediated trans-repression of genes is more complicated and sometimes involves multiple mechanisms.

Gene repression mediated through nGRE

The association of ligand bound GR with a gene promoter without a consensus GRE, gave rise to the concept of negative elements or nGREs. The first example was of that of the prolactin gene. The down regulation of prolactin gene expression by ligand bound-GR is mediated through an nGRE[64]. Subsequent studies identified many genes with such nGREs. For example the pro-

opiomelanocortin gene (*POMC*), which encodes the precursor protein for ACTH, is repressed by GRs through nGREs present in the promoter[65]. In this mechanism, nGREs recruit ligand activated GR and favor transcriptional repression (Figure 6D). Another example of such nGRE mediated repression includes the thymic stromal lymphopoietin gene (TSLP) promoter[66]. Many other genes show the presence of putative nGREs such as osteocalcin, keratin, Rev-ErbA, insulin and insulin receptor gene in human and mouse[66-68]. GR also represses genes by preventing binding of transcription factors such as CREB to the gene promoter. In case of glycoprotein hormone α subunit gene, which is positively regulated by cAMP mediated activation, GR prevents binding of CREB to the promoter[69]. Prevention of CREB binding by GR leads to gene repression[69]. This mechanism operates via a competitive nGRE (Figure 6E).

GR can also lead to the negative regulation of a gene via the interaction with other transcription factors. Reporter assay studies on collagenase I promoter have shown that GR interaction with activator protein (AP)-1 results in repression of GR regulated genes[70-72]. The GR mediated repression of inflammatory genes such as interleukin 6 is also mediated through this mechanism, where GR interacts with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and represses inflammatory genes[73]. In this mechanism GR represses a gene without direct DNA binding and through interaction with AP-1 or NF-kB[70-73] (Figure 6F)

Interestingly, many genes which are negatively regulated by GR are also regulated by DNA methylation. For example, the osteocalcin gene, which is repressed by GR through nGRE, is regulated by methylation of CpG nucleotides in its promoter[67,74]. Conversely reduced CpG methylation is associated with increased expression of osteocalcin gene in rat osteoblasts. A second example includes the TSLP gene, which is transcriptionally activated by 5-Azacytidine mediated inhibition of DNA methylation[75]. A third example includes *crh* in the PVN

of the hypothalamus. This gene is derepressed by decreased methylation of CpG nucleotides and repressed by GR in the hypothalamus[41,42].

Remarkably, there is no study that has examined the relation between DNA methylation and GR mediated negative regulation of these genes. Here, we demonstrate the interdependence of GR action and promoter DNA methylation. The findings of this study reveal a novel of molecular mechanisms of GR mediated negative regulation of *crh*.

1.f Importance and significance

Depression is an important public health problem because of its impact on socioeconomic status and physical health. According to Centers for Disease Control and Prevention (CDC), one in 20 adults in the United States is affected by a depressive disorder[76]. Major depression is among top mental disorders in U.S. adolescents and its prevalence is three times higher in females as compared to males[77-79].

An over activated HPA axis is tightly associated with major depressive disorder. The association of a dysregulated HPA axis and depression was documented over half a century ago. The combined Dex/CRH test on depressed individuals and other clinical studies corroborate that GR mediated negative regulation is impaired in depressed individuals[80]. Though the failure of GR-mediated negative regulation, which maintains the HPA axis, is tightly associated with depressive disorders, the focus of treatment for depression has been the levels of monoamines. Currently available drugs mainly act by increasing levels of monoamine such as nor epinephrine, dopamine and serotonin in the brain[81] and not directly targeting HPA axis. Furthermore, 40-50% of depressed patients do not respond to conventional medicines this group is called as treatment-refractory depression[82]. Understanding the molecular mechanisms by which GR regulates *crh*

expression, which is a major component of HPA axis, will reveal newer targets for development of medicines for treatment of depression.

The purpose of this study was to advance our understanding of the molecular basis of GR mediated *crh* regulation. In this project we have investigated the molecular mechanisms by which GR regulates *crh.* The overarching hypothesis of this study is that GR-mediated repression of *crh* involves formation of a co-repressor complex and chromatin modifications. We have investigated the role of DNA methylation and how it affects the ability of GR to repress *crh*. We first examined the formation of GR mediated co-repressor complex and determined its role in *crh* repression. Then we examined the role of MeCP2 in Dex mediated repression and maintenance of basal *crh* levels. Finally, we investigated the role of DNA methylation in regulating *crh* and how it affects the GR mediated repression of *crh*. Our findings reveal a novel molecular mechanism by which GR regulates *crh*.

Figures and legends

Figure 1. Schematic representation of Hypothalamic-Pituitary-Adrenal axis.

Schematic representation of HPA axis and negative feedback regulation. Black arrows represent activation, blue lines represent inhibition. CRH, Corticotropin releasing hormone; ACTH, Adrenocorticotrophic hormone; PVN, Paraventricular nucleus of hypothalamus

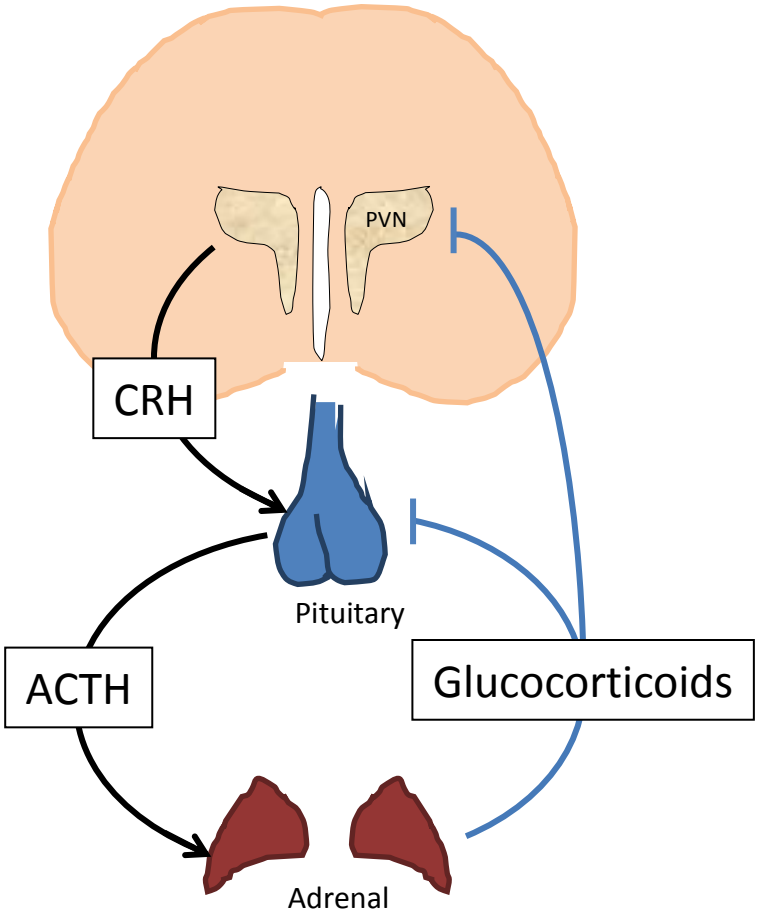
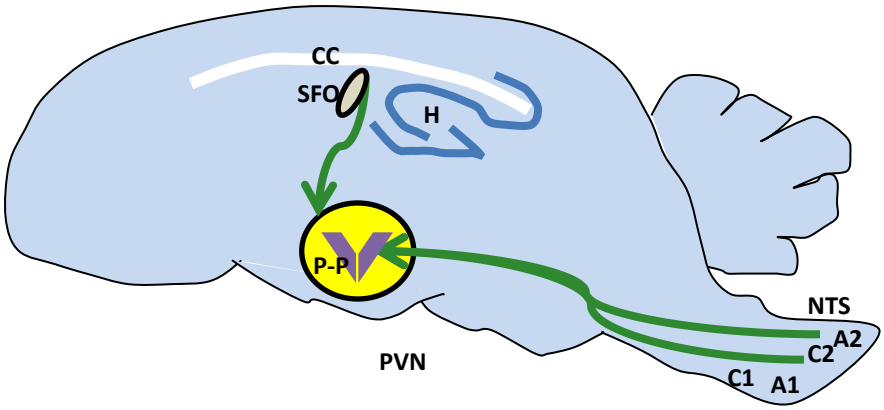

PVN
CRH
Pituitary
ACTH
Glucocorticoids
Adrenal

Figure 2. Neuronal inputs to the Corticotropin Releasing Hormone neurons.

A, Schematic of a rat sagittal brain section showing direct neuronal innervations (green lines) to CRH neurons (purple) in the PVN from other areas of the brain (Adapted from Aguilera G, 2012). B, A model showing direct and indirect neuronal inputs to CRH neurons in the PVN (Adapted from Levy B. and Tasker J., 2011). (+) sign indicates activation, (-) sign indicates inhibition. cc, Corpus callosum; HI, Hippocampus; SFO, subfornical organ; PVN, Paraventricular nucleus of hypothalamus; P-P, peri-PVN; NTS, nucleus of the solitary tract; 3V, 3rd Ventricle; CRH, Corticotropin releasing hormone; NE, Norepinephrine; GLU, Glutamate; GABA, ɤ-amino butyric acid.

A



B

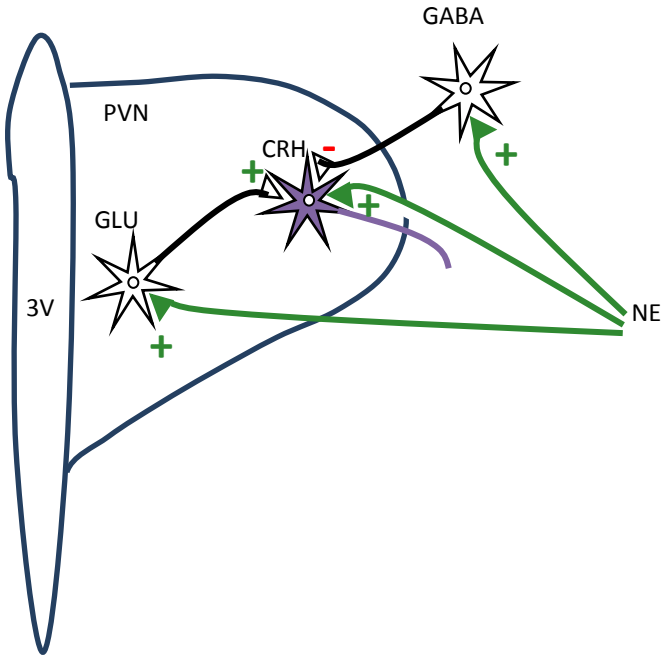
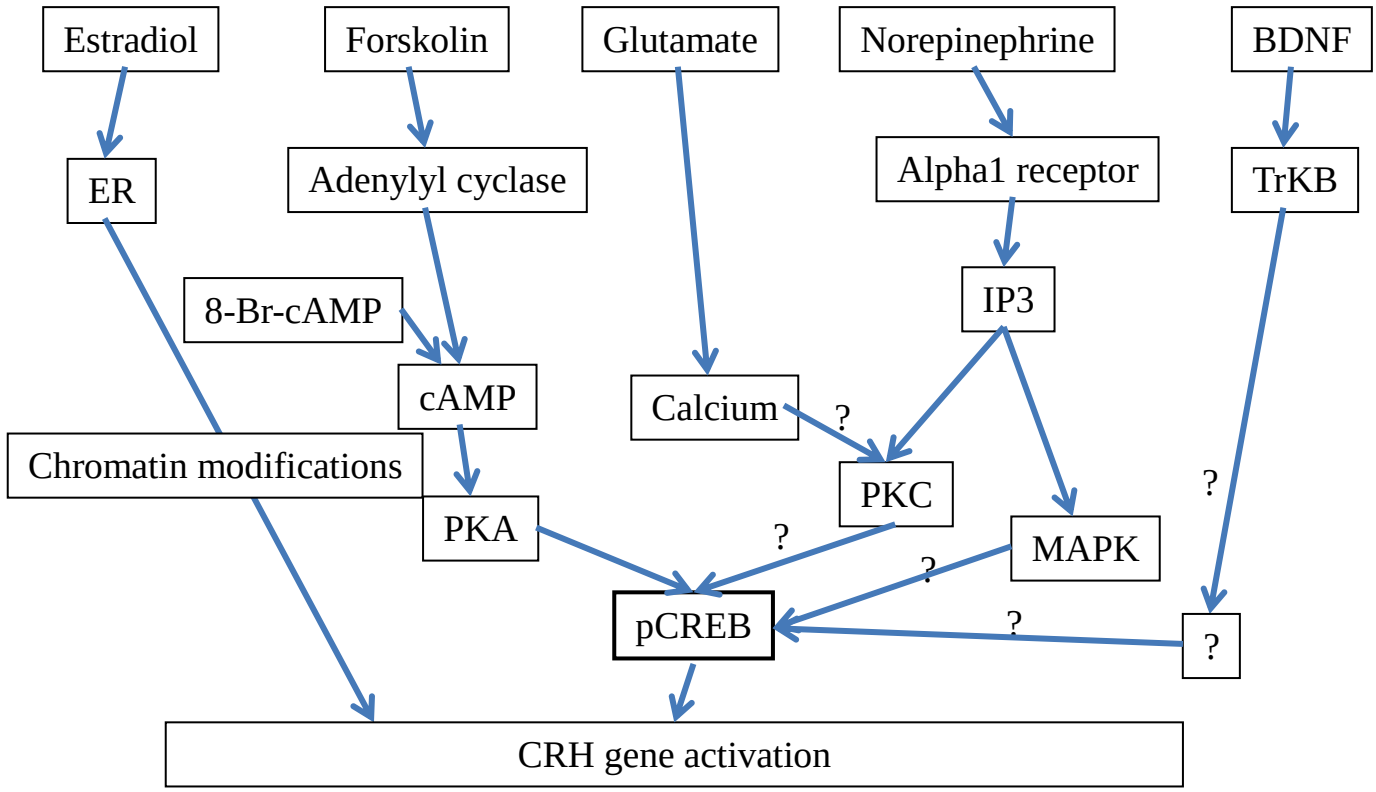


Figure 3. Positive regulation of CRH gene.

A, External stimuli and cellular pathways those lead to the activation of CRH gene in hypothalamus. Question marks indicate possible, but unknown links. Arrows indicate activation or increased levels. B, cAMP mediated molecular pathway depicting activation of CRH gene. BDNF, Brain derived neurotrophic factor; ER, Estrogen receptor; TrKB, Tyrosine-related kinase B receptor; cAMP, cyclic Adenosine mono-phosphate; 8-Br-cAMP, 8-Bromo cAMP; IP3, Inositol tri-phosphate; PKC, Protein Kinase C; MAPK, Mitogen activated protein kinase; PKA, protein kinase A; CREB, cAMP response element (CRE) binding protein; CBP, CREB binding protein; CRTC, CREB protein regulated transcription co-activator; P: phosphorylated form.

A


Estradiol
Forskolin
Glutamate
Norepinephrine
BDNF
ER
Adenylyl cyclase
Alpha1 receptor
TrKB
8-Br-cAMP
IP3
cAMP
Calcium
?
Chromatin modifications
PKC
?
PKA
?
MAPK
?
pCREB
?
?
CRH gene activation

B

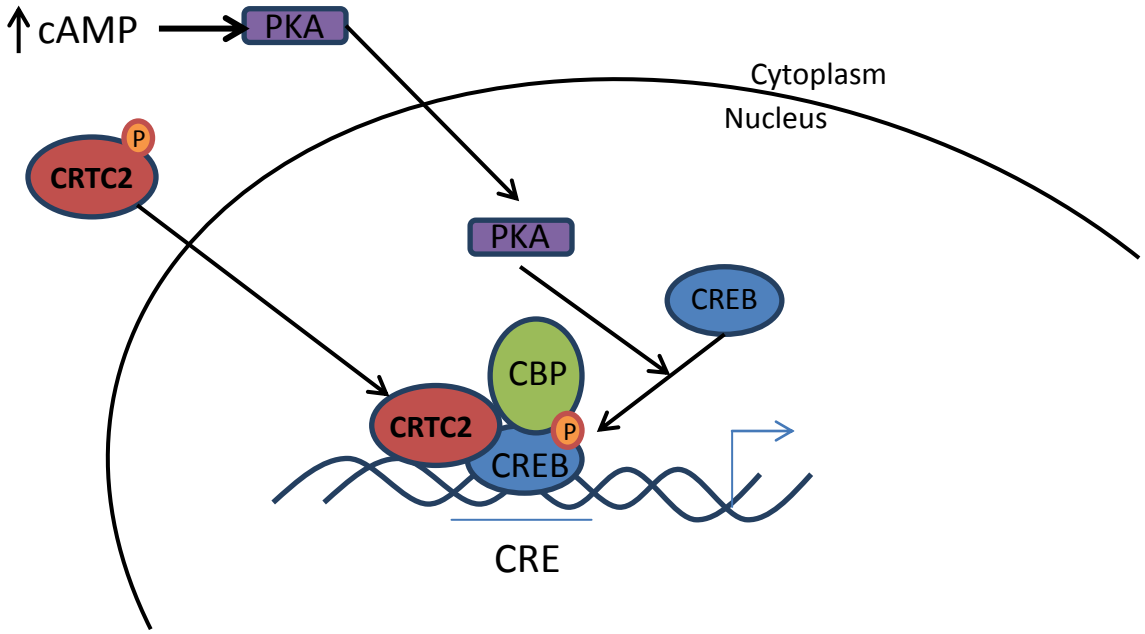

cAMP
PKA
Cytoplasm
Nucleus
P
CRTC2
PKA
CREB
CBP
CRTC2
P
CREB
CRE

Figure 4. CRH proximal promoter region.

A, Proximal promoter sequence of CRH gene showing response elements and CpG di-nucleotides. B, Graph showing GC percentage of DNA in CRH proximal promoter with CpG island (Blue). nGRE, Negative Glucocorticoid response elements; CRE, cAMP response elements; TSS, Transcription start site.

A

AATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTTTAGGGCT**CG**[1] TTGA**CG**[2] TCACCAAG

nGRE

CRE

GAGG**CG**[3] ATAAATATCTGTTGATATAATTGGATGTGAGATTCAGTGTTGAAATAGCAGAACCCTGTCC

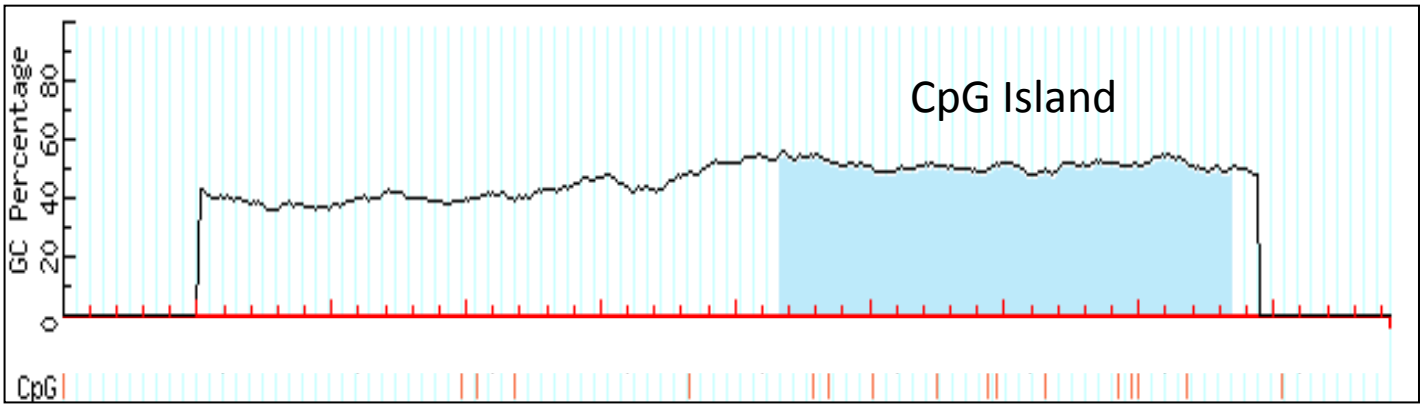
CT**CG**[4] CTCCTTGGCAGGGCCCTATTATTTATGCAGGAGCAGAGGCAGCA**CG**[5] CAAT**CG**[6] AGCTGT

CAAGAGAG**CG**[7] TCAGCTTATTAGGCAAATGCTG**CG**[8] TGCTTTCTGAAGAGGGT**CG**[9] A**CG**[10] TTA

TAAAATCTCACTC**CG**[11] GGCTCTGGTGTGG**AGAA**

TSS

B



-277bp +4bp

crh proximal promoter

Figure 5. Structural aspects of glucocorticoid receptor protein.

Diagram of different regions of Glucocorticoid receptor protein, including the zinc finger region in the DNA binding domain (DBD). LBD, ligand binding domain; AF1, ligand binding independent activation domain; AF2, ligand binding dependent activation domain; N- Amino terminal, C- Carboxy terminal. DBD showing two zinc fingers which interact with DNA.

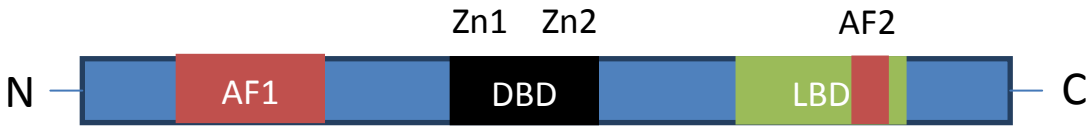
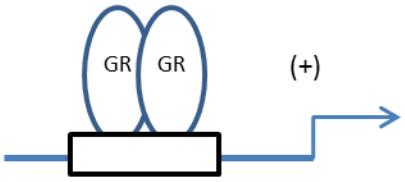

Zn1
Zn2
AF2
N
AF1
DBD
LBD
C

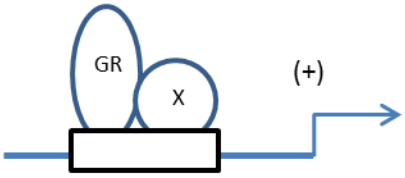
Figure 6. Mechanisms of glucocorticoid receptor mediated gene regulation.

A and C, transactivation; D-G, transrepression. GR, Glucocorticoid receptors; X, Transcription factors and co-regulators. A, Classical GR mediated activation of gene through binding of dimerized GR to GRE. B, C, GR interacts with regulatory proteins thus activating gene through composite GRE or tethering mechanism; D, Dimerized GR repressing gene through nGRE binding; E, GR preventing activator from DNA binding, hence inhibiting the gene by competitive inhibition; F, G, GR interacts with other regulatory proteins and thus repressing gene through tethering or composite mechanism.(Adapted from Newton R, *Thorax*, 2000)

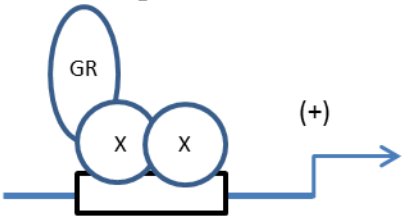
A Simple GRE



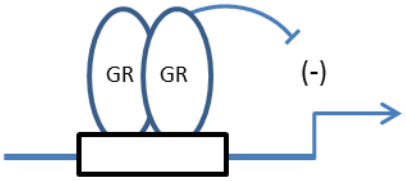
B Composite GRE



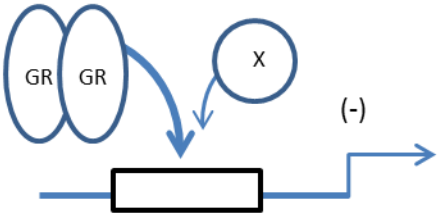
C Tethering GRE



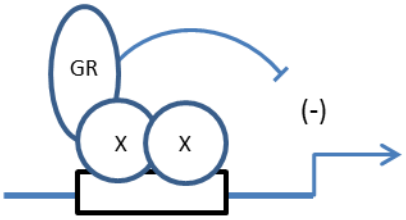
D Simple nGRE



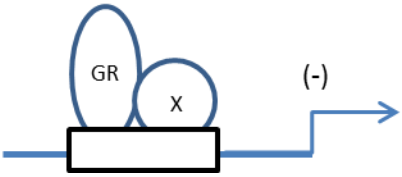
E Competitive inhibition



F Tethering nGRE



G Composite nGRE



CHAPTER 2

Glucocorticoid receptor mediated CRH gene repression involves formation of a co-repressor complex

a. Introduction

The focus of the first part of this study was to determine whether GR can form a co-repressor complex which mediates *crh* repression. A study by Miller et al. suggested that HDAC1 participates in the GR mediated repression of *crh* and not HDAC3[37]. Studies have shown that HDAC1 forms a co-repressive complex with MeCP2 causing repression of many genes[43,44]. Thus, we tested the hypothesis that GR forms a putative complex with HDAC1 and MeCP2 in context of *crh* repression and that the interaction is ligand dependent. Since *crh* expression is regulated by promoter methylation[41,42] and MeCP2 occupancy[45,83], we further asked if DNA methylating enzymes such as DnMT3a and 3b are part of this complex.

The second part of this study was designed to investigate the role of MeCP2 in GR mediated *crh* regulation. MeCP2 plays critical role in maintaining the basal level of *crh* expression. Increased as well as decreased functional availability of MeCP2 is associated with up regulation of *crh*[45,83,84]. What role MeCP2 plays in the GR mediated maintenance of the *crh* gene is not known. siRNA mediated MeCP2 knockdown was utilized to understand the role of MeCP2 in GR mediated regulation of *crh* expression.

The data suggest that GR forms a complex with HDAC1, MeCP2 and DnMT3b in ligand dependent manner. GR interaction with the DnMT3b isoform was specific and ligand dependent. The results also indicate that MeCP2 which is part of the putative complex is essential for maintenance of basal levels of *crh* and repression of *crh* mediated by GR.

Choice of cell line

The hypothalamus has a heterogeneous population of cells. Cells in different nuclei have different characteristics. In most cases the boundary between these nuclei is blurred with a mixed population of cells[85]. Thus, there are very few cell lines derived from the hypothalamus that represent PVN nucleus. Of these, one of the earliest developed was the IVB cell line. IVB cells are derived from hypothalamus of 18 day male rat embryo. These cells were immortalized by transfecting them with SV-40 large T antigen followed by clonal selection. These cells have properties of the parvocellular neurons of the PVN region of the hypothalamus[86]. IVB cells express CRH and AVP mRNA. They show immunoreactivity for GR, and express functional type 1 CRH receptors[87]. IVB cells have been used previously to study the *crh* regulation[33,37,38,50,88]. They were used in all the experiments described in this dissertation.

b. Results

Dex Treatment Decreases the Expression of *crh* in IVB Cells.

First the effect of GR agonist (Dex) on the CRH expression was re-assessed. The previously reported concentration (10^{-7}M) of Dex and duration of exposure (2hrs) were used[37]. The *crh* expression was measured by RT-qPCR analysis. The data indicate that Dex decreases the CRH mRNA expression level by 40%, (Figure 7A) and hnRNA levels by 30%, (Figure 7B). For all future experiments hnRNA levels were used as a measure of gene expression. To understand whether Dex effect is limited to transcriptional regulation, we analyzed whether Dex also decreases the CRH protein levels. Immunocytochemistry (ICC) reveals that following 2 hrs exposure to Dex, CRH immunoreactivity was decreased (Figure 7C).

Localization of GR, MeCP2, and HDAC1

GR translocation from cytoplasm to nucleus in the presence of ligand is well known. To confirm this and to determine if Dex alters the cellular translocation of HDAC1 and MeCP2, western blot analysis was done on nuclear and cytoplasmic fractions of cell extracts. As expected, Dex exposure led to translocation of GR from the cytoplasm to the nucleus. HDAC1 and MeCP2 were nuclear irrespective of Dex treatment (Figure 8). Western blot analysis of MeCP2 revealed two bands. The MeCP2 gene is known to be alternatively spliced in humans[89,90] and rats[91], and the bands are presumed to correspond to previously reported isoforms, MeCP2-e1 and MeCP2-e2[91,92].

Dex treatment increases the association of GR with HDAC1 and MeCP2.

To determine the degree to which GR associates with HDAC1or MeCP2, to form a complex, co-immunoprecipitation analyses were performed. These analyses revealed that Dex enhanced interactions of GR with HDAC1 by more than two fold, (Figure 9A) and with MeCP2 by more than three fold, (Figure 9B). This indicates that Dex induces a formation of complex consisiting of GR, HDAC1 and MeCP2 which are recruited to the *crh* promoter region[39].

Dex Induces GR Interaction with DnMT3b and not DnMT3a

Given that MeCP2 binds to methylated CpG dinucleotides and DnMTs are involved in such methylation[93], so we sought to determine whether or not DnMTs are associated with the GR and/or are present in the same region of the *crh* proximal promoter as evaluated above. An interaction between GR and DnMT3a was not observed. The bands in Figure 10A, IP, are not specific. In contrast, GR interacted with DnMT3b, and the interaction was increased by treatment with Dex by more than two fold, (Figure 10B). Consistent with Co-IP data, ChIP analysis showed that Dex increased association of DnMT3b and not -3a with *crh* proximal promoter region as reported in Sharma et al. 2013

siRNA mediated MeCP2 protein knockdown

In order to understand the importance of MeCP2 in GR mediated repression, short interference (si) RNA mediated protein knockdown was used. We tested three different siRNA sequences provided by the manufacturer (OriGene) for their efficiency to knock down MeCP2 protein expression. A scrambled RNA duplex was used as control. All three siRNA sequences (Table 3) significantly reduced protein expression of MeCP2 72 hrs post transfection. siRNA labeled as

siMECP2C showed maximum (~50%) reduction in protein levels (Figure 11). Scramble control had no effect on MeCP2 protein levels. This siRNA (relabeled as siMeCP2) was used in all subsequent experiments.

MeCP2 knockdown is associated with increased *crh* expression

To examine the effect of MeCP2 protein depletion on *crh* expression, we measured CRH hnRNA levels in cells transfected with scrambled or siMeCP2 by RT-qPCR. Decreased MeCP2 protein levels (Figure 12A) are associated with an almost 3.5 fold increase in the levels of *crh* hnRNA transcripts, (Figure 12B).

MeCP2 knockdown leads to failure of Dex to mediate *crh* repression

The effect of MeCP2 knockdown on Dex mediated repression of *crh* was examined by treating scrambled or siMeCP2 transfected cells with Dex. siMeCP2 transfection increased expression of CRH hnRNA irrespective of the presence of Dex by at least 2 fold (Figure 13). Dex failed to repress the hnRNA expression in the presence of scramble siRNA. This may be due to transfection process.

c. Discussion

The results suggest that the GR holoreceptor forms a putative complex with HDAC1 and MeCP2 along with DnMT3b. The study by Sharma et al. showed that the formation of this complex coincides with increased recruitment of GR, HDAC1, MeCP2 and DnMT3b to the *crh* promoter region[39]. These findings are consistent with previous reports that indicate involvement of HDAC1 and MeCP2 in *crh* regulation[37,45,83]. The formation of this putative complex at the *crh*

promoter occurs in parallel with a decreased *crh* expression at mRNA, hnRNA as well as protein level. These data indicate that GR mediated repression of *crh* involves ligand induced formation of the complex. The results from the MeCP2 knockdown experiments suggest that it is required for maintenance of basal levels of *crh*. Furthermore, loss of MeCP2 prevents Dex mediated repression of *crh* suggesting that it is necessary for GR mediated action. The results underscore the significance of MeCP2 in the regulation of *crh* expression[45,83]. The formation of co-repressor complex and its recruitment to the *crh* promoter is associated with increased methylation of histone residues[39]. The results from this study support the findings of McGill et al. which suggest that MeCP2 is associated with methylated histone[45].

The sequence of formation of this complex and its assembly at the *crh* promoter is not known. The CpG dinucleotides as well as nGRE present in *crh* proximal promoter may participate in formation of assembly. One scenario could involve the binding of MeCP2 to the methyl CpGs followed by GR and HDAC1 recruitment. This may be preceded by GR bound-DnMT3b mediated methylation of CpGs. In this proposed molecular mechanism GR does not directly interact with DNA, but may stabilize the interaction of MeCP2 with HDAC1. Since MeCP2 knock down prevents Dex mediated *crh* repression, MeCP2 may be necessary for GR recruitment to the promoter. Furthermore, MeCP2 can interact with DnMT1[94] and thus whether MeCP2 knockdown changes the methylation of *crh* promoter and alter GR holoreceptor recruitment remains unknown. A study by McGill, using a mouse model with truncated MeCP2 suggests that recruitment of MeCP2 to the *crh* promoter does not change promoter methylation[45].

A second possibility is composite GR action that GR interacts with nGRE in a complex with MeCP2 which is recruited to methyl CpGs. The HDAC1 would then be recruited to the complex which would results in the repression of the gene. The DnMT3b interaction with

GR would lead to methylation of CpGs inturn would set the stage for MeCP2 recruitment. Taken together, the results suggest that GR mediated *crh* repression involves formation of a co-repressor complex and that MeCP2 is required for the complex function.

d. Materials and Methods

Cell Culture

Cells were grown in 5.5% CO_2 at 37^0C, in phenol red free media. DME/Ham's F12 1:1 customized media from Hyclone was used in all experiments. The media was supplemented with newborn calf serum (NCS), 10%. Media was also supplemented with 1mM l-glutamine, 1mM Sodium Pyruvate and 100IU/ml of Penicillin and Streptomycin. The volume of media used is indicated in Table 1.

Growing cells from frozen stocks: Frozen cells vials were stored in liquid nitrogen. Media was warmed in a water bath prior to the vial being removed from liquid nitrogen tank. The vial was taken out and warmed immediately in the water bath and then the cells were added to the Petri plate/dish (Nunc, Thermo Fisher) containing the media. The plate was swirled gently for an even distribution of cells. The cells were allowed to attach and grow for 4-6 hrs before changing the media. The cells were then used for future experiments.

Passaging cells: Cell cultures were split at 80-90% confluence. For this, the culture media was removed and cells were washed once with phosphate buffer saline (PBS) (10mM phosphate, pH 7.4; 0.15M NaCl)) once. After removing PBS, trypsin was added onto the cells and allowed to spread over the entire surface area. The plate was incubated for 2-3 min at 37^0C. Trypsin was

then neutralized by the addition of 4-5 times volume of media. The cell suspension was centrifuged at 600*g* for 2 min. The supernatant was removed and the cell pellet was re-suspended in the medium. The cell suspension was then divided and cells were plated as per requirements.

Cryopreservation of cells: After obtaining a single cell suspension Dimethyl sulfoxide (DMSO) was added to achieve the final concentration of 10%. The cell suspension was then divided into cryostatic vials each containing 1-1.5 ml of cell suspension. The vials were labeled and immediately frozen at -80^0C for 24 hrs and then transferred into liquid nitrogen.

Cell counting and cell plating for experiments: The single cell suspension was used for counting number of cells per ml. 10µl of cell suspension was stained with equal volume of Trypan blue dye (Sigma). 10µl of this was added to cell counting chamber. The total cells in 4 corner squares were counted and cell/ml was calculated by adjusting dilutions. Desired numbers of cells were plated for consistency of results.

Dexamethasone treatment: A 10^{-4}M Dexamethasone stock solution was prepared in 100% ethanol. The appropriate volume of Dex solution in ethanol was directly added to the media to achieve 10^{-7}M concentration. Equal volumes of ethanol served as a negative control. To avoid the effect of steroids in serum, cells were incubated in media with SS, 24 hrs prior to Dex treatment.

Cytoplasmic and nuclear fractionation: Harvested cells were centrifuged at 600*g* for 2 min, then washed with PBS and centrifuged again at 600*g* for 2 min. Supernatant was discarded and

the cell pellet was re-suspended in buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 1mM DTT, 1mM, 0.5% NP-40, Phenylmethanesulfonylfluoride (PMSF), 10µl/ml Protease inhibitor cocktail (PIC) (Thermo Scientific)) by pipetting several times. It was then centrifuged at 15000*g* for 3 min. Supernatant (cytoplasmic fraction) was carefully transferred to a fresh tube. The pellet was then re-suspended in buffer B (20mM HEPES, 400mM NaCl, 1mM EDTA, 10% Glycerol, 1.5% NP-40, 1mM DTT, 10mM PMSF, PIC 10µl/ml) by pipetting several times and then incubated for 5 min on ice. Then it was centrifuged at 17000*g* for 10 min and the supernatant (nuclear fraction) was transferred to a fresh tube. The total protein content of nuclear and cytoplasmic fractions was measured using Thermo Scientific Micro BCA Protein Assay Kit.

Immunocytochemistry

Cells were plated in the Lab-Tek chamber slides, 2000 cells per well. After treatment, cells were washed with ice cold PBS three times. Cells were then fixed with 0.25 ml 4% PFA for 30 minutes at 2-8^{0}C. The fixing solution was removed and cells were washed 3 times with cold PBS-T (1XPBS with 0.1% Triton-X 100). The fixed cells were then incubated with blocking solution (5% Normal Goat Serum (NGS), 5% Bovine serum albumin (BSA), 0.1% Triton X-100 in 1X PBS at pH 7.4) for 30 minutes. Cell were then incubated with primary antibody diluted in antibody diluent (2% NGS, 2% BSA, 0.1% Triton X-100 in 1X PBS pH 7.4 and incubated at 4^{0}C overnight. Next day the cells were washed 3 times with antibody diluent at room temperature (RT) and incubated with a second antibody (Alexa Fluor), diluted in antibody diluent with Hoechst (DNA stain), for one hour at RT. Finally, cells were washed 3 times in PBS-T at RT (10-20 min for each wash) and mounted using Aqua PolyMount (Polyscience) and viewed under fluorescent microscope next day.

Co-Immunoprecipitation

Nuclear protein fractions were obtained as described above. 600µg (Final Volume 750µl) of nuclear protein was incubated with 3µg of GR (Santa Cruz) antibody for overnight at 4°C. Then on the next day 30µl of Protein A/G PLUS-Agarose (Santa Cruz) beads were added to each sample and incubated for 4 hrs at 4°C. The samples were then centrifuged at 2000rpm for 1 min. Supernatant was discarded and beads were gently washed 3 times with buffer B. The proteins were then eluted by incubating beads in 25µl of elusion buffer (1% SDS) for 5 min at RT. Eluted protein was then subjected to western blotting.

Western Blotting

Relative protein expression was measured by western blotting. Following treatment, the cells were trypsinized and collected by centrifugation at 600*g* for 2min. The cells were then lysed using RIPA buffer (10mM Tis-HCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.5% SDS, and 140mM NaCl) and insoluble contents were removed by centrifugation at 13000*g* for 15 min at 4^0C. The total protein content was measured using a bicinchonicic acid (BCA) protein assay kit from Thermo Scientific. Samples were prepared by boiling cell lysate containing 30µg of total protein and Laemmli buffer containing 5% β-mercaptoethanol. Samples were cooled and then loaded on 12% MINI protean precast gel from Bio-rad. After electrophoretic separation proteins were transferred to PVDF membrane (Bio-rad). The membranes were stained with Ponceau stain (Sigma) to confirm even transfer and equal loading. The membranes were then blocked with 5% non-fat milk solution for at least 1hr. Following blocking, membranes were incubated with primary antibodies overnight on a rocker at 4^0C. Next

day the membranes were washed at least 3 times with 5% non-fat milk and then probed with appropriate Horseradish peroxidase conjugated secondary antibodies for 1hr at RT. A dilution of 1:15000 was used for secondary antibody. The membranes were then washed with PBS at least 3 times 10 min each. Membranes were then exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) in the dark for 5 min. The bands were visualized using UVP Biospectrum 500. The images were analyzed by ImageJ software for semi quantitative analysis.

RNA isolation and RT-qPCR

Total RNA was isolated using TRI reagent (Molecular Biology). TRI reagent was directly added to the cell culture well after removing media. Cell lysate in TRI reagent was collected and the aqueous phase was separated after addition of chloroform. RNA was precipitated from the aqueous phase by the addition of isopropanol. The RNA pellet was washed with 70% ethanol and air dried. RNA was re-suspended and dissolved in nuclease free water. The quantity and quality of RNA were measured by measuring absorption at 260 and 280nM wavelengths in the Synergy H4 analyzer. The 260/280 ratio of more than 1.7 was set as acceptable criterion. One microgram of total RNA was converted into cDNA using a Thermo Scientific cDNA kit or a iScript reverse transcription kit from Bio-Rad. One micro liter of cDNA was used for PCR amplification using gene specific primers (Table 2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was measured as loading control.

RNAi transfection

siRNA transfection was performed in 6 well plates. The cells were allowed to grow to 50-60% confluence before transfection. The media was replaced with DME Ham's F12 media without

supplements. The transfection mixture was prepared in following manner: 100µl of DME media per reaction was added to a 1.5 ml microcentrifuge tube. Six µl of Lipofectamine 2000 was added and mixed. To this mixture 2µl of 10µM siRNA (OriGene) was added and mixed gently. The mixture was incubated at RT for 20-30 min. This mixture was then layered on top of the cells and plates were swirled gently to mix evenly. The cells were then incubated at 37^0C for 5-6 hrs. After incubation the media was removed and serum supplemented media was added. The cells were allowed to grow for 48-72 hrs before harvesting or treatment.

Statistical analysis

Student T-test: For comparison of two groups unpaired Students T-test was performed. The significance level was set at 95% ($P<0.05$).

Analysis of variance: For experiments with more than two groups ANOVA was performed.

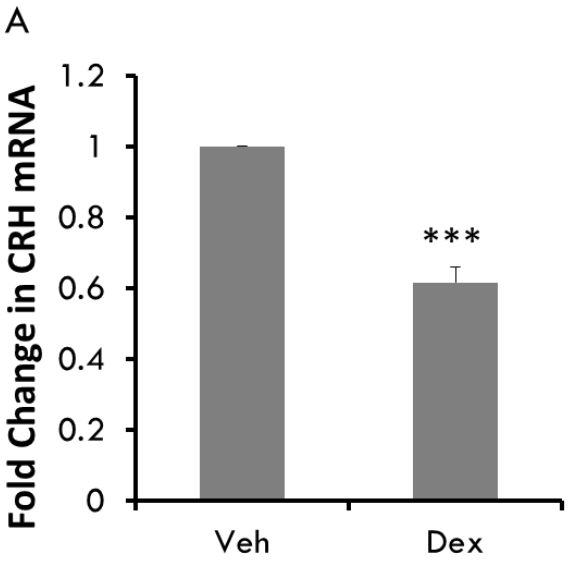
Post-hoc analysis: The pairwise comparisons were performed to identify the significant difference among groups. Fischer's Least Significant test was used to detect statistical significance at 95% confidence interval ($P<0.05$).

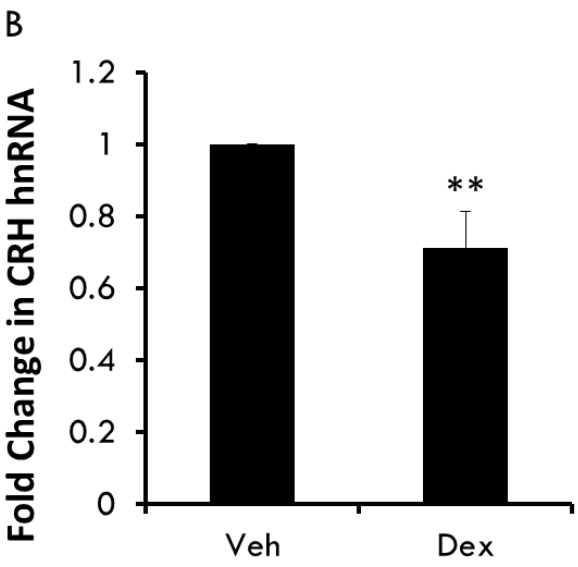
SPSS software was used for all statistical analyses.

Figures and legends

Figure 7. Effect of Dex on the expression of *crh* in IVB cells.

A, CRH mRNA and B, CRH hnRNA expression in IVB cells after incubation in media containing stripped NCS for 24 hours and then treated with Dex (10^{-7}M) for 2 hours. Expression of CRH mRNA (N=3) and hnRNA (N=11) was measured by real time RT-PCR. Student's T test, The data represented as mean ± SEM ***P= 0.0008**, P = 0.0085. C, Immunocytochemistry (ICC) on formaldehyde fixed cells. ICC detection of CRH was performed with a polyclonal antibody (Abnova, Taipei, Taiwan) at a 1:250 dilution. ir, immunoreactivity


A
Fold Change in CRH mRNA
1.2
1
0.8
0.6
0.4
0.2
0
***
Veh
Dex


B
Fold Change in CRH hnRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
Veh
Dex

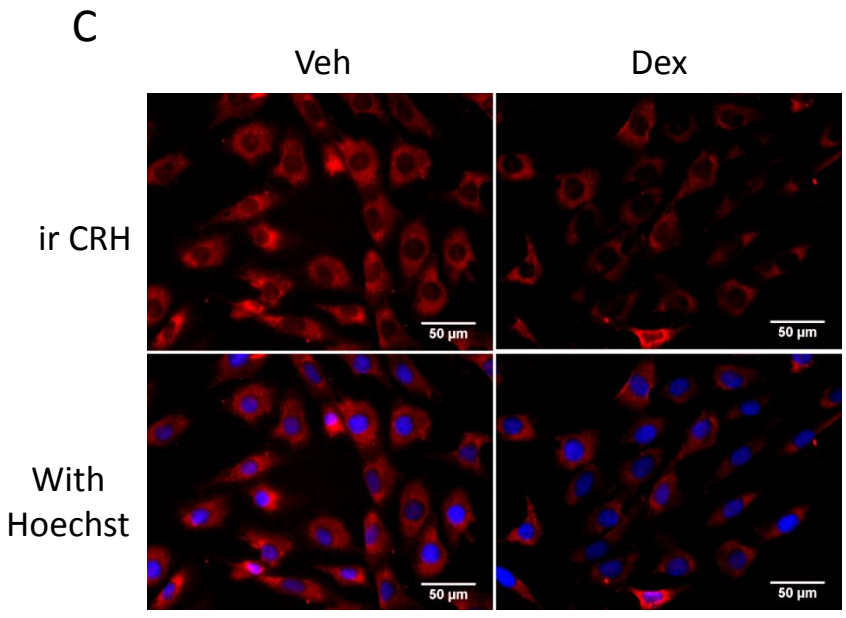

C
Veh
Dex
ir CRH
With Hoechst
50 µm
50 µm
50 µm
50 µm

Figure 8. Effect of Dex on subcellular localization of GR, MeCP2, and HDAC1.

A representative western blot image of GR, MeCP2, and HDAC1. Antibodies used were as follows: monoclonal anti-GR (Thermo/Pierce), monoclonal anti-MeCP2 (Sigma); monoclonal anti-HDAC1 (Abcam); and anti-Actin monoclonal (Santa Cruz). All the antibodies were used in 1:1000 dilutions. Cyto, cytoplasmic; Nucl, nuclear; Dex, Dexamethasone; Veh, vehicle.

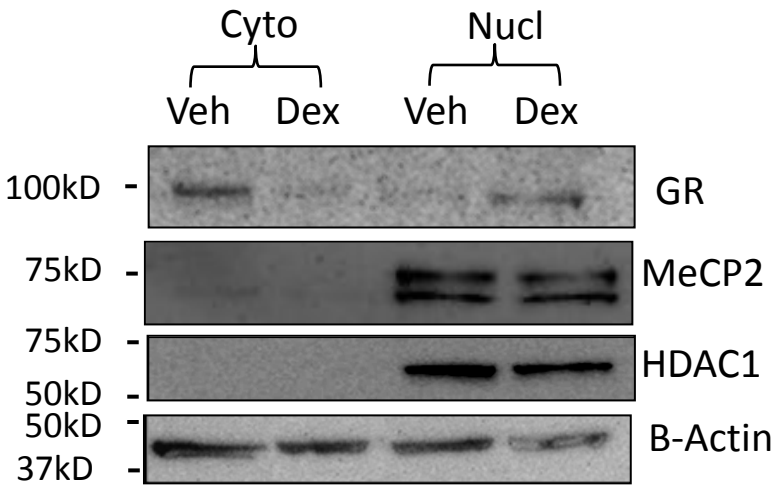
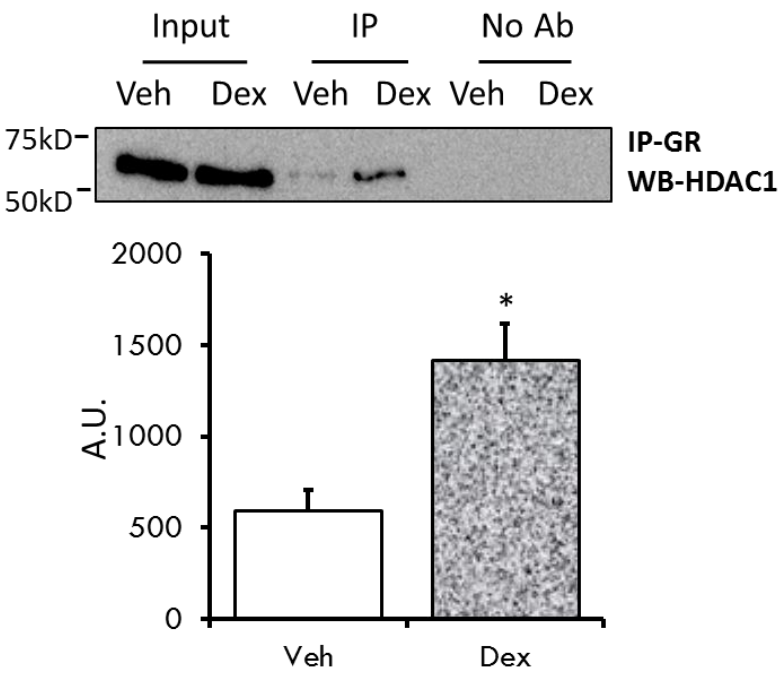

Cyto
Nucl
Veh
Dex
Veh
Dex
100kD
GR
75kD
MeCP2
75kD
HDAC1
50kD
50kD
B-Actin
37kD

Figure 9. Dex increases the association of GR with MeCP2 and HDAC1.

Co-immunoprecipitation analysis of A; GR:HDAC1, and B; GR: MeCP2. Cell extracts were prepared and subjected to immunoprecipitation as indicated. Antibodies used are as follows: A, polyclonal GR (Santa Cruz) and monoclonal HDAC1 (Abcam); B, polyclonal GR (Santa Cruz) and monoclonal MeCP2 (Sigma); N=3, Student's T test, The data represented as mean ± SEM. **P*= 0.01 (GR:HDAC1); ***P*= 0.003 (GR:MeCP2). A.U., arbitrary units; ab, antibody; IP, immunoprecipitation; Veh, vehicle; WB, Western blot.

A


Input
IP
No Ab
Veh
Dex
Veh
Dex
Veh
Dex
75kD
50kD
IP-GR
WB-HDAC1
2000
1500
1000
500
0
A.U.
*
Veh
Dex

B

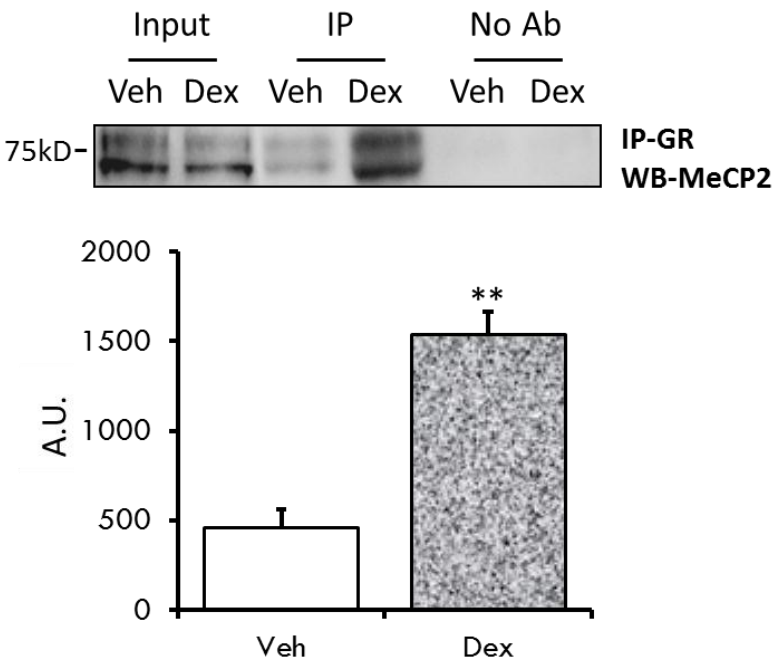
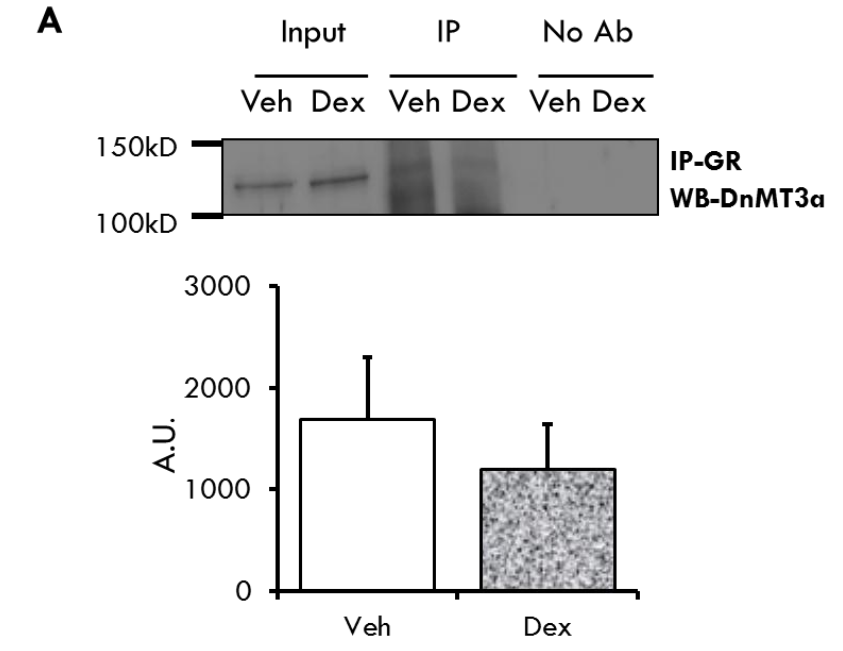

Input
IP
No Ab
Veh
Dex
Veh
Dex
Veh
Dex
75kD
IP-GR
WB-MeCP2
2000
1500
1000
500
0
A.U.
**
Veh
Dex

Figure 10. Dex Induces GR Interaction with DnMT3b.

Co-immunoprecipitation analysis of A; GR-DnMT3a and B; GR-DnMT3b interactions. Antibodies used were monoclonal GR (Thermo/Pierce), DnMT3a and -3b (Santa Cruz) N = 3, Student's T test, The data represented as mean ± SEM and are represented as the fold difference of the Veh. **P*= 0.011 (GR:DnMT3b). A.U., arbitrary units; ab, antibody; IP, immunoprecipitation; Veh, vehicle; WB, Western blot.


A
Input
IP
No Ab
Veh Dex Veh Dex Veh Dex
150kD
100kD
IP-GR
WB-DnMT3a
3000
2000
1000
0
A.U.
Veh
Dex

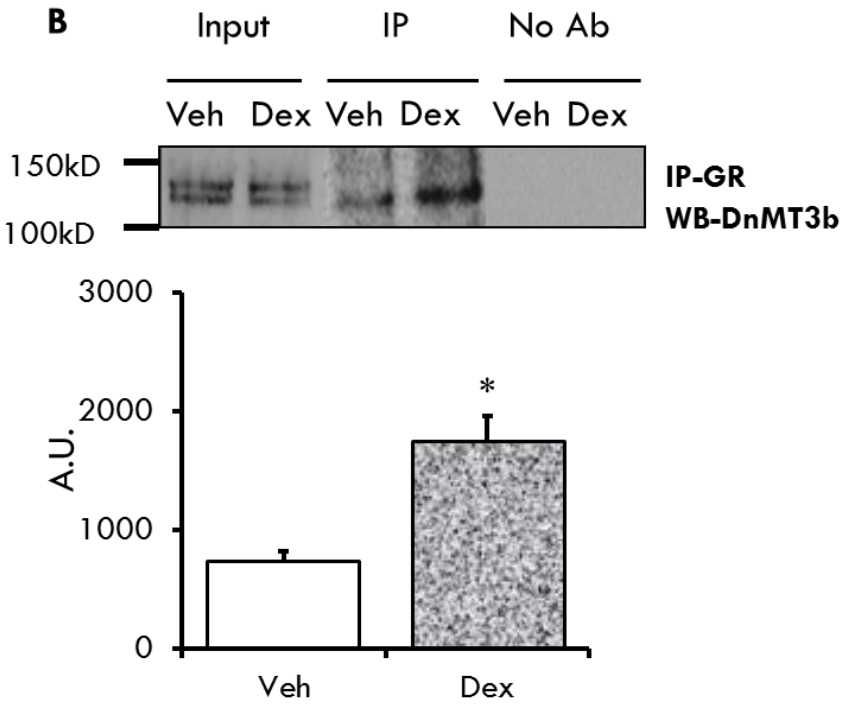

B
Input
IP
No Ab
Veh Dex Veh Dex Veh Dex
150kD
100kD
IP-GR
WB-DnMT3b
3000
2000
1000
0
A.U.
*
Veh
Dex

Figure 11. siRNA mediated MeCP2 knockdown.

Western blot and densitometric quantitation showing levels of MeCP2 and Actin, 72 hrs after transient transfection with siRNA against MeCP2. Monoclonal MeCP2 (Abcam) and Polyclonal Actin (Cell Signaling) antibodies were used in 1:1000 dilution. N=3, Student's T test compared to control, The data represented as mean ±SEM , * $P< 0.05$, *** $P< 0.0005$. Veh; Vehicle, Dex; Dexamethasone

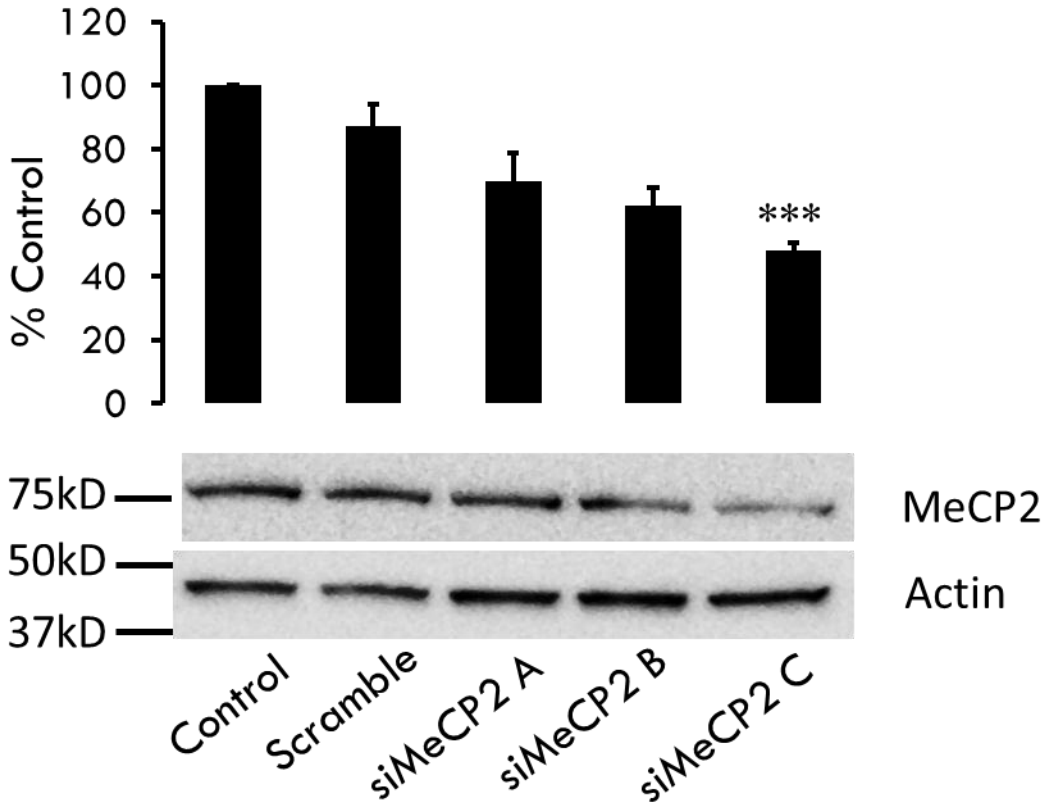
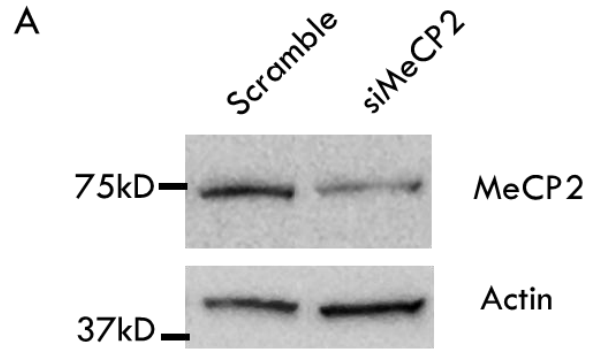

120
100
80
60
40
20
0
% Control
***
75kD
50kD
37kD
MeCP2
Actin
Control
Scramble
siMeCP2 A
siMeCP2 B
siMeCP2 C

Figure 12. MeCP2 depletion is associated with increased *crh* expression.

A; Western blot showing relative protein levels of MeCP2 and Actin (N=3) and B; CRH hnRNA levels (N=6), 72 hrs post transfection with scrambled control or siMeCP2. Monoclonal MeCP2 (Abcam) and Polyclonal Actin (Cell Signaling) antibodies were used in 1:1000 dilution. Student's T test, The data represented as mean ± SEM, **P*= 0.0163


A
Scramble
siMeCP2
75kD
MeCP2
37kD
Actin

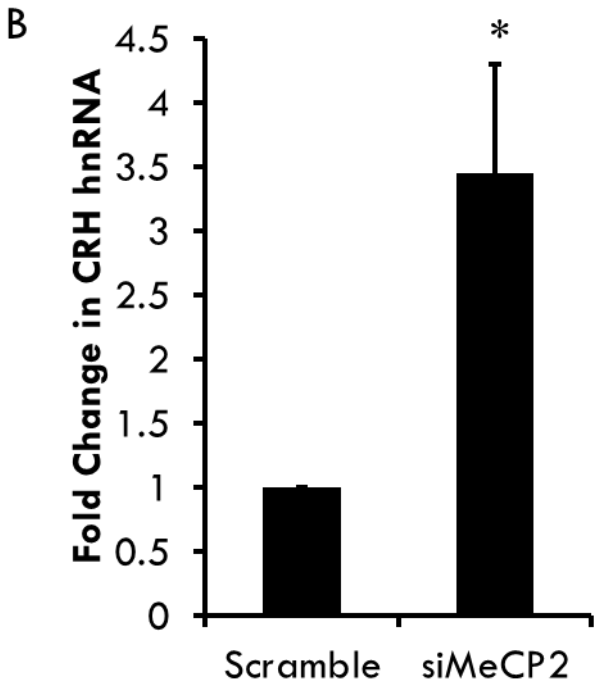

B
Fold Change in CRH hnRNA
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Scramble
siMeCP2

Figure 13. MeCP2 contributes to the maintenance of basal *crh* expression, and required for Dex mediated repression of CRH hnRNA

Decreased MeCP2 levels increases basal CRH hnRNA levels and prevent Dex-mediated reduction of *crh* expression levels. N≥6, One way ANOVA, $P= 0.024$, protected Fischer's Least Significance difference Post hoc test as compared to scramble/Veh treated group, The data represented as mean ±SEM, $**P= 0.005$, $*P= 0.05$. Veh; Vehicle, Dex; Dexamethasone.

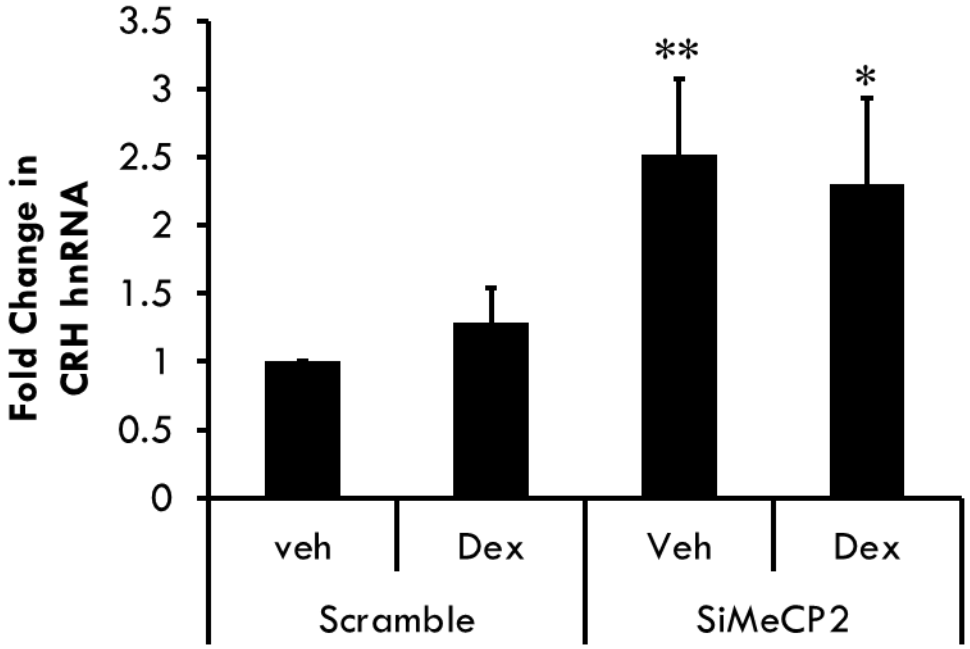

Fold Change in CRH hnRNA
3.5
3
2.5
2
1.5
1
0.5
0
**
*
veh
Dex
Veh
Dex
Scramble
SiMeCP2

Table 1: Specifications of cell culture dishes and media volume used in experiments

No	Plate Size	Surface area (cm^2)	Volume of media (ml)	No. of cells per well/plate
1	Chamber Slide	0.7	0.4	$0.01X10^5$
2	12 well plate	3.5	1	$0.2\text{-}0.8X10^5$
3	6 well plate	9.6	2	$0.2\text{-}0.8X10^5$
4	10 cm plate	56.7	10	$0.5\text{-}1X10^5$
5	15 cm plate	145	20	$2\text{-}4X10^5$

Table 2. List of primers used for relative expression of gene by RT-qPCR

No	Transcript	Forward primer	Reverse Primer	Product size (bp)
1	hnCRH	TCAATCCAATCTGCCACTCA	TAAGCTATTCGCCCGCTCTA	155
2	mCRH	TGCCCACGCTTAGTTTCTATGTGC	ACAACTGGGTGACTTCCATCTGCT	112
3	mGAPDH	TGGAGTCTACTGGCGTCTT	GCTGACAATCTTGAGGGAG	158

Table 3. Sequences of siRNA against MeCP2

r= Ribonuclic acid phosphor-diester bond

siRNA	Sequence
siMeCP2A	rArGrCrUrUrArArArCrArGrArGrGrArArGrUrCrUrGrGrUCG
siMeCP2B	rCrGrCrArArArGrArCrArUrUrGrUrUrUrCrArUrCrCrUrCCA
siMeCP2C	rUrCrGrCrUrCrUrArArArGrUrArGrArArUrUrGrArUrUrGCA

CHAPTER 3

DNA methylation is required for glucocorticoid receptor mediated CRH gene repression

a. Introduction

Methylation of CpG dinuceotides present in the *crh* promoter plays an important role in the regulation of gene expression[41,42]. The putative co-repressor complex formed by GR contains DnMT3b, which is recruited to the *crh* promoter region[39]. Furthermore MeCP2, which is recruited by methylated CpG[43,92], is part of the complex and is necessary for repression of *crh* (Chapter 2). This part of the study was dedicated to understanding the role of proximal promoter methylation in the GR mediated regulation of *crh*. Previous studies have shown that stress induces site specific hypomethylation of *crh* promoter[41,42]. Thus we first tested whether Dex increases *crh* promoter methylation and if this is site specific. Then we tested the role of promoter methylation in the regulation of *crh* expression and GR recruitment to the promoter.

Like *crh*, many genes that are repressed by GR are also regulated by DNA methylation. The interdependence of these two regulatory aspects in not known. In this part of the study, we investigated the role of DNA methylation in GR mediated *crh* repression. We analyzed whether DNA methylation is required for GR to repress *crh* and whether it alters GR binding to the *crh* promoter.

b. Results

Dex increases methylation of CpG islands in *crh* proximal promoter

Sharma et al. demonstrated that Dex mediated regulation of *crh* involves DNA methyltransferse (DNMT3b) and methylated DNA binding protein (MeCP2)[39]. Furthermore, the authors found that Dex increases DNA methylation of CpG island present in the *crh* promoter. Dex increases overall methylation of *crh* promoter by more than 10%, (Figure 14A, B). Further analysis of individual CpGs revealed that this increase was attributed to two specific sites. CpG No. 9 and 10 showed more than 30% increase in the level of methylation following Dex treatment (Figure 14C).

5-AzaDC mediated inhibition of DNA methylation is associated with increased CRH hnRNA expression

To determine whether DNA methylation is required for *crh* gene repression and/or maintenance of basal levels, the cells were treated with DnMT inhibitor, 5-AzaDC. The levels of CRH hnRNA were measured following four day of 5-AzaDC treatment. Concentrations of 0.25, 0.5 and 1.0 µM led to a significant increase in CRH hnRNA, (Figure 15). 0.5µM led ot the

maximum increase of 2.7 fold in *crh* hnRNA level and this concentration was used for remainder of the experiments.

5-AzaDC decreases the overall methylation of the *crh* proximal promoter

To analyze whether increased *crh* expression is associated with decreased DNA methylation, the DNA methylation status following 0.5 µM 5-AzaDC exposure was analyzed. The level of methylation in the region of *crh* promoter was measured by bisulfite conversion followed by methylated DNA specific PCR and TOPO-TA cloning. The 0.5µM concentration of 5-AzaDC led to decreased overall methylation of *crh* promoter by over 10%, (Figure 16A, B).

5-AzaDC mediated inhibition of *crh* promoter methylation is site specific

Since Dex mediated increase in promoter methylation is site specific[39], 5-AzaDC mediated inhibition of DNA methylation was analyzed for site-specific changes. We measured the level of methylation at individual CpGs. To our surprise, 5-AzaDC reduced DNA methylation at CpG site No. 10. This decrease in methylation was more than 20%, (Figure 17). This was one of the two sites at which Dex increased the methylation (Figure 14).

Dex fails to repress *crh* in cells pretreated with 5-AzaDC

To answer our primary question, whether DNA methylation is necessary for GR to repress *crh*, we exposed 5-AzaDC treated cells to Dex. The corresponding CRH hnRNA was measured. Even though, Dex maintained the repressed levels of *crh*, it failed to repress the *crh* in cells pre-treated with 5-AzaDC. To our surprise Dex actually stimulated *crh* in cells pre-treated with 5-AzaDC with a more than two fold increase in CRH hnRNA levels, (Figure 18). On the other

hand, contrary to previous experiment (Figure 15), 5-AzaDC failed to increase the *crh* hnRNA expression.

5-AzaDC mediated inhibition of *crh* promoter methylation is necessary but not sufficient for *crh* activation.

To understand this contradiction in the results we measured the effect of 5-AzaDC on CRH hnRNA levels in charcoal stripped NCS and non-stripped NCS supplemented media. We found that in the presence of stripped NCS, 5-AzaDC failed to activate *crh* (Figure 19). This suggests that 5-AzaDC mediated inhibition of DNA methylation is necessary but not sufficient for *crh* activation.

GR and MeCP2 protein levels are not affected by 5-AzaDC and Dex exposure.

To understand the mechanism by which the Dex effect is altered in the presence of 5-AzaDC, we measured the changes in protein levels of GR and MeCP2. Since previous studies have reported that 5-AzaDC increased the protein levels of GR in B and T lymphocyes[95] and MeCP2 in neuronal stem cells[96] we wanted to determine that this was not the case here. Indeed, in our conditions the protein levels of GR and MeCP2 were not affected by either 5-AzaDC or Dex exposure (Figure 19A). Semi-quantitative analysis revealed no significant changes in protein levels when compared to the loading control- Actin (Figure 20).

Localization of GR and MeCP2 with 5-AzaDC+/- Dex

Next we analyzed the effect of 5-AzaDc and Dex treatment on the cellular localization of GR and MeCP2. As expected, GR translocates from the cytoplasm into the nucleus in the presence of

Dex. The subcellular localization of GR in the presence of 5-AzaDc was inconclusive. This may be due to less number of cells and shrunken cell body. The MeCP2 on the other hand was exclusively nuclear. Although its cytoplasmic vs nuclear distribution was not affected by Dex. 5-AzaDC exposure led to the shrunken nuclei (Figure 21).

Dex fails to recruit GR and MeCP2 to the *crh* promoter in the presence of 5-AzaDC.

Lastly, we examined the recruitment of GR and MeCP2 to the *crh* promoter. Since the protein levels and subcellular distribution was not altered with 5-AzaDC, we analyzed whether 5-AzaDC mediated inhibition of DNA methylation alters the presence of GR and MeCP2 at the *crh* promoter. Consistent with our previous findings, Dex increased the recruitment of GR as well as MeCP2 to the *crh* promoter region by more than 1.5 fold. On the other hand, Dex failed to recruit the GR and MeCP2 to the *crh* promoter in cells in which methylation was inhibited; that is pre-treated with 5-AzaDC (Figure 22).

c. Discussion

There are three reports that indicate a role for DNA methylation in the regulation of *crh* expression [41,42,45]. A study by Elliot et al., suggest that in mouse hypothalamic N42 cells 5-AzaDC mediated inhibition of DNA methylation is associated with increased *crh* expression[42]. Our results are consistent with these findings. Decreased site specific methylation due to 5-AzaDC is associated with increased expression of CRH hnRNA (Figure 15, 16 and 17).

The studies by Elliot et al. and Chen et al. indicate that, in the context of *in vivo* stress models, hypomethylation following stressor is associated with increased expression of *crh*.[41,42]. Furthermore, Chen et al suggests that this change in DNA methylation and *crh* expression is

specific to the hypothalamus. Both these studies reveal that changes in DNA methylation occur in the region of CRE. This is in contrast to the data presented here. Dex exposure leads to increased methylation of CpG No. 9 and 10 present at -36bp and -33bp. In distinction CpG No. 10 is hypomethylated following 5-AzaDC exposure. Neither site 9 nor 10 show any alteration in methylation in either *in vivo* studies. These contrasting findings may be due different species. Rat and mouse *crh* promoters are slightly different. While CpG No. 10 is absent in mouse promoter, it has an extra CpG dinucleotide at -236bp[39,41]. Also the rat promoter has a CpG No. 4 uniquely hypomethylated. This site is also hypomethylated in mouse along with other sites[39,41]. Furthermore the data presented here and the one from *in vivo* studies suggest that the stimulation and repression of gene involves methylation of different CpG sites. Thus, different patterns of methylation exist in depending upon the activated or repressed status of the *crh*. It will be interesting to investigate the role of individual CpG in responses to stress and elevated GCs.

crh promoter methylation also alters recruitment of activator proteins such as pCREB. Increased methylation at CpG No.2, present within CRE is associated with decreased pCREB binding[41]. We found that decreased methylation, following 5-AzaDC exposure, is associated with diminished GR and MeCP2 binding to the *crh* promoter in the presence of Dex. Whether changes in methylation at CpG No.9 and 10 alter pCREb binding is not known.

The results also suggest that GR requires DNA methylation for *crh* repression. In other words DNA methylation sets the stage for GR-mediated gene repression. It is well known that GR binding to the promoter depends on the DNA sequence and orientation[97,98]. Our results suggest that methylation also plays a role. Hypomethylation prevents GR and MeCP2 binding to the *crh* promoter. This may be due to changes in the chromatin environment. Whether GR

binding to the *crh* promoter is direct or mediated through MeCP2 recruitment remains to be determined.

d. Material and methods

In addition to the techniques described in Chapter 2, following techniques were used

5-AzaDC treatment: For experiments involving 5-AzaDC, the IVB cells were incubated in media supplemented with NCS containing 0.0, 0.25, 0.5, 1.0, 2.5, and 5 µM of 5-AzaDC for 4 days. The cell culture media was replaced every 24 hrs with fresh media containing the desired concentration of 5-AzaDC. Media containing equal volumes of solvent (DMSO) was used as a control.

5-AzaDC and Dex co-treatment: In the experiments involving 5-AzaDC and Dex treatment, IVB cells were incubated in media supplemented with NCS containing 0.5µM 5-AzaDC for 3 days followed by 24 hrs incubation in media supplemented with charcoal stripped NCS containing 5-AzaDC, followed by 2hrs Dex treatment.

Chromatin Immunoprecipitation

At the end of treatment, cells were fixed by the addition of 37% formaldehyde directly to cell media to a final concentration of 1%. The cells were fixed for 10 min with swirling at RT. The fixation was stopped by adding 1.25M Glycine solution to achieve a final concentration of 0.125M for 5min. The media was removed and cells were washed with ice cold PBS twice and collected in cold PBS by scraping. The cells were pelleted by centrifugation at 600*g* for 5 min. The cell pellets were re-suspended in 20 times the pellet volume of cell lysis buffer(50mM

HEPEs-KOH, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.5% SDS) and incubated on ice for 10 min.

Sonication

Chromatin fragmentation by sonication was optimized to achieve the sheared chromatin fragments in the range of 200- to 800-bp. Sonication condition of 80% amplitude and 10 pulses each of 30 Sec with 45 Sec in between was found to be optimal. For sonication, Misonix sonicator Q700 with cup horn (QSonica, LLC, CT, USA) was used.

After sonication, chromatin was centrifuged at 13000*g* for 15 min at 4^0C, to remove the cell debris. Thirty microliter of chromatin was diluted 10 times with IP buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 0.5%NP-40, 1% Triton X-100) and incubated overnight with primary antibody (2-5ug) against the target protein. The next day 20µl of pre-blocked Pierce Protein A/G Magnetic Beads (Thermo Scientific) were added to each reaction and incubated for 2hrs at 2-8^0C using a nutator. Beads were washed 4 times with RIPA buffer (50mM HEPES KOH, 1mM EDTA, 0.5% NP-40, 0.5% Na-Deoxycholate) with 0.25M LiCl for 5 min each with a final wash with TE buffer (10mM Tris-HCl, 1mM Na-EDTA) containing 0.25M NaCl. To each sample and input fraction 100µl of 10% Chelex-100 (Bio-rad) slurry was added. The chromatin cross-linking was reversed by boiling for 10 min. Samples were cooled and RNAase A added and the samples incubated for 1 hr at 55^0C. Subsequently, proteinase K was added and samples were incubated for 30 min at 55^0C. The enzymes were inactivated by boiling the samples for 15 min. Finally, samples were centrifuged at 13000*g* for 5 min. Supernatants containing DNA were removed and placed in fresh tubes and used for real time qPCR.

Genomic DNA extraction

Cells were collected following treatment and lysed with RIPA buffer. Genomic DNA was extracted and purified using phenol-chloroform extraction with a final wash of 70% ethanol.

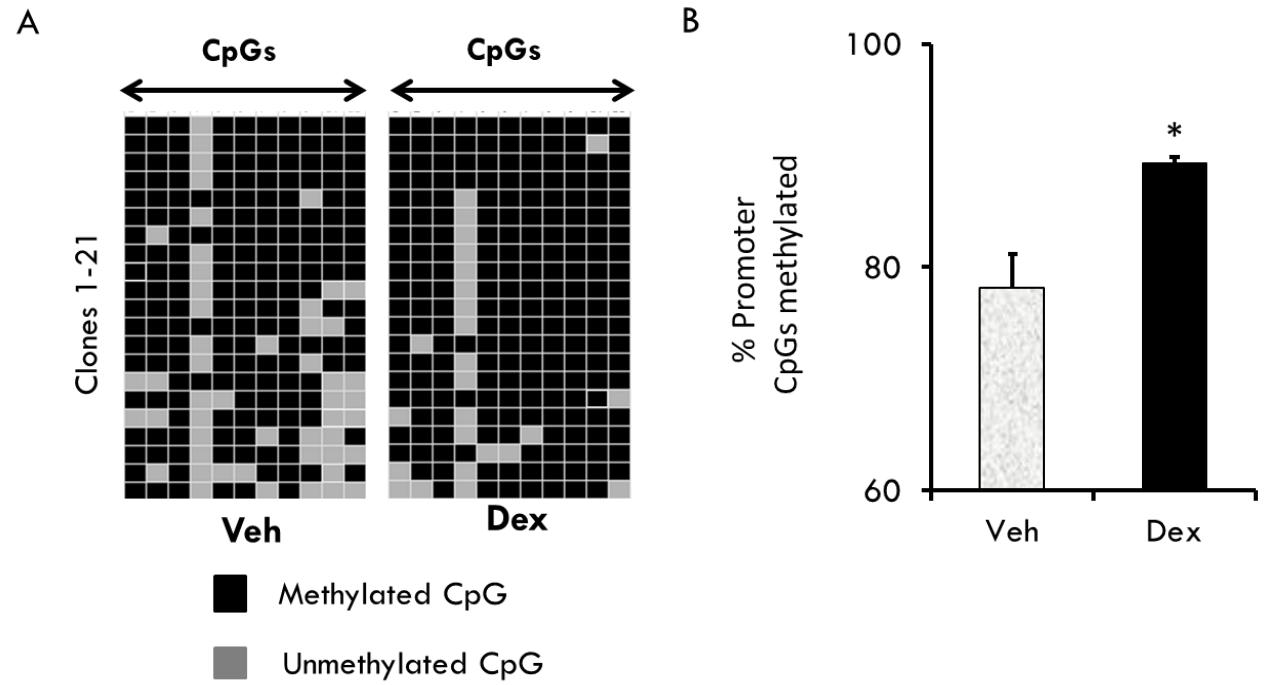
DNA methylation analysis

Genomic DNA (1000 ng) was modified with bisulfite treatment using EZ DNA methylation-Gold kit (Zymogen) according to the manufacturer's instructions. Modified DNA was subjected to the first round of PCR amplification using primers listed in table 1. The protocol involves an initial denaturation at 95°C for 5 minutes, followed by 34 cycles of denaturation (95°C, 60 Sec), annealing (45°C, 60 Sec), and extension (72°C, 60 Sec) with a final extension cycle (72°C, 10 min). The PCR product (406 bp) was purified and 5µL was used as a template for the second round of PCR using nested primers (Table 4). The 294-bp PCR product was purified and cloned into pCR4-TOPO vector using TOPO-TA Cloning Kit (Life Technologies). A total of 32 recombinant clones from 5 independent experiments were sequenced from Eurofins MWG Operon. Sequence information was analyzed and methylation data were compiled using the BISMA software available online (http://biochem.jacobs-university.de/BDPC/BISMA).

Figures and legends

Figure 14. Dex increases *crh* promoter methylation at specific CpG sites.

A, Raw sequencing data for promoter methylation. Each row represents an independent clone. B, Analysis of overall methylation across all sites. C, Percent methylation at specific sites. Analysis of *crh* promoter DNA methylation was performed by sequencing of PCR clones derived from sodium bisulfite- treated genomic DNA, N =3, Student's T test, The data represented as mean ± SEM. *, P <0.05. Veh, vehicle; Dex, Dexamethasone. (This experiment was done by Dr. D. Sharma)


A
CpGs
CpGs
Clones 1-21
Veh
Dex
Methylated CpG
Unmethylated CpG
B
100
80
60
% Promoter CpGs methylated
*
Veh
Dex

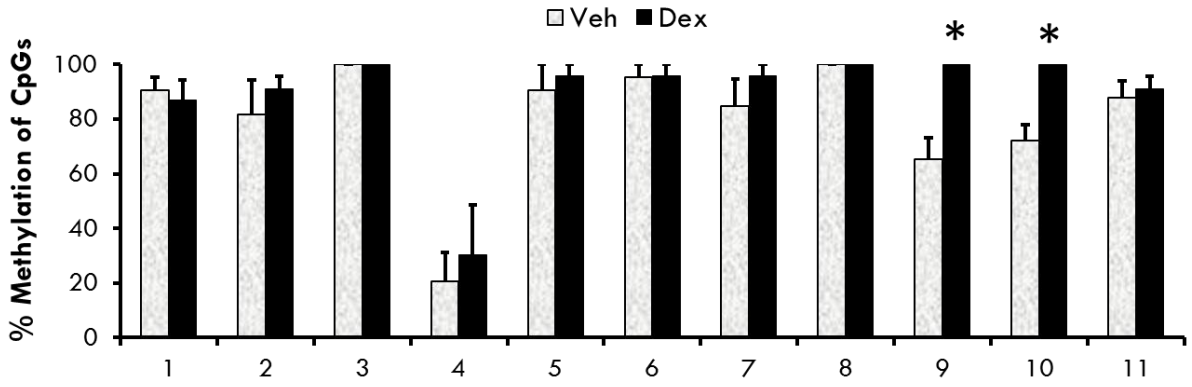

Veh
Dex
% Methylation of CpGs
100
80
60
40
20
0
*
*
1
2
3
4
5
6
7
8
9
10
11

Figure 15. 5-AzaDC mediated inhibition of promoter methylation increases *crh* hnRNA expression.

A, 5-AzaDC - inhibited promoter methylation increases basal levels of *crh* expression. Cells were treated with indicated concentrations of 5-AzaDC (Solid line) or DMSO (Dotted line) for four days, N≥3, One way ANOVA, $P = 0.016$, protected Fischer's Least Significance difference post hoc test. The data is represented as mean ± SEM $^{**}P = 0.003$, $^{*}P < 0.05$.

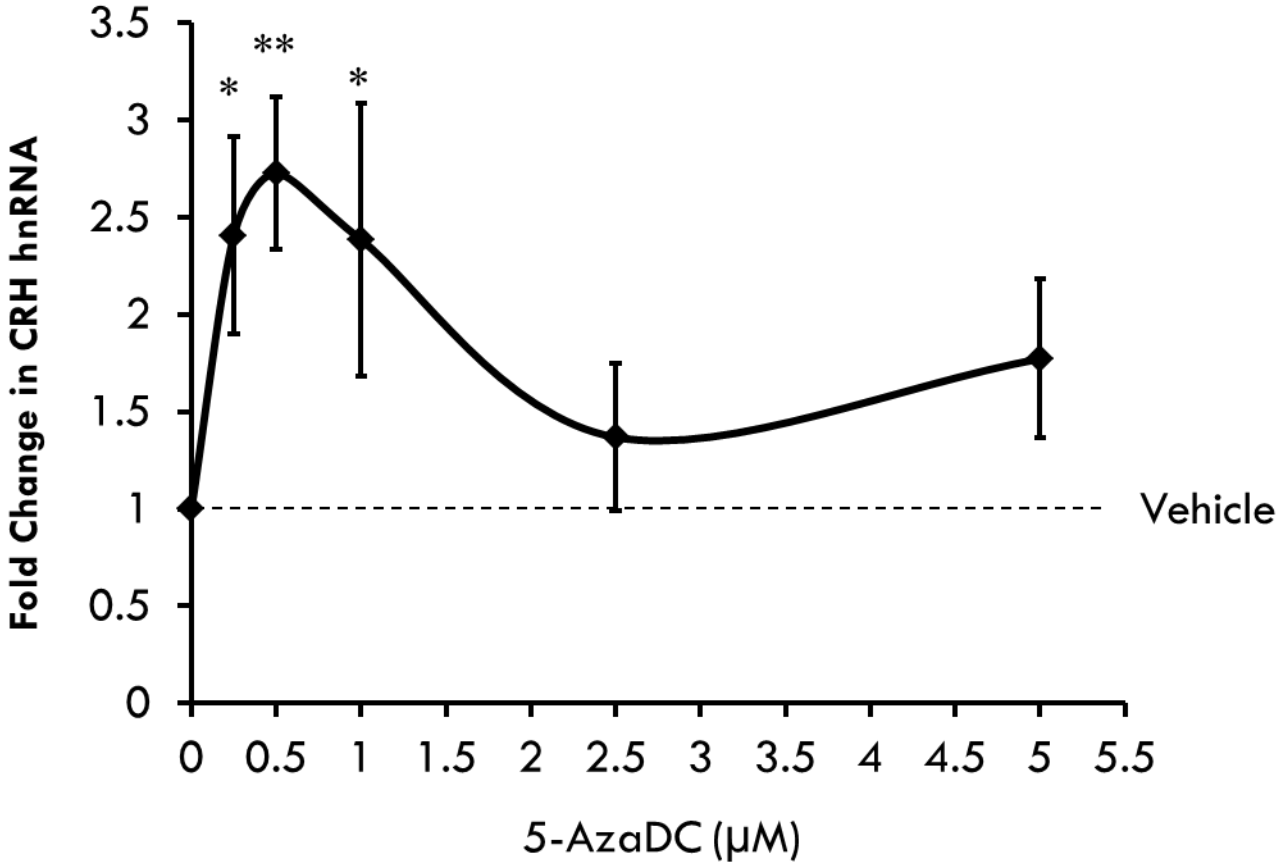

Fold Change in CRH hnRNA
3.5
3
2.5
2
1.5
1
0.5
0
*
**
*
Vehicle
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
5-AzaDC (μM)

Figure 16. 5-AzaDC inhibits overall DNA methylation of *crh* promoter.

A, Raw promoter methylation sequence data. Each row represents an independent clone, and each column represents a CpG site. B, Overall promoter methylation following 5-AzaDC exposure, N=5, Student's T test, The data represented as mean ± SEM, **P*= 0.0106.

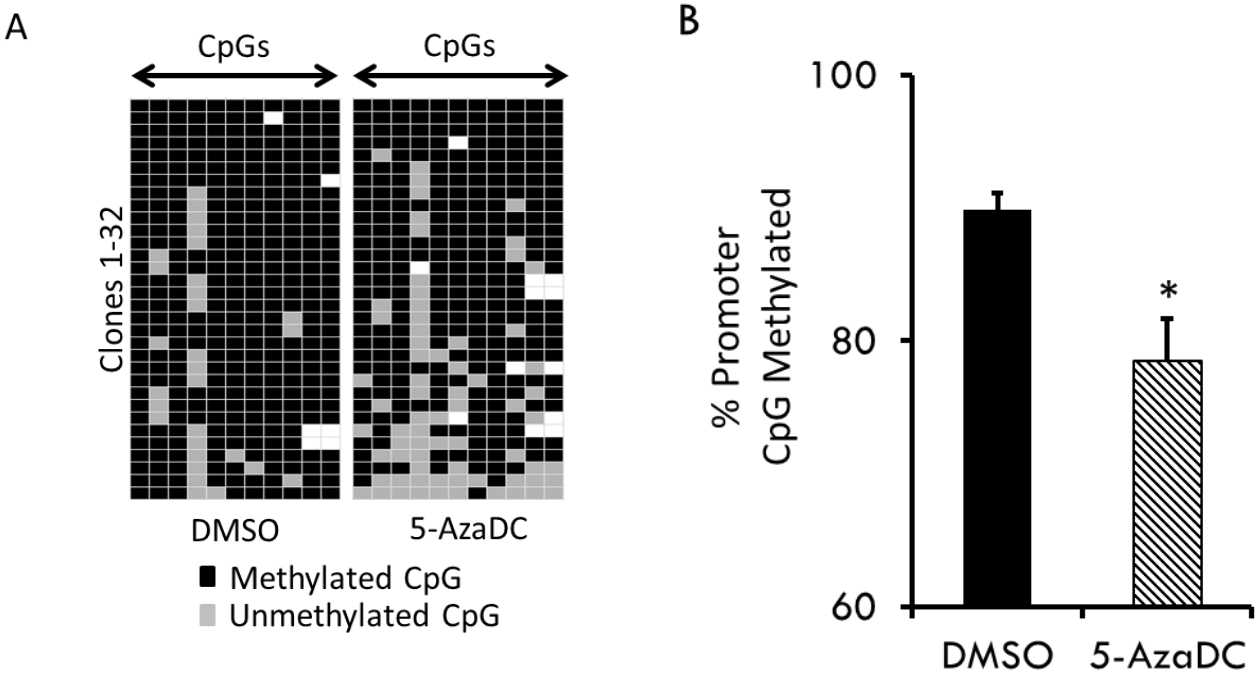

A
CpGs
CpGs
Clones 1-32
DMSO
5-AzaDC
Methylated CpG
Unmethylated CpG
B
100
80
60
% Promoter CpG Methylated
*
DMSO
5-AzaDC

Figure 17. 5-AzaDC inhibits methylation at specific sites in *crh* promoter.

Percent methylation of individual CpG sites. CpG No. 10 shows a significant decrease in methylation. N=5, Student's T test, The data represented as mean ± SEM, **P*= 0.0309.

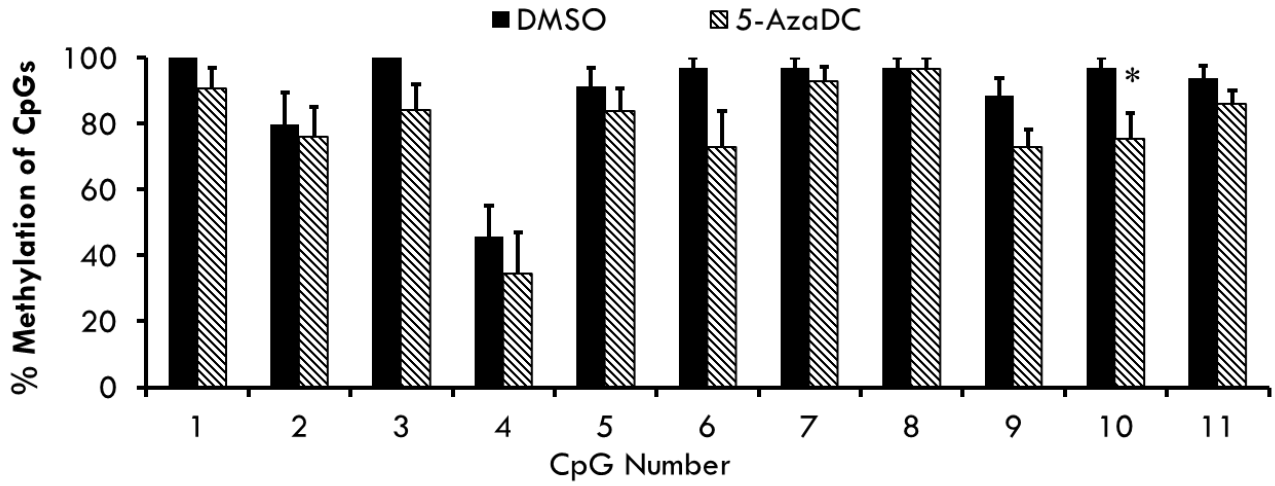

DMSO
5-AzaDC
% Methylation of CpGs
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
CpG Number
*

Figure 18. The *crh* promoter methylation is required for Dex mediated gene repression

A, 5-AzaDC abrogates Dex-induced *crh* repression, N≥6, One way ANOVA, *P*= 0.001, Protected Fischer's Least Significance difference Post hoc test, The data represented as mean ± SEM, ***P*= 0.002. Veh; vehicle, Dex; Dexamethasone.

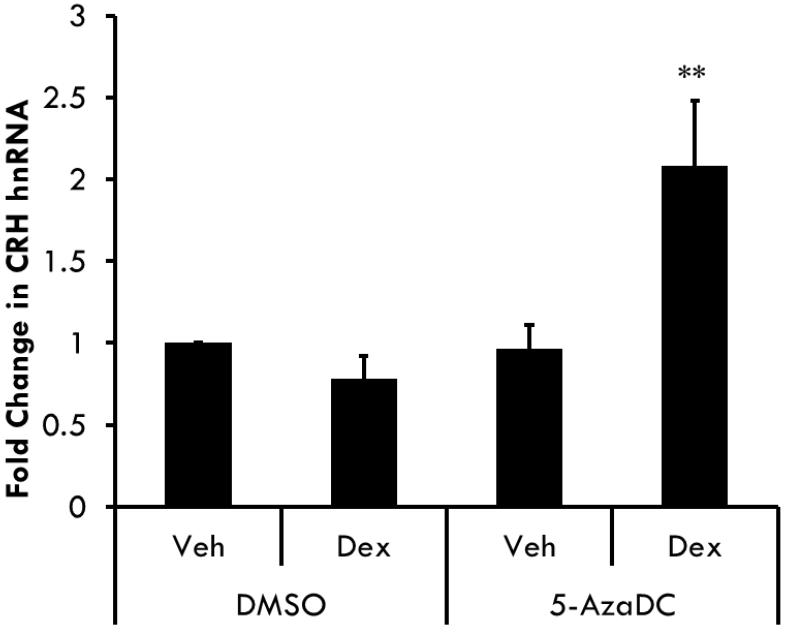

Fold Change in CRH hnRNA
3
2.5
2
1.5
1
0.5
0
**
Veh
Dex
Veh
Dex
DMSO
5-AzaDC

Figure 19. Effect of 5-AzaDC on *crh* expression in stripped and non-stripped serum media. 5-AzaDC - inhibited promoter methylation increases basal levels of *crh* expression in cells incubated in NCS media (solid line) but not in cell incubated in Stripped NCS media (Dotted line). Cells were treated with indicated concentrations of 5-AzaDC for four days, N≥3, One way ANOVA, $P = 0.016$, protected Fischer's Least Significance difference post hoc test, The data represented as mean ± SEM, $^{**}P = 0.003$, $^{*}P < 0.05$. The NCS trace is same as shown in figure 15.

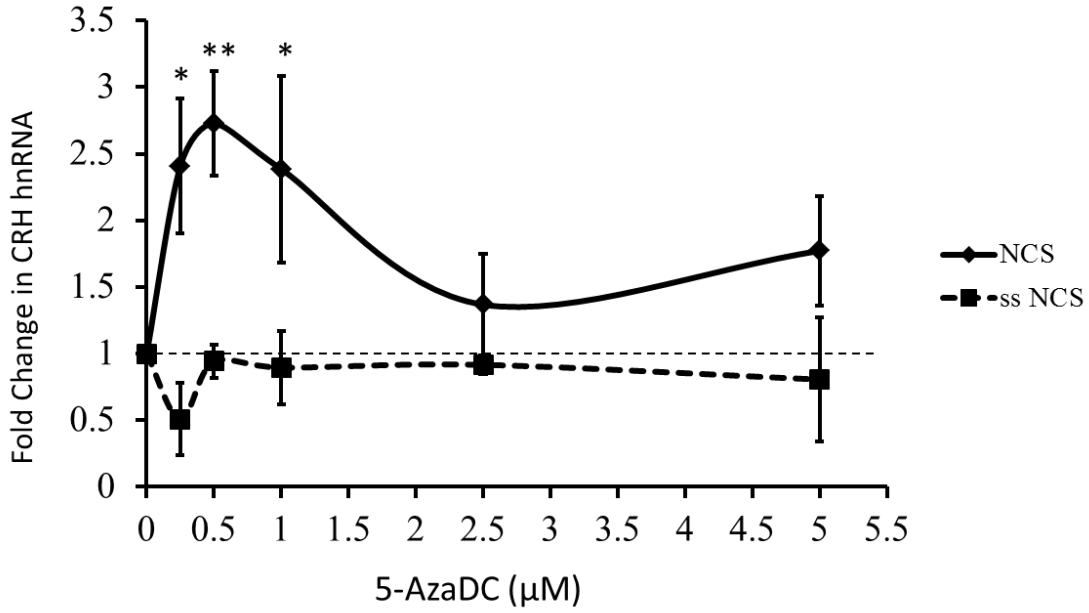
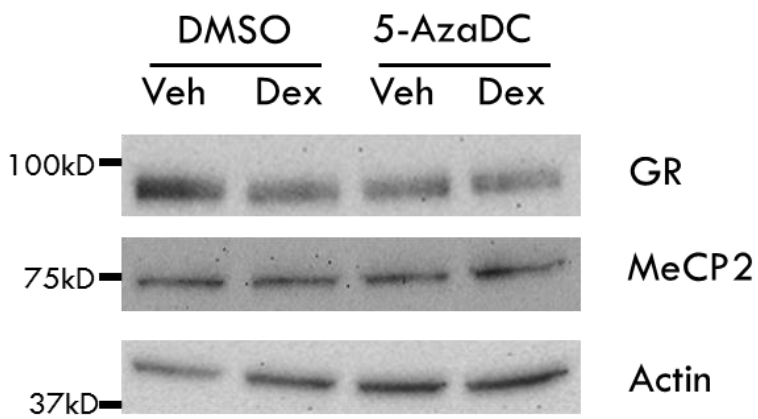

3.5
3
2.5
2
1.5
1
0.5
0
*
**
*
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
Fold Change in CRH hnRNA
5-AzaDC (µM)
NCS
ss NCS

Figure 20. 5-AzaDC and Dex do not alter GR and MeCP2 protein levels.

A; Representative western blot showing protein levels of GR and MeCP2 in IVB whole cell extracts after 5-AzaDC and Dex exposure. B; GR and MeCP2 western blot quantitation corrected for loading control-Actin. N=3, One way ANOVA, The data represented as mean ± SEM. Veh; vehicle, Dex; Dexamethasone.

A


DMSO
5-AzaDC
Veh
Dex
Veh
Dex
100kD
75kD
37kD
GR
MeCP2
Actin

B

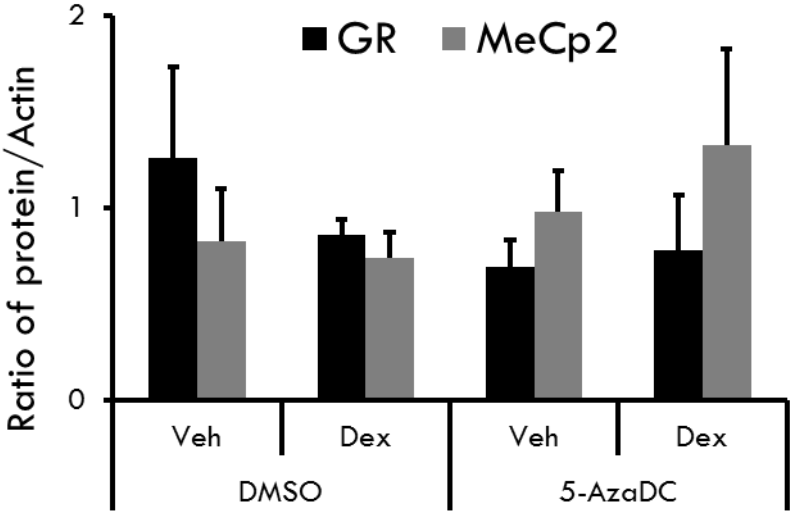

GR
MeCp2
Ratio of protein/Actin
2
1
0
Veh
Dex
Veh
Dex
DMSO
5-AzaDC

Figure 21. Effect of 5-AzaDC and Dex on cellular localization of GR and MeCP2.

Immunocytochemistry analysis shows ligand dependent translocation of GR in the nucleus. There was no detectable effect of 5-AzaDC on GR and MeC2. MeCP2 is exclusively in nucleus irrespective of treatment. Polyclonal GR (Thermo Fisher) and monoclonal MeCP2 (Sigma) antibodies were used in 1:250 dilution.

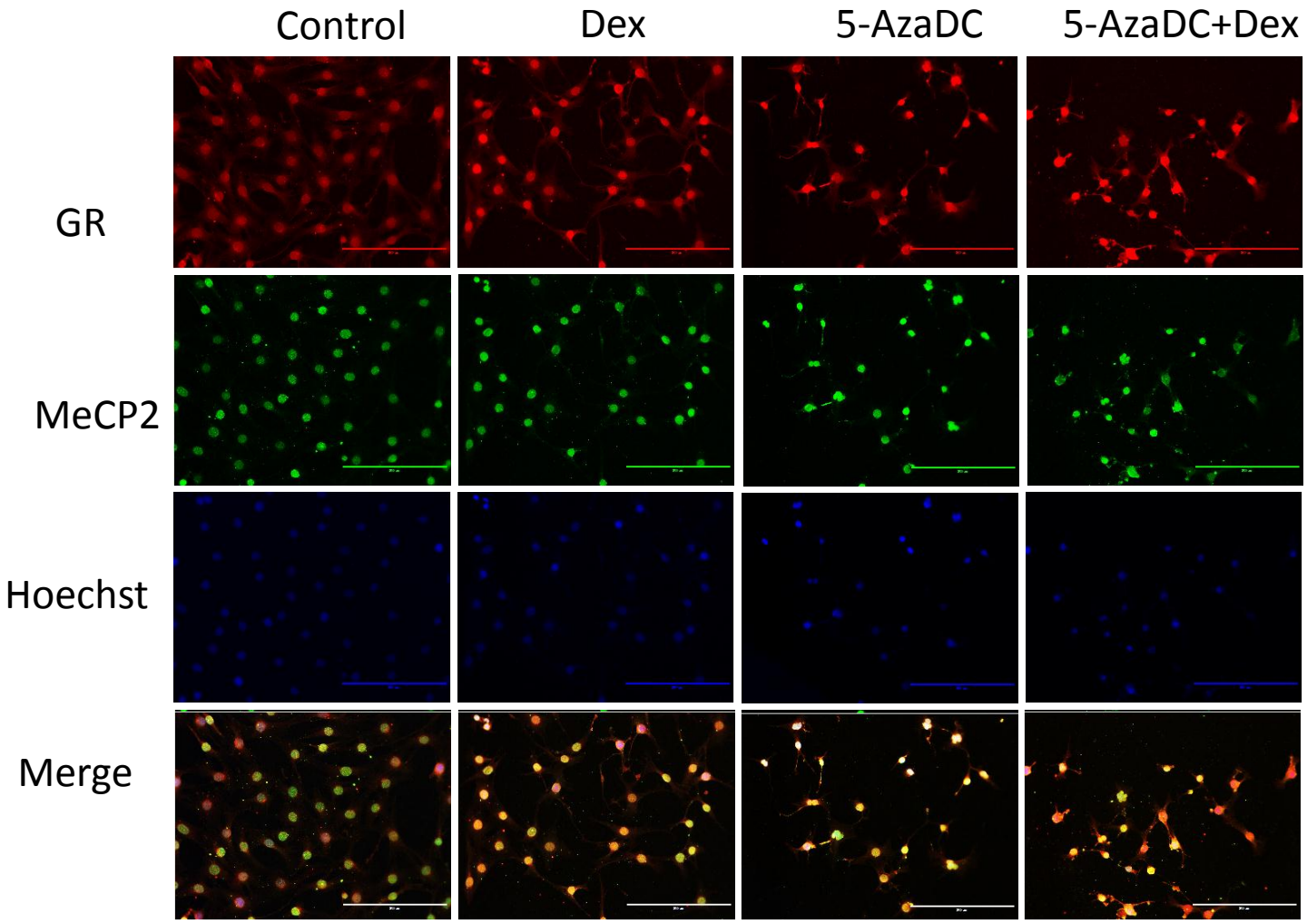

Control
Dex
5-AzaDC
5-AzaDC+Dex
GR
MeCP2
Hoechst
Merge

Figure 22. Site specific promoter methylation is necessary for Dex-induced GR and MeCP2 occupancy at the *crh* promoter

Chromatin immunoprecipitation (ChIP) analysis shows the relative enrichment of the *crh* promoter by GR and MeCP2. In the presence of 5-AzaDC, Dex fails to recruit GR and MeCP2 to the *crh* promoter region. N≥3, Fischer's Least Significance difference Post hoc test, The data represented mean ± SEM, **P*<0.05.

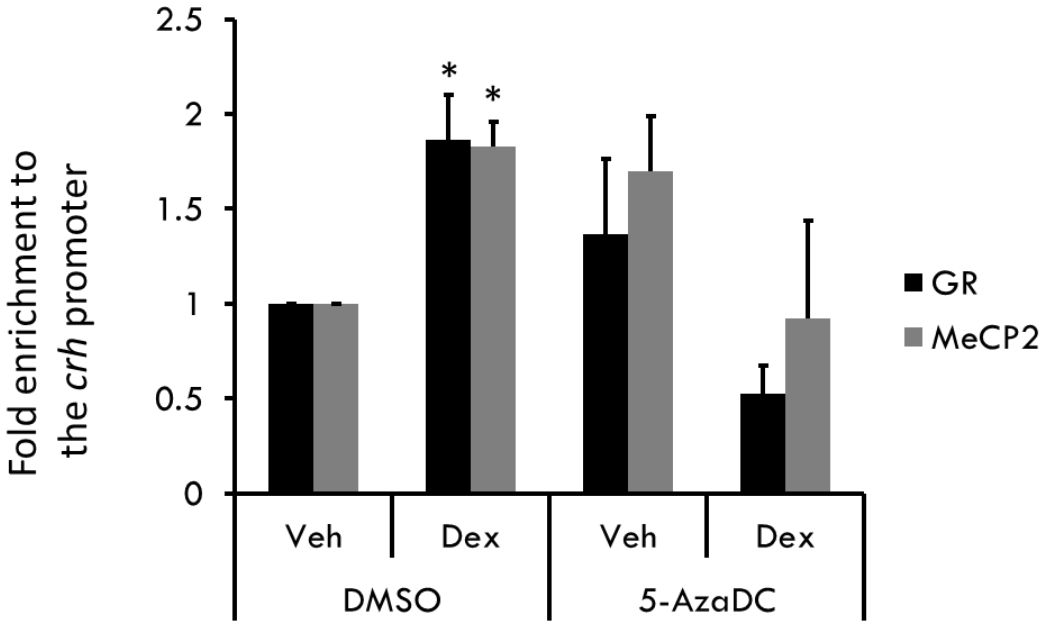

Fold enrichment to the *crh* promoter
2.5
2
1.5
1
0.5
0
*
*
Veh
Dex
Veh
Dex
DMSO
5-AzaDC
GR
MeCP2

Table 4. List of primers used for DNA methylation analysis and chromatin immunoprecipitation.

No	Promoter Region	Forward primer	Reverse Primer	Product size (bp)
1	Bisulfite converted *crh* proximal promoter	TTTTTTTTGGTTTGTATTTGGTT	ACCTTTCCCCTTTCTCTTCAAT	406
2	Bisulfite converted *crh* proximal promoter (nested)	AATTTTTGTTAATGGATAAGT	AACTCTAAATTTCTCCACACCA	294
3	*crh* proximal promoter	TCAGTATGTTTTCCACACTTGGAT	TTTATCGCCTCCTTGGTGAC	112

CHAPTER 4

Effect of repeated dexamethasone exposure on CRH gene expression

a. Introduction

Dysregulation of the HPA axis is associated with many neuropsychiatric disorders[8-11,99]. The failure of negative feedback mediated through GR is often associated with dysregulation of the HPA axis[8,23]. This imbalance in HPA regulation axis leads to the elevated levels of circulating glucocorticoids[5]. The purpose of this study was to understand the effect of repeated exposure of glucocorticoid on expression of *crh* and glucocorticoid receptors. We designed the experiment such that IVB cells were exposed to Dex intermittently (Figure 23). This simulates the high levels and increased number of glucocorticoid spikes observed in depressed individuals[100-102]. How this repeated exposure leads to changes in GR and *crh* levels is unknown.

b. Results and Discussion

The *crh* expression data reveals some interesting aspects. We found that though single 2hr exposure to Dex leads to decreased *crh* expression levels this does not continue with subsequent exposures. After the 2^{nd} and 4^{th} Dex exposures there are marked increases in *crh* levels as compared to vehicle treated samples. Furthermore the 3^{rd} exposure to Dex failed to alter the *crh* expression. The results indicate that while the shorter duration of exposure represses *crh* expression, repeated Dex results in increased expression of *crh* (Figure 24). We then tested if these changes in *crh* expression correlate with changes in GR protein levels. We found that GR protein levels remain unchanged by repeated Dex exposure (Figure 25). Further experiments are required to understand the underlying mechanism. It will be interesting to analyze histone and DNA methylation changes occurring after each Dex exposure. Our prediction is that the chromatin changes due to first Dex exposure affect the subsequent Dex treatment. Further experiments are needed to understand the mechanism by which repeated GC exposure changes the chromatin, which results in alteration of gene expression leading to susceptibility of the HPA axis to dysregulation.

c. Materials and methods

All the techniques used are described in Chapter 2 and 3.

To mimic the high GC environment, cells were intermittently exposed to 10^{-7}M Dex for 2hrs. In all, cells were exposed to four such Dex treatments. As shown in Figure 23, cells were first incubated in charcoal stripped NCS supplemented media for 24 hrs before first Dex exposure. Then the media was replaced with stripped NCS media for 2 hrs before the next Dex exposure.

This sequence was repeated for a total of four Dex exposures. Cells were collected after each Dex treatment for analysis.

Figures and legends

Figure 23. Experimental design for repeated Dex exposure.

IVB cells were plated in media containing NCS and allowed to grow for 24hrs (Red line). Cells were then incubated in media containing charcoal stripped NCS (ss media) for 24 hrs (Black line). On the 3rd day cells were treated with 10^{-7}M Dex or Veh for 2hrs (Blue line). After treatment, cells were incubated in media containing charcoal stripped NCS for 2hrs (Black line). This sequence of 3rd day was repeated three more times as shown. The cells were collected after every Dex/Veh treatment as indicated by arrows.

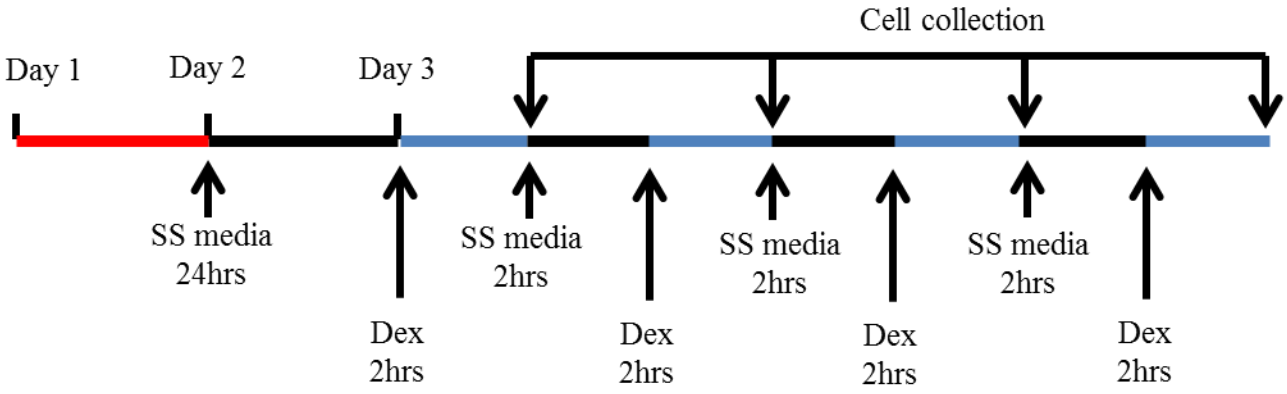

Cell collection
Day 1
Day 2
Day 3
SS media
24hrs
Dex
2hrs
SS media
2hrs
Dex
2hrs
SS media
2hrs
Dex
2hrs
SS media
2hrs
Dex
2hrs

Figure 24. The *crh* expression following repeated Dex exposure.

CRH hnRNA expression measured by RT-qPCR following repeated Dex treatments (Gray bars) presented as fold change of corresponding Veh treated samples (Black bars). The first 2hrs of Dex exposure decreases *crh* expression while the 2^{nd} and 4^{th} Dex exposure increases it. $N \geq 3$, Student T test compared to Veh, The data are represented as mean ±SEM, $^{*}P<0.05$.

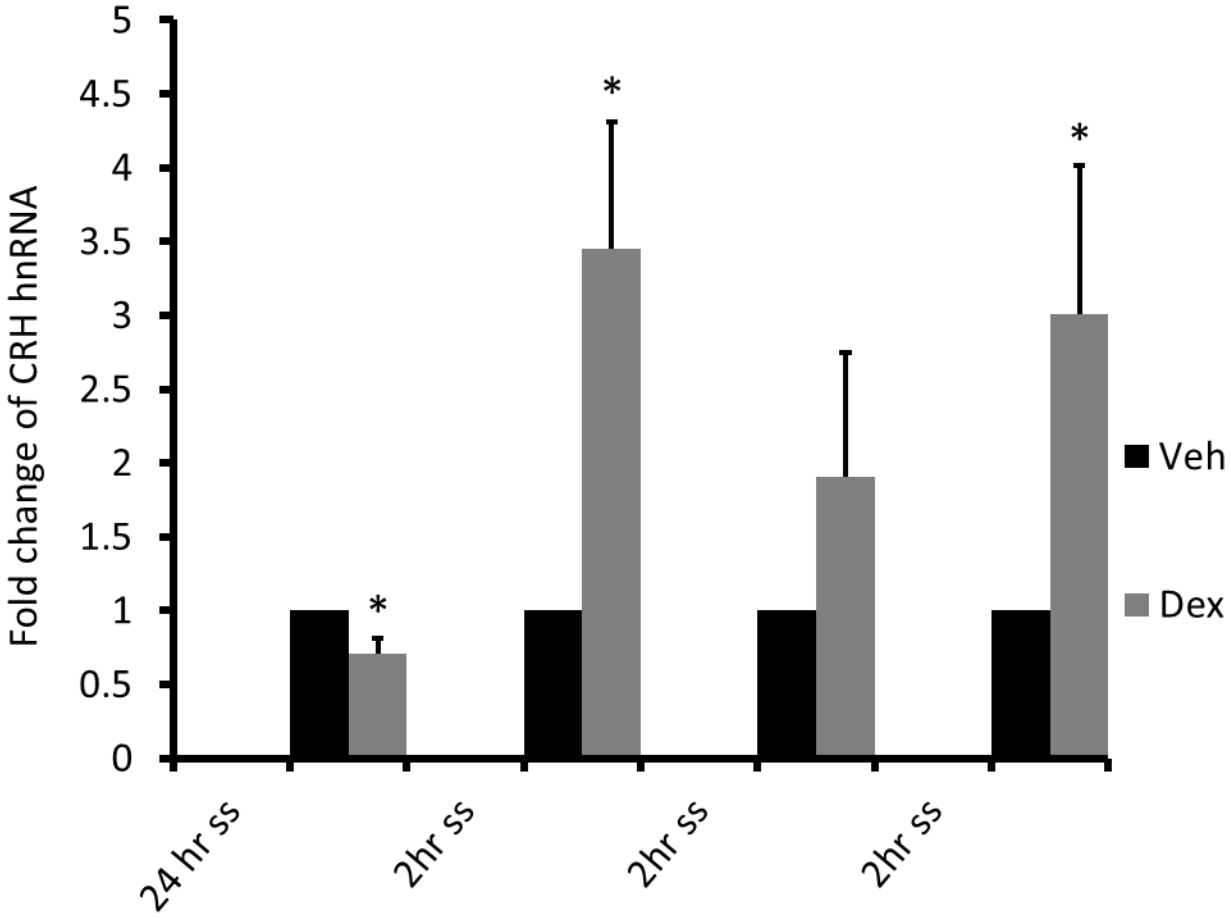
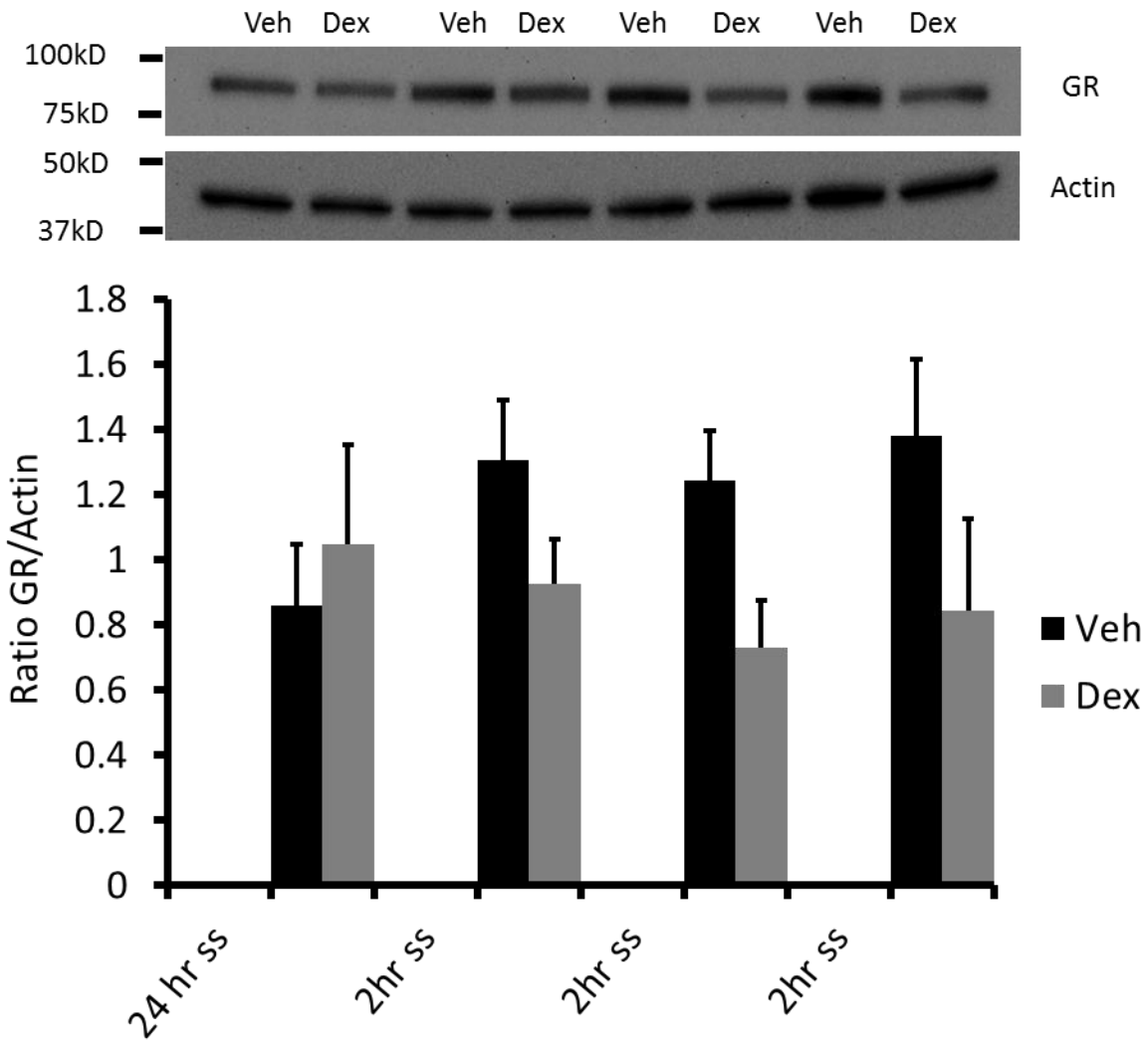

Fold change of CRH hnRNA
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
Veh
Dex
24 hr ss
2hr ss
2hr ss
2hr ss

Figure 25. The relative expression of glucocorticoid receptor following repeated Dex exposure.

Western blot showing relative GR and Actin protein expression following repeated Dex treatments. No significant difference was observed in Dex treated samples when compared with corresponding Veh treated samples. Polyclonal GR (Santa Cruz) and polyclonal Actin (Cell Signaling) antibodies were used in 1:1000 dilution. N=3, Student T test. The data are represented as mean ±SEM.


Veh
Dex
Veh
Dex
Veh
Dex
Veh
Dex
100kD
75kD
50kD
37kD
GR
Actin
Ratio GR/Actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24 hr ss
2hr ss
2hr ss
2hr ss
Veh
Dex

CHAPTER 5

Discussion

The regulation of *crh* is a major component of the negative feedback mechanism which is important in homeostasis of the HPA axis[23]. The molecular mechanism by which *crh* expression is finely tuned dtermines the susceptibility of the HPA axis to stress[42]. The CRH neurons in the PVN of hypothalamus receive various signals which converge onto the CRH gene[5,15,16]. The balance between stimulatory and inhibitory signals determines the HPA axis activity[16]. The molecular changes that maintain this balanced state are not well understood. Glucocorticoids secreted by adrenal glands act through GRs inhibiting HPA axis at various levels. In the PVN of the hypothalamus they inhibit *crh*[21] activity and in pituitary glucocorticoids inhibit Pro-opiomelanocortin (POMC) gene, a precursor for ACTH[23].

Studies over the years have suggested different mechanisms by which *crh* is regulated in the PVN. This study was aimed at investigating different molecular aspects by which GR represses *crh* in hypothalamic cells.

Formation of a GR mediated putative complex

Our findings suggest that GR interacts with chromatin modifying proteins to form a complex in a ligand dependent manner[39]. Previous finding suggested that HDAC1 plays an important role in GR mediated repression of *crh*[37]. Consistent with this, we found that GR interacts with HDAC1 and this interaction increases in the presence of Dex. It was also shown that Dex increases the occupancy of GR and HDAC1 at the *crh* promoter region in the presence of Dex. A depressive state is associated with dysregulated HPA axis activity and increased CRH levels[8,103]. Interestingly, studies have shown that conventional antidepressants have HDAC inhibitory action[104,105]. Also sodium butyrate, a known HDAC inhibitor, shows antidepressant like action[105]. Whether antidepressant mediated inhibition of HDAC affects the *crh* and HPA axis activity is not known. Taken together, the results from this study are consistent with previous findings suggesting a role of HDAC in depression related pathophysiology.

We also analyzed the interaction of GR with MeCP2. The role of MeCP2 in the regulation of *crh* is evident from the findings of McGill et al.[45]. The mice with truncated MeCP2 have higher levels of *crh* in the PVN[45]. We found that similar to HDAC1, The degree of GR-MeCP2 interaction is also a ligand dependent. Furthermore, Dex increased the recruitment of MeCP2 to the *crh* promoter region. This interaction and promoter enrichment was associated with decreased *crh* expression[39]. McGill et al. reported that the mutation in MeCP2 decreases its

ability to occupy the *crh* promoter region[45]. These two findings are consistent and point to the role of MeCP2 in repressing *crh*.

We then further investigated the role of MeCP2 in the maintenance of basal *crh* activity. A 50% reduction in the MeCP2 protein level is associated with 3.5 fold increased expression of *crh* in hypothalamic cells. This is consistent with findings by previous reports, which suggests that functional deficits in MeCP2 level lead to elevated levels of CRH mRNA in the rat PVN [45], however levels of MeCP2 and *crh* are not always inversely correlated[84]. Transgenic mice that over-express MeCP2, as observed in the MeCP2 duplication syndrome, also express increased CRH mRNA in the PVN[83]. Thus, MeCP2 expression level is not always inversely related to *crh* expression. This is explained in a comprehensive review by Chao and Zoghbi[86]. Complete MeCP2 functionality is necessary for normal phenotype. Increased as well as subnormal MeCP2 protein functionality is detrimental[84].

Next we demonstrated that MeCP2 knock down leads to failure of Dex to repress *crh* to basal levels. This suggests that the GR mediated repression and maintenance of the basal *crh* level requires 100% availability of MeCP2. MeCP2 also acts as a link between DNA and histone methylation[106]. The results from this study and one by Sharma et al.[39]. suggest that Dex induced recruitment of MeCP2 to the *crh* promoter is associated with increased H3K9 di and tri-methylation[39]. Whether MeCP2, in context of *crh*, interacts with the histone methylating enzyme remains unknown.

Role of Promoter methylation

The *crh* promoter has a CpG island which plays an important role in the regulation of the gene[41,42]. *In vivo* studies in mice have shown that exposure to stress is associated with decreased

methylation of the *crh* promoter and subsequent increased gene expression[41,42]. Also, CRH mRNA levels are elevated following treatment with the DNA methyltransferase inhibitor 5-AzaDC in mouse N42 hypothalamic cells[42]. The role of DNA methylation in *crh* regulation is also evident through the maternal deprivation (MD) model of stress. According to Chen et al., animals exposed to MD after birth have elevated levels of hypothalamic but not amygdalar CRH mRNA following restraint stress in adulthood. These elevated levels of CRH mRNA correspond to reduced methylation of CpG dinucleotides in the *crh* promoter[41].

The findings from this study are consistent with previous reports. The results indicate that the Dex mediated repression of *crh* coincides with overall and a site specific increase in DNA methylation (Figure 7 and 14). The results presented here also suggest that increased methylation is mediated through DnMT3b, which forms a complex with GR and is recruited at the *crh* promoter region in the presence of Dex[39]. The Dex-mediated site specific methylation takes place at CpG No. 9 and 10. The studies presented here were performed to determine the requirement of DNA methylation in down-regulation of *crh* expression. The findings suggest that this is indeed the case (Figure 15). This inhibition of methylation by DnMT inhibitor 5-AzaDC is also site specific. The findings suggest that methylation of CpG dinucleotide No. 10 plays a critical role in regulation of basal *crh* expression (Figure 16 and 17). Interestingly, this was one of the two sites at which Dex exposure increases the methylation (Figure 14)[39].

It is interesting to note that other studies that examined *crh* promoter methylation also reported site specific changes in methylation. The studeis by Chen et al., and Elliot et al. reported that under stressful conditions methylation at CpG No. 2, which overlaps the CRE,

decreases, resulting in increased *crh* expression. Contrary to this, we do not see any change in methylation of CpG No. 2, either in presence of Dex or 5-AzaDC.

IVB cells, used in this study, are derived from rat embryos and show a distinct *crh* promoter methylation pattern. In this pattern the CpG at -147 (No.4) is notably hypomethylated as compared to other sites. This is in keeping with our previous report[39] (Figures 14C and 17). This site is also hypomethylated in *in vivo* analysis along with other sites as reported by Chen et al.[41]. The methylation at this site is not affected by stress[41], Dex[39] or 5-AzaDC (Figure 17).

The *in vivo* study on rats in maternal deprivation model of stress also revealed that the changes in DNA methylation following stress are specific to PVN of the hypothalamus. The amygdala, which also plays an important role in stress response and expresses *crh* showed no changes in the methylation of *crh* promoter[41]. This indicates that the changes in DNA methylation are cell specific and may be the reason for differential response to glucocorticoids as reported previously[28-30].

Though the effects of glucocorticoids and DNA methylation on *crh* had been known, there had been no prior study which had examined their interdependence. This is the first study to analyze the action of glucocorticoid down-regulation of *crh* in relation to DNA methylation. Some of our findings were unexpected. We found that Dex alone was able to repress or maintain the repressed levels of *crh*. But when used in combination with 5-AzaDC, it actually stimulated expression (Figure 18). It should be noted that in the conditions used, 5-AzaDC failed to stimulate *crh* on its own. This was unexpected and contradictory to our concentration response curve (Figure 15). We believe that this effect is due to charcoal stripping of NCS. While the concentration response curve (Figure 15) was done with cells in media supplemented with NCS for all 5 days, the 5-AzaDC±Dex experiment (Figure 18) was

performed with media supplemented with charcoal stripped NCS for 24 hrs prior to Dex exposure. To test whether charcoal stripping of NCS indeed alters the response, the concentration response curve was done in the presence of charcoal stripped NCS. The 5-AzaDC failed to stimulate *crh* in media containing charcoal stripped NCS (Figure 19). This suggests that inhibition of DNA methylation by 5-AzaDC is required but not sufficient for activation of *crh*. These findings also indicate that Dex bound GR has different effects on a gene depending upon the methylation status of the promoter. As mentioned earlier, this could also explain the opposite effects of glucocorticoids in the PVN and amygdala, both of which express GR.

Changes in DNA methylation by 5-AzaDC are known to increase the protein levels of GR in B and T lymphocytes [95] and MeCP2 in Neuronal stem cells[96]. Our results suggest that the failure of Dex to repress the gene is not accounted for by changes in GR or MeCP2 protein levels (Figure 20). This suggests that the availability of regulatory proteins is not compromised due to treatment. This was an important experiment as changes in the levels of regulatory proteins can have a direct impact on gene expression. The negative immunocytochemical data corroborates the lack of changes in protein levels. Furthermore data suggest that the findings are not due to changes in sub-cellular localization. Dex and/or 5-AzaDC do not alter the sub-cellular distribution of GR and MeCP2 (Figure 21).

To further understand the mechanism of Dex failure to repress *crh*, we tested the hypothesis that, decreased methylation prevents the binding of GR and/or MeCP2 to the promoter region. Indeed results from ChIP analysis suggest that demethylation mediated by 5-AzaDC, restricts the Dex mediated enrichment of GR and MeCP2 to the *crh* promoter region indicating that promoter methylation allows recruitment of GR holoreceptor and MeCP2 (Figure 22).

Data from the last study presented suggest that repeated Dex exposure leads to stimulation of *crh* after initial repression (Figure 24). To explain this further, investigation is needed to understand the chromatin structure due to repeated Dex exposure. One possibility could be that, initial Dex exposure alters the chromatin and sets the stage for next Dex exposure. Whether repeated Dex exposure changes DNA methylation and thus alters GR recruitment to the promoter remains unknown.

Taken together, these findings add novel insight into the molecular mechanism by which GR regulates *crh*. This will enable us to better understand the reasons by which HPA axis becomes dysfunctional in the case of depression. Depressed individuals have an imbalanced HPA axis mainly because of failed GR mediated negative feedback[8]. The study by Elliott et al., suggests that imipramine, a widely used antidepressant, is able to reverse the DNA methylation changes that are associated with social avoidance model of depression[42]. It may be that, antidepressants restore the *crh* promoter methylation that allows GR-mediated down-regulation of *crh* expression, thus restoring physiologic HPA axis function.

In the context of GR and other NR signaling:

The mechanisms by which nuclear receptors (NR) mediate repression of a gene remain elusive. Initial findings suggested that the gene repression mediated through NRs is through the recruitment of co-repressors by unliganded receptors. For example, the unliganded thyroid hormone receptor recruits the silencing mediator of retinoid and thyroid hormone receptor (SMRT)[107]. It also recruits nuclear receptor co-repressor (NCoR)[108], in association with HDAC3[109]. Other studies indicated that NR mediated negative regulation of a gene is mediated through negative response elements present in gene promoter. This mechanism is still under

scrutiny since there is a substantial deviation in the response element sequence. Also the presence of half sites adds doubt to the association of NRs to corresponding negative response elements.

GR is a major ligand activated transcription factor and negatively regulates the expression of many genes. The *crh* promoter lacks the consensus GREs but contains a composite half site for GRE and AP-1 as reported by Maloski and Droin[35]. The association of GR to the *crh* promoter was first demonstrated through electromobility shift assays[36]. The authors concluded that this interaction was mediated through GR half sites. Whether the GR holoreceptor interacts with the *crh* promoter remains controversial. Evans et al.[47] reported that GR does not interact with *crh* promoter region containing half sites[47] others contradict these findings[36-39]. This contradiction may be due to difference in test models. While studies in a rat stress model reveal no GR-*crh* promoter interaction following stress and increased glucocorticoids[47], *in vitro* studies show ligand dependent interaction[37-39]. Our findings indicate that GR interacts with the *crh* promoter and that this interaction is ligand dependent.

As reported by Sharma et al., there are two possible ways in which GR can interact with *crh* promoter (Figure 26A, B).The first scenario is that GR interacts with the nGRE half sites in the *crh* promoter. This interaction is coupled with GR-MeCP2 and GR-DnMT3b interactions. DnMT3b then leads to the methylation of the promoter and MeCP2 recruitment to the methylated CpGs. This complex then recruits HDAC1. We predict that GR-MeCP2 interaction stabilizes their respective interactions with DNA. This is also supported by our findings which suggest that MeCP2 knock down and inhibition of CpG methylation prevents GR mediated *crh* repression. Furthermore, Dex fails to recruit GR and MeCP2 to the de-methylated promoter (Figure 22). This mechanism is similar to the one suggested in figure 6G.

As mentioned earlier some studies have reported that GR does not interact with *crh* promoter directly[47]. One study done on $GR^{dim/dim}$ mice suggests that GR-DNA interaction may not be necessary for *crh* gene regulation. The $GR^{dim/dim}$ mice carry a mutation in GR protein that prevents its binding to the DNA. The $GR^{dim/dim}$ mice show no change in CRH mRNA levels in the median eminence as compared to wild type[46]. This suggests that direct interaction of GR may not be necessary for *crh* regulation. On the other hand, in the same study there was a marked increase in *POMC* expression as compared to the wild type, suggesting that GR-DNA interaction is necessary for its negative regulation.

Figure 26B shows a working model in which GR may not interact with DNA directly but through its interaction with MeCP2. Such an alternate or tethering pathway mechanism was first described with respect to AP-1 binding proteins[70-72]. In those studies AP-1 prevented GR binding to GRE and acted as a potent inhibitor of gene activation. For example the repression of collagenase gene by GR does not involve direct binding of GR to the promoter but through possible interaction with the AP-1 site[71,72]. Such trans-repression mediated by GR is also implicated in the genes activated by NF-kB. The physical association of GR holoreceptor with NF-kB prevents it from binding to target gene promoter. This results in repression of gene without direct GR-DNA interaction[73,110,111]. Thus GR mediated repression of *crh* is possible without direct GR-DNA interaction.

Here the molecular mechanism proposed by Sharma et al. was further analyzed to begin to determine which components of the putative complex are required for its assembly. The inhibition of methylation revealed that site specific methylation is necessary for maintaining repressed levels of *crh* and recruitment of components of complex. As shown in figure 27B, demethylation increases the expression of *crh* in parallel to the decreased presence of GR and

MeCP2 at the promoter. We predict that the methylation pattern sets the stage for GR action. Perhaps in case of site specific inhibition of methylation, GR activates a gene, by recruiting co-activators. On the other hand, in presence of CpG methylation GR and MeCP2 are recruited and thus cause gene repression.

It has been demonstrated that a DNA sequence determines GR recruitment, interaction, conformation and activity[98,112]. In addition, recent crystallographic study revealed that the binding of GR to a novel nGRE is unique as compared to GRE. Generally GR interacts with GRE as a dimer while its interaction with these novel nGRE is in such a way that it prevents GR dimerization[97]. These findings underscore GR:DNA binding depends on DNA sequence and structural orientation. Another study by Meijsing et al. suggests that the activity of GR can change due to changes in the response elements by as little as single nucleotide. That crystallographic study revealed that distinct binding site sequences induce structural changes in GR that lead to different activity[98]. Taken together, the course of GR action depends on sequence of DNA and chromatin architecture, thus changes in the methylation of promoter CpG will alter the course of GR action.

Since MeCP2 binds to methylated CpGs and is a part of GR-mediated putative co-repressor complex, we tested if MeCP2 is necessary for GR action. Our results indicate that GR fails to repress *crh* in the absence of MeCP2 (Figure 13 and 27). Whether depletion of MeCP2 affects GR occupancy to the *crh* promoter remains unknown. The results from the MeCP2 knock down study differ from that of inhibition of methylation. In cells incubated in charcoal stripped NCS, 5-AzaDC failed to increase *crh* expression (Figure 18). On the other hand MeCP2 knock down alone was able to increase the *crh* expression and Dex failed to repress

it (Figure 13). These findings suggest that inhibition of methylation is necessary but not sufficient to activate the gene, while MeCP2 knockdown is sufficient to activate the gene.

In summary, GR mediated regulation of *crh* in the PVN involves chromatin changes and formation of a complex. While the DNA methylation pattern sets the stage for the course of GR action, MeCP2 plays a critical role in its execution. The studies presented advanced our knowledge about the role of chromatin and epigenetics in the regulation of *crh* and hence regulation of the stress response. These mechanistic insights should help us understand the development of depressive conditions.

Relevance to other areas

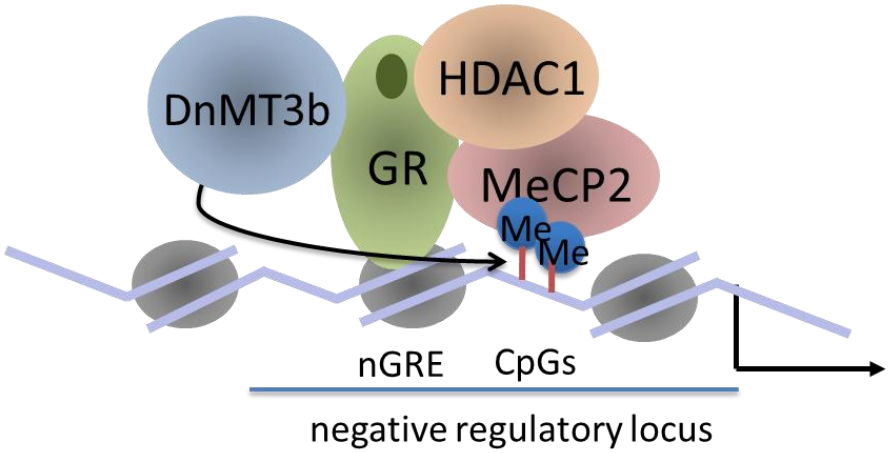
Our findings add a new dimension to the regulation of *crh* gene and a novel mechanism of GR mediated gene regulation. To our knowledge, there is no study that has investigated the role of DNA methylation in NR mediated gene regulation and NR-DNA binding. These findings will likely have significant implications in a wider array of areas. For example, the difference in gene promoter methylation can explain the opposing effects of hormonal ligands in different tissues and in diseased and normal state. It could also explain the resistance to hormonal therapies observed in certain cases of breast, cervical and prostate cancer which have a major hormonal component.

Figures and legends

Figure 26. Working model of co-repressor complex recruitment

A, GR and MeCP2 interact directly with DNA elements and form a putative complex with DnMT3b and HDAC1. B, The Methylation of DNA at CpG dinucleotides is induced by GR-DnMT3b complex. Then the methylated DNA recruits MeCP2, GR and HDAC1 and the complex which represses gene expression. In this case GR does not bind to the DNA directly but is present at the *crh* promoter as a part of the complex. Me; Methylated CpGs. (From Sharma et al. *Mol. Endo*. 2013)

A


DnMT3b
HDAC1
GR
MeCP2
Me
Me
nGRE
CpGs
negative regulatory locus

B

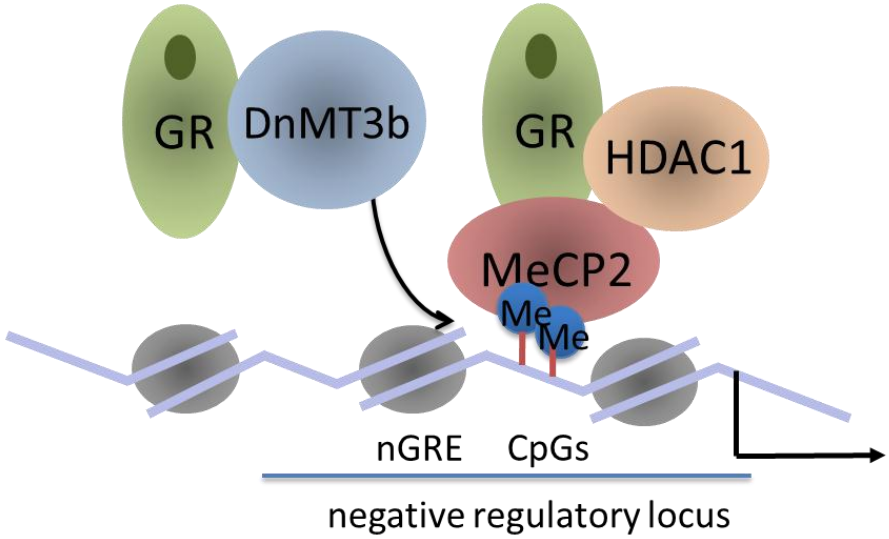
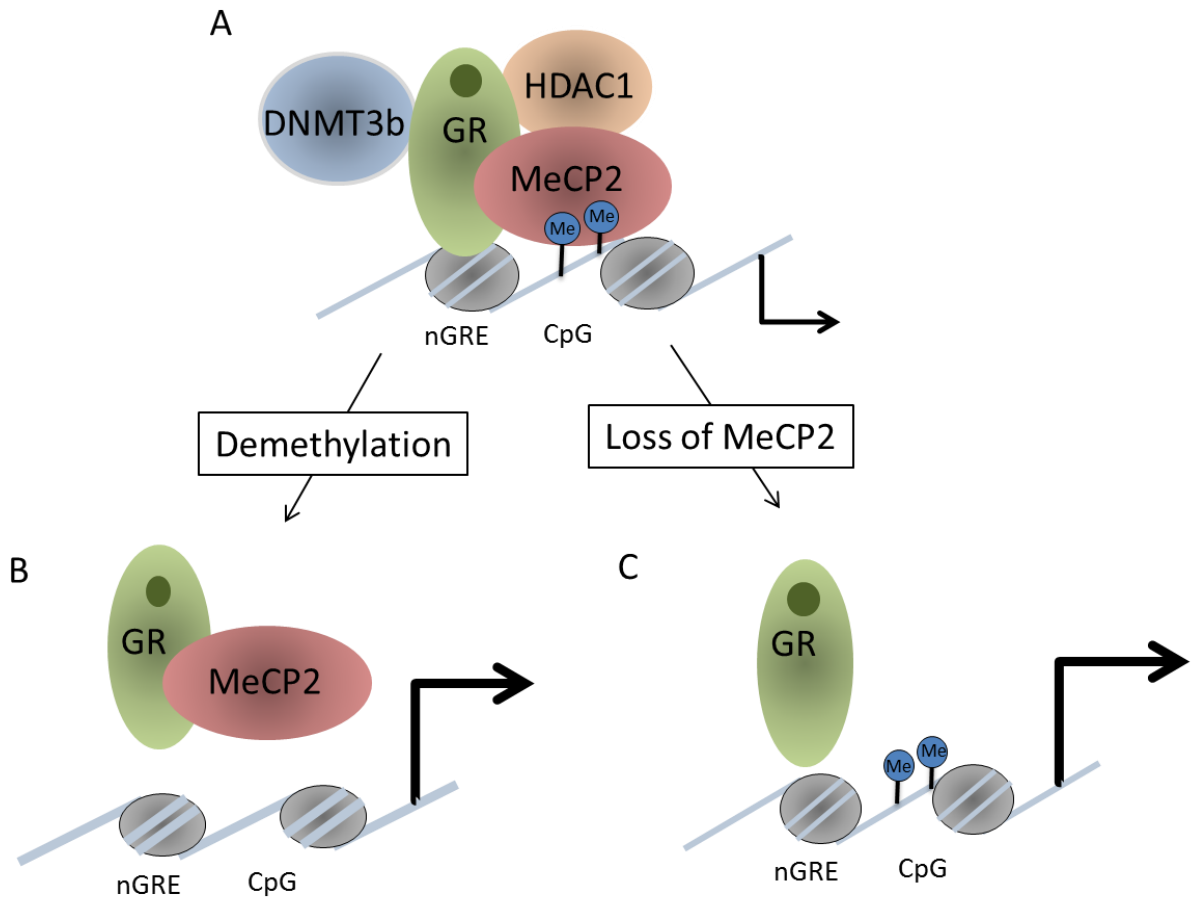

GR
DnMT3b
GR
HDAC1
MeCP2
Me
Me
nGRE
CpGs
negative regulatory locus

Figure 27. Contributions of MeCP2 and CpG methylation to repression.

A, Ligand-bound GR induces formation of a GR-mediated co-repressor complex that is recruited to the proximal promoter. **B**, Demethylation of CpG site 10, diminishes occupancy of GR and MeCP2, which in turn leads to increased gene expression. **C**, The absence of MeCP2 permits gene expression, even though DNA methylation is not altered. The fate of DnMT3b and HDAC1 is not known for either scenario (B and C). Me; Methylated CpGs.


A
DNMT3b
GR
HDAC1
MeCP2
Me
Me
nGRE
CpG
Demethylation
Loss of MeCP2
B
GR
MeCP2
nGRE
CpG
C
GR
Me
Me
nGRE
CpG

CHAPTER 6

References

1. Miller DB, O'Callaghan JP. Neuroendocrine aspects of the response to stress. *Metabolism*. 2002;51(6 Suppl 1):5-10. doi: ameta05100s5 [pii].

2. Selye H. A syndrome produced by diverse nocuous agents. 1936. *J Neuropsychiatry Clin Neurosci*. 1998;10(2):230-231.

3. Szabo S, Tache Y, Somogyi A. The legacy of hans selye and the origins of stress research: A retrospective 75 years after his landmark brief "letter" to the editor# of nature. *Stress*. 2012;15(5):472-478. doi: 10.3109/10253890.2012.710919 [doi].

4. Denver RJ. Structural and functional evolution of vertebrate neuroendocrine stress systems. *Ann N Y Acad Sci*. 2009;1163:1-16. doi: 10.1111/j.1749-6632.2009.04433.x [doi].

5. Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci*. 2006;8(4):383-395.

6. Johnson EO, Kamilaris TC, Chrousos GP, Gold PW. Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. *Neurosci Biobehav Rev*. 1992;16(2):115-130. doi: S0149-7634(05)80175-7 [pii].

7. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci*. 2009;10(6):397-409. doi: 10.1038/nrn2647 [doi].

8. Carroll, Curtis GC, Mendels J. Neuroendocrine regulation in depression. I. limbic system-adrenocortical dysfunction. *Arch Gen Psychiatry*. 1976;33(9):1039-1044.

9. Swaab DF, Bao AM, Lucassen PJ. The stress system in the human brain in depression and neurodegeneration. *Ageing Res Rev*. 2005;4(2):141-194. doi: S1568-1637(05)00004-8 [pii].

10. Brown ES, Rush AJ, McEwen BS. Hippocampal remodeling and damage by corticosteroids: Implications for mood disorders. *Neuropsychopharmacology*. 1999;21(4):474-484. doi: S0893133X99000548 [pii].

11. Thompson KN, Phillips LJ, Komesaroff P, et al. Stress and HPA-axis functioning in young people at ultra high risk for psychosis. *J Psychiatr Res*. 2007;41(7):561-569. doi: S0022-3956(06)00109-9 [pii].

12. Lechan RM, Toni R. Functional anatomy of the hypothalamus and pituitary. In: De Groot LJ, Beck-Peccoz P, Chrousos G, et al, eds. *Endotext.* South Dartmouth (MA): MDText.com, Inc; 2000. NBK279126 [bookaccession].

13. Uht RM. *Mechanisms of glucocorticoid receptor (GR) mediated corticotropin releasing hormone gene expression.* INTECH Open Access Publisher; 2012.

14. Herman JP, Figueiredo H, Mueller NK, et al. Central mechanisms of stress integration: Hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol*. 2003;24(3):151-180. doi: S0091302203000293 [pii].

15. Aguilera G, Liu Y. The molecular physiology of CRH neurons. *Front Neuroendocrinol*. 2012;33(1):67-84. doi: 10.1016/j.yfrne.2011.08.002 [doi].

16. Levy BH, Tasker JG. Synaptic regulation of the hypothalamic-pituitary-adrenal axis and its modulation by glucocorticoids and stress. *Front Cell Neurosci*. 2012;6:24. doi: 10.3389/fncel.2012.00024 [doi].

17. Sawchenko PE, Imaki T, Potter E, Kovacs K, Imaki J, Vale W. The functional neuroanatomy of corticotropin-releasing factor. *Ciba Found Symp*. 1993;172:5-21; discussion 21-9.

18. Imaki T, Xiao-Quan W, Shibasaki T, et al. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *J Clin Invest*. 1995;96(1):231-238. doi: 10.1172/JCI118026 [doi].

19. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev*. 2000;21(1):55-89. doi: 10.1210/edrv.21.1.0389 [doi].

20. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev*. 1984;5(1):25-44. doi: 10.1210/edrv-5-1-25 [doi].

21. Herman JP, McKlveen JM, Solomon MB, Carvalho-Netto E, Myers B. Neural regulation of the stress response: Glucocorticoid feedback mechanisms. *Braz J Med Biol Res*. 2012;45(4):292-298. doi: S0100-879X2012007500041 [pii].

22. Keller-Wood ME, Dallman MF. Corticosteroid inhibition of ACTH secretion. *Endocr Rev*. 1984;5(1):1-24. doi: 10.1210/edrv-5-1-1 [doi].

23. Kretz O, Reichardt HM, Schutz G, Bock R. Corticotropin-releasing hormone expression is the major target for glucocorticoid feedback-control at the hypothalamic level. *Brain Res*. 1999;818(2):488-491. doi: S0006-8993(98)01277-3 [pii].

24. Jacobson L, Sapolsky R. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev*. 1991;12(2):118-134. doi: 10.1210/edrv-12-2-118 [doi].

25. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*. 1981;213(4514):1394-1397.

26. Baigent SM. Peripheral corticotropin-releasing hormone and urocortin in the control of the immune response. *Peptides*. 2001;22(5):809-820. doi: S0196-9781(01)00395-3 [pii].

27. Grino M, Chrousos GP, Margioris AN. The corticotropin releasing hormone gene is expressed in human placenta. *Biochem Biophys Res Commun*. 1987;148(3):1208-1214. doi: S0006-291X(87)80261-9 [pii].

28. Schulkin J, Gold PW, McEwen BS. Induction of corticotropin-releasing hormone gene expression by glucocorticoids: Implication for understanding the states of fear and anxiety and allostatic load. *Psychoneuroendocrinology*. 1998;23(3):219-243. doi: S0306453097000991 [pii].

29. Schulkin J, McEwen BS, Gold PW. Allostasis, amygdala, and anticipatory angst. *Neurosci Biobehav Rev*. 1994;18(3):385-396. doi: 0149-7634(94)90051-5 [pii].

30. Swanson LW, Simmons DM. Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: A hybridization histochemical study in the rat. *J Comp Neurol*. 1989;285(4):413-435. doi: 10.1002/cne.902850402 [doi].

31. Liu Y, Aguilera G. Cyclic AMP inducible early repressor mediates the termination of corticotropin releasing hormone transcription in hypothalamic neurons. *Cell Mol Neurobiol*. 2009;29(8):1275-1281. doi: 10.1007/s10571-009-9423-1 [doi].

32. Liu Y, Coello AG, Grinevich V, Aguilera G. Involvement of transducer of regulated cAMP response element-binding protein activity on corticotropin releasing hormone transcription. *Endocrinology*. 2010;151(3):1109-1118. doi: 10.1210/en.2009-0963 [doi].

33. Liu Y, Poon V, Sanchez-Watts G, Watts AG, Takemori H, Aguilera G. Salt-inducible kinase is involved in the regulation of corticotropin-releasing hormone transcription in hypothalamic neurons in rats. *Endocrinology*. 2012;153(1):223-233. doi: 10.1210/en.2011-1404 [doi].

34. Jeanneteau FD, Lambert WM, Ismaili N, et al. BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. *Proc Natl Acad Sci U S A*. 2012;109(4):1305-1310. doi: 10.1073/pnas.1114122109 [doi].

35. Malkoski SP, Dorin RI. Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. *Mol Endocrinol*. 1999;13(10):1629-1644. doi: 10.1210/mend.13.10.0351 [doi].

36. Malkoski SP, Handanos CM, Dorin RI. Localization of a negative glucocorticoid response element of the human corticotropin releasing hormone gene. *Mol Cell Endocrinol*. 1997;127(2):189-199. doi: S0303-7207(96)04004-X [pii].

37. Miller L, Foradori CD, Lalmansingh AS, Sharma D, Handa RJ, Uht RM. Histone deacetylase 1 (HDAC1) participates in the down-regulation of corticotropin releasing hormone gene (crh) expression. *Physiol Behav*. 2011;104(2):312-320. doi: 10.1016/j.physbeh.2011.03.026 [doi].

38. Przybycien-Szymanska MM, Mott NN, Pak TR. Alcohol dysregulates corticotropin-releasing-hormone (CRH) promoter activity by interfering with the negative glucocorticoid response element (nGRE). *PLoS One*. 2011;6(10):e26647. doi: 10.1371/journal.pone.0026647 [doi].

39. Sharma D, Bhave S, Gregg E, Uht R. Dexamethasone induces a putative repressor complex and chromatin modifications in the CRH promoter. *Mol Endocrinol*. 2013;27(7):1142-1152. doi: 10.1210/me.2013-1079 [doi].

40. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007;128(4):693-705. doi: S0092-8674(07)00184-5 [pii].

41. Chen J, Evans AN, Liu Y, Honda M, Saavedra JM, Aguilera G. Maternal deprivation in rats is associated with corticotrophin-releasing hormone (CRH) promoter hypomethylation and

enhances CRH transcriptional responses to stress in adulthood. *J Neuroendocrinol*. 2012;24(7):1055-1064. doi: 10.1111/j.1365-2826.2012.02306.x [doi].

42. Elliott E, Ezra-Nevo G, Regev L, Neufeld-Cohen A, Chen A. Resilience to social stress coincides with functional DNA methylation of the crf gene in adult mice. *Nat Neurosci*. 2010;13(11):1351-1353. doi: 10.1038/nn.2642 [doi].

43. Nan X, Ng HH, Johnson CA, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*. 1998;393(6683):386-389. doi: 10.1038/30764 [doi].

44. Jones PL, Veenstra GJ, Wade PA, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet*. 1998;19(2):187-191. doi: 10.1038/561 [doi].

45. McGill BE, Bundle SF, Yaylaoglu MB, Carson JP, Thaller C, Zoghbi HY. Enhanced anxiety and stress-induced corticosterone release are associated with increased crh expression in a mouse model of rett syndrome. *Proc Natl Acad Sci U S A*. 2006;103(48):18267-18272. doi: 0608702103 [pii].

46. Reichardt HM, Kaestner KH, Tuckermann J, et al. DNA binding of the glucocorticoid receptor is not essential for survival. *Cell*. 1998;93(4):531-541. doi: S0092-8674(00)81183-6 [pii].

47. Evans AN, Liu Y, Macgregor R, Huang V, Aguilera G. Regulation of hypothalamic corticotropin-releasing hormone transcription by elevated glucocorticoids. *Mol Endocrinol*. 2013;27(11):1796-1807. doi: 10.1210/me.2013-1095 [doi].

48. Ma XM, Camacho C, Aguilera G. Regulation of corticotropin-releasing hormone (CRH) transcription and CRH mRNA stability by glucocorticoids. *Cell Mol Neurobiol*. 2001;21(5):465-475.

49. Shepard JD, Liu Y, Sassone-Corsi P, Aguilera G. Role of glucocorticoids and cAMP-mediated repression in limiting corticotropin-releasing hormone transcription during stress. *J Neurosci*. 2005;25(16):4073-4081. doi: 25/16/4073 [pii].

50. Liu Y, Kalintchenko N, Sassone-Corsi P, Aguilera G. Inhibition of corticotrophin-releasing hormone transcription by inducible cAMP-early repressor in the hypothalamic cell line, 4B. *J Neuroendocrinol*. 2006;18(1):42-49. doi: JNE1383 [pii].

51. Newton R. Molecular mechanisms of glucocorticoid action: What is important? *Thorax*. 2000;55(7):603-613.

52. Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM. Functional domains of the human glucocorticoid receptor. *Cell*. 1986;46(5):645-652. doi: 0092-8674(86)90339-9 [pii].

53. Hollenberg SM, Evans RM. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell*. 1988;55(5):899-906. doi: 0092-8674(88)90145-6 [pii].

54. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev*. 1996;17(3):245-261. doi: 10.1210/edrv-17-3-245 [doi].

55. Kolber BJ, Wieczorek L, Muglia LJ. HPA axis dysregulation and behavioral analysis of mouse mutants with altered GR or MR function. *Stress*. 2008;11(5):321-338. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2744095/. doi: 10.1080/10253890701821081.

56. Morimoto M, Morita N, Ozawa H, Yokoyama K, Kawata M. Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: An immunohistochemical and in situ hybridization study. *Neurosci Res*. 1996;26(3):235-269. doi: S0168-0102(96)01105-4 [pii].

57. Bamberger CM, Bamberger AM, de Castro M, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest*. 1995;95(6):2435-2441. doi: 10.1172/JCI117943 [doi].

58. Beato M, Truss M, Chavez S. Control of transcription by steroid hormones. *Ann N Y Acad Sci*. 1996;784:93-123.

59. Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J. Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. *J Biol Chem*. 1991;266(5):3107-3112.

60. Strahle U, Klock G, Schutz G. A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. *Proc Natl Acad Sci U S A*. 1987;84(22):7871-7875.

61. Rose AJ, Vegiopoulos A, Herzig S. Role of glucocorticoids and the glucocorticoid receptor in metabolism: Insights from genetic manipulations. *J Steroid Biochem Mol Biol*. 2010;122(1-3):10-20. doi: 10.1016/j.jsbmb.2010.02.010 [doi].

62. Smoak KA, Cidlowski JA. Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev*. 2004;125(10-11):697-706. doi: S0047-6374(04)00161-7 [pii].

63. Reichardt HM, Schutz G. Glucocorticoid signalling--multiple variations of a common theme. *Mol Cell Endocrinol*. 1998;146(1-2):1-6.

64. Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson JA, Rottman FM, Yamamoto KR. Hormone-mediated repression: A negative glucocorticoid response element from the bovine prolactin gene. *Genes Dev*. 1988;2(9):1144-1154.

65. Drouin J, Trifiro MA, Plante RK, Nemer M, Eriksson P, Wrange O. Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. *Mol Cell Biol*. 1989;9(12):5305-5314.

66. Surjit M, Ganti KP, Mukherji A, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell*. 2011;145(2):224-241. doi: 10.1016/j.cell.2011.03.027 [doi].

67. Morrison N, Eisman J. Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. *J Bone Miner Res*. 1993;8(8):969-975. doi: 10.1002/jbmr.5650080810 [doi].

68. Torra IP, Tsibulsky V, Delaunay F, et al. Circadian and glucocorticoid regulation of rev-erbalpha expression in liver. *Endocrinology*. 2000;141(10):3799-3806. doi: 10.1210/endo.141.10.7708 [doi].

69. Akerblom IE, Slater EP, Beato M, Baxter JD, Mellon PL. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. *Science*. 1988;241(4863):350-353.

70. Jonat C, Rahmsdorf HJ, Park KK, et al. Antitumor promotion and antiinflammation: Down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell*. 1990;62(6):1189-1204. doi: 0092-8674(90)90395-U [pii].

71. Yang-Yen HF, Chambard JC, Sun YL, et al. Transcriptional interference between c-jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell*. 1990;62(6):1205-1215. doi: 0092-8674(90)90396-V [pii].

72. Schule R, Rangarajan P, Kliewer S, et al. Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. *Cell*. 1990;62(6):1217-1226. doi: 0092-8674(90)90397-W [pii].

73. Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A*. 1994;91(2):752-756.

74. Villagra A, Gutierrez J, Paredes R, et al. Reduced CpG methylation is associated with transcriptional activation of the bone-specific rat osteocalcin gene in osteoblasts. *J Cell Biochem*. 2002;85(1):112-122. doi: 10.1002/jcb.10113 [pii].

75. Luo Y, Zhou B, Zhao M, Tang J, Lu Q. Promoter demethylation contributes to TSLP overexpression in skin lesions of patients with atopic dermatitis. *Clin Exp Dermatol*. 2014;39(1):48-53. doi: 10.1111/ced.12206 [doi].

76. Pratt LA, Brody DJ. Depression in the united states household population, 2005-2006. *NCHS Data Brief*. 2008;(7)(7):1-8.

77. Merikangas KR, He JP, Burstein M, et al. Lifetime prevalence of mental disorders in U.S. adolescents: Results from the national comorbidity survey replication--adolescent supplement (NCS-A). *J Am Acad Child Adolesc Psychiatry*. 2010;49(10):980-989. doi: 10.1016/j.jaac.2010.05.017 [doi].

78. Kessler RC. Epidemiology of women and depression. *J Affect Disord*. 2003;74(1):5-13. doi: S0165032702004263 [pii].

79. Kessler RC, Berglund P, Demler O, et al. The epidemiology of major depressive disorder: Results from the national comorbidity survey replication (NCS-R). *JAMA*. 2003;289(23):3095-3105. doi: 10.1001/jama.289.23.3095 [doi].

80. Ising M, Holsboer F. Genetics of stress response and stress-related disorders. *Dialogues Clin Neurosci*. 2006;8(4):433-444.

81. Linde K, Kriston L, Rucker G, et al. Efficacy and acceptability of pharmacological treatments for depressive disorders in primary care: Systematic review and network meta-analysis. *Ann Fam Med*. 2015;13(1):69-79. doi: 10.1370/afm.1687 [doi].

82. Berman RM, Narasimhan M, Charney DS. Treatment-refractory depression: Definitions and characteristics. *Depress Anxiety*. 1997;5(4):154-164. doi: 10.1002/(SICI)1520-6394(1997)5:4<154::AID-DA2>3.0.CO;2-D [pii].

83. Samaco RC, Mandel-Brehm C, McGraw CM, Shaw CA, McGill BE, Zoghbi HY. Crh and Oprm1 mediate anxiety-related behavior and social approach in a mouse model of MECP2 duplication syndrome. *Nat Genet*. 2012;44(2):206-211. doi: 10.1038/ng.1066 [doi].

84. Chao HT, Zoghbi HY. MeCP2: Only 100% will do. *Nat Neurosci*. 2012;15(2):176-177. doi: 10.1038/nn.3027 [doi].

85. Daniel PM. Anatomy of the hypothalamus and pituitary gland. *J Clin Pathol Suppl (Assoc Clin Pathol)*. 1976;7:1-7.

86. Sawchenko PE, Swanson LW, Vale WW. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. *Proc Natl Acad Sci U S A*. 1984;81(6):1883-1887.

87. Kasckow J, Mulchahey JJ, Aguilera G, et al. Corticotropin-releasing hormone (CRH) expression and protein kinase A mediated CRH receptor signalling in an immortalized hypothalamic cell line. *J Neuroendocrinol*. 2003;15(5):521-529. doi: 1026 [pii].

88. Liu Y, Kamitakahara A, Kim AJ, Aguilera G. Cyclic adenosine 3',5'-monophosphate responsive element binding protein phosphorylation is required but not sufficient for activation of corticotropin-releasing hormone transcription. *Endocrinology*. 2008;149(7):3512-3520. doi: 10.1210/en.2008-0052 [doi].

89. Kriaucionis S, Bird A. The major form of MeCP2 has a novel N-terminus generated by alternative splicing. *Nucleic Acids Res*. 2004;32(5):1818-1823. doi: 10.1093/nar/gkh349 [doi].

90. Mnatzakanian GN, Lohi H, Munteanu I, et al. A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to rett syndrome. *Nat Genet*. 2004;36(4):339-341. doi: 10.1038/ng1327 [doi].

91. Dastidar SG, Bardai FH, Ma C, et al. Isoform-specific toxicity of Mecp2 in postmitotic neurons: Suppression of neurotoxicity by FoxG1. *J Neurosci*. 2012;32(8):2846-2855. doi: 10.1523/JNEUROSCI.5841-11.2012 [doi].

92. Meehan RR, Lewis JD, Bird AP. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res*. 1992;20(19):5085-5092.

93. Wade PA. Methyl CpG binding proteins: Coupling chromatin architecture to gene regulation. *Oncogene*. 2001;20(24):3166-3173. doi: 10.1038/sj.onc.1204340 [doi].

94. Kimura H, Shiota K. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. *J Biol Chem*. 2003;278(7):4806-4812. doi: 10.1074/jbc.M209923200 [doi].

95. Piotrowska H, Jagodzinski PP. Trichostatin A, sodium butyrate, and 5-aza-2'-deoxycytidine alter the expression of glucocorticoid receptor alpha and beta isoforms in hut-78 T- and raji B-lymphoma cell lines. *Biomed Pharmacother*. 2007;61(7):451-454. doi: S0753-3322(07)00067-4 [pii].

96. Liyanage VR, Zachariah RM, Rastegar M. Decitabine alters the expression of Mecp2 isoforms via dynamic DNA methylation at the Mecp2 regulatory elements in neural stem cells. *Mol Autism*. 2013;4(1):46-2392-4-46. doi: 10.1186/2040-2392-4-46 [doi].

97. Hudson WH, Youn C, Ortlund EA. The structural basis of direct glucocorticoid-mediated transrepression. *Nat Struct Mol Biol*. 2013;20(1):53-58. doi: 10.1038/nsmb.2456 [doi].

98. Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR. DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science*. 2009;324(5925):407-410. doi: 10.1126/science.1164265 [doi].

99. Watson S, Owen BM, Gallagher P, Hearn AJ, Young AH, Ferrier IN. Family history, early adversity and the hypothalamic-pituitary-adrenal (HPA) axis: Mediation of the vulnerability to mood disorders. *Neuropsychiatr Dis Treat*. 2007;3(5):647-653.

100. Holsboer F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology*. 2000;23(5):477-501. doi: S0893-133X(00)00159-7 [pii].

101. Stokes PE. The potential role of excessive cortisol induced by HPA hyperfunction in the pathogenesis of depression. *Eur Neuropsychopharmacol*. 1995;5 Suppl:77-82. doi: 0924977X9500039R [pii].

102. Oquendo MA, Echavarria G, Galfalvy HC, et al. Lower cortisol levels in depressed patients with comorbid post-traumatic stress disorder. *Neuropsychopharmacology*. 2003;28(3):591-598. doi: 10.1038/sj.npp.1300050 [doi].

103. Raadsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, Swaab DF. Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. *Neuroendocrinology*. 1994;60(4):436-444.

104. Covington HE,3rd, Maze I, LaPlant QC, et al. Antidepressant actions of histone deacetylase inhibitors. *J Neurosci*. 2009;29(37):11451-11460. doi: 10.1523/JNEUROSCI.1758-09.2009 [doi].

105. Yamawaki Y, Fuchikami M, Morinobu S, Segawa M, Matsumoto T, Yamawaki S. Antidepressant-like effect of sodium butyrate (HDAC inhibitor) and its molecular mechanism of action in the rat hippocampus. *World J Biol Psychiatry*. 2012;13(6):458-467. doi: 10.3109/15622975.2011.585663 [doi].

106. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem*. 2003;278(6):4035-4040. doi: 10.1074/jbc.M210256200 [doi].

107. Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*. 1995;377(6548):454-457. doi: 10.1038/377454a0 [doi].

108. Horlein AJ, Naar AM, Heinzel T, et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*. 1995;377(6548):397-404. doi: 10.1038/377397a0 [doi].

109. Jones PL, Shi YB. N-CoR-HDAC corepressor complexes: Roles in transcriptional regulation by nuclear hormone receptors. *Curr Top Microbiol Immunol*. 2003;274:237-268.

110. Mukaida N, Morita M, Ishikawa Y, et al. Novel mechanism of glucocorticoid-mediated gene repression. nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *J Biol Chem*. 1994;269(18):13289-13295.

111. Nissen RM, Yamamoto KR. The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev*. 2000;14(18):2314-2329.

112. Rogatsky I, Wang JC, Derynck MK, et al. Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proc Natl Acad Sci U S A*. 2003;100(24):13845-13850. doi: 10.1073/pnas.2336092100 [doi].