Chapter 1

Mechanisms of Glucocorticoid Receptor (GR) Mediated Corticotropin Releasing Hormone Gene Expression

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1. Introduction

Normal physiologic functioning is dependent on the maintenance of homeostasis in the face of numerous stressors. Responses to stress include the fight or flight reaction and activation of the sympathetic nervous and endocrine systems. The central component of the endocrine response is the hypothalamic-pituitary-adrenal (HPA) axis, which when activated leads to increased levels of circulating glucocorticoids. Indeed, an increase in glucocorticoids has been used as an operational definition of stress.

The HPA axis is activated by a wide range of stimuli which includes perception of danger, pain, sepsis, and others. These stimuli are integrated at points throughout the central nervous system and ultimately impinge on the HPA axis motor neurons in the paraventricular nucleus of the hypothalamus (PVH). HPA neurons synthesize and secrete corticotropin releasing factors (crfs), the best known of which is the 41 amino acid peptide corticotropin releasing hormone (CRH);(Vale et al 1981)). CRH travels through the hypothalamic portal circulation to the anterior pituitary where it binds CRH receptors. This in turn leads to adrenocorticotrophic hormone (ACTH) secretion into the systemic circulation which stimulates the adrenal cortex to secrete glucocorticoids.

Glucocorticoids elicit gluconeogenesis, which increases circulating levels of glucose. Although this mechanism is adaptive in the face of a homeostatic challenge, glucocorticoids can also have deleterious effects. Dysregulation of the HPA axis underlies classic endocrine disorders, such as Cushing's disease, and is highly correlated with a number of psychiatric disorders, including post-traumatic stress disorder, anorexia nervosa, and depression. In addition, high circulating levels of glucocorticoids lead to osteopenia and immunosuppression. Thus, regulation of the HPA axis must be exquisitely controlled.

There are numerous components to HPA axis down-regulation; one of the most significant of these is the end product of HPA axis regulation itself – glucocorticoids. Glucocorticoids down-regulate axis activity by acting at numerous loci in the HPA axis and in extra-hypothalamic regions of the brain, such as the hippocampus(de Kloet et al 2005, (Sapolsky et al 1984). Glucocorticoid regulation in the hippocampus, extra-hypothalamic sites and pituitary are reviewed below.

1.1. Glucocorticoidregulation of the hippocampal-hypothalamic pathway

Glucocorticoid receptors (GRs) are most densely concentrated in the hippocampus of the central nervous system (CNS), and in fact it is in the hippocampus where glucocorticoid binding sites were first detected(McEwen et al 1979). GRs were first classified by their binding characteristics. Two types were identified, distinguished in part by their binding characteristics to corticosterone and the synthetic glucocorticoid dexamethasone (Dex) (de Kloet et al 1975). After steroid receptor cloning it became apparent that the two receptors correlate to the mineralocorticoid receptor (MR) and the GR. The GR is recruited in the presence of high levels of circulating glucocorticoids elicited in the face of stress (de Kloet et al 2005).

Down-regulatory signals from the hippocampus are processed through a multisynaptic pathway. Hippocampal projections to the subiculum elicit excititory signals in the form of glutamatergic synapses in the basal nucleus of the stria terminalis (BNST). These stimulate inhibitory output from the BNST, which in turn down regulates the HPA axis. Thus, damage to the hippocampus leads to loss of HPA axis inhibition (Choi et al 2007, Herman et al 2003).

1.2. Glucocorticoid regulation at the level of the pituitary

One of the best studied components of glucocorticoid down-regulation of the HPA axis is ligand-bound GR-mediated down-regulation of the gene that codes for the ACTH precursor, pre-pro-opiomenlanocortin (POMC)(Bicknell 2008). As is the case in other cells, glucocorticoids gain entry to the cyptoplasm and bind the cytoplasmic GR. Ligand binding activates the receptor, a process that includes dissociation from the heat shock protein 90 (hsp90) as reviewed by Pratt and Dittmar(Pratt & Dittmar 1998). The ligand-bound receptor is transported into the nucleus where it interacts with numerous nuclear proteins and chromatin to regulate transcription. Prototypically, the GR binds to glucocorticoid response elements that are inverted palindromes; however, the regulatory region of *pomc* does not have such elements. Rather, it has a negative glucocorticoid response elements (nGREs) – hybrid elements also called composite elements. GRs bind these sites as monomers and interact with monomers of other transcription factors to down-regulate *pomc* transcription. GRs also repress transcription in the absence of direct DNA binding by modulating the activity of other transcription factors. In these aspects *pomc* regulation is similar to regulation of *crh*.

A significant difference between the synthesis of CRH and ACTH is the relative contribution of post-translational enzymatic processing. In the case of CRH synthesis, one peptide is produced, thus, the majority of regulatory steps are pre-translational. Conversely, numerous

peptides are generated from *pomc.* These include ACTH, beta-endorphin, and alpha-melanocyte stimulating hormone. Thus, pre-pro-POMC enzymatic processing plays a major role in determining levels of functional ACTH.

1.3. Hypothalamic crfs

CRH-expressing parvocellular neurons are the final common integrators of humeral and synaptic input. Located in the mpPVH, they receive inputs from numerous sites in the CNS: the hippocampus, brainstem, amygdala, intrahypothalamic sites, and PVH interneurons. (Swanson & Sawchenko 1980).

Although CRH is the most potent and best known crf, it is only one of several. Prior to the biochemical characterization of CRH(Vale et al 1981), arginine vasopressin (AVP) and oxytocin were also known to have crf properties(Gibbs 1986). Perhaps the most studied of these is AVP, best known for its activity as an anti-diuretic hormone. AVP arises from PVH magnocellular neurons whose terminals secrete AVP directly into the systemic circulation.The AVP that acts as a crf is synthesized in parvocellular neurons of the PVH. Interestingly, all parvocellular neurons that express AVP also express CRH. Furthermore, all of these express GRs(Cintra et al 1987, Uht et al 1988), and their function is measured by the ability to translocate into the nucleus in the presence of Dex(Uht et al 1988).

2. Glucocorticoid receptors

Many biochemical and pharmacologic properties of GRs were characterized prior to cloning (Gustafsson et al 1987). The existence of the MR and the GR was determined pharmacologically. In the absence of ligand the GR was present in a cytoplasmic complex. In the presence of ligand, the GR was present in the nucleus. Furthermore, ligand bound GR had been shown to bind specific sites in DNA, which came to be known as glucocorticoid response elements (GREs). Thus, before cloning, the fundamental differences between a steroid receptor and receptors for other hormones had been determined. The GR was an intracellular receptor rather than a plasma membrane bound receptor and its mechanism of action involved binding DNA. Hence the term that evolved -- ligand activated nuclear receptors.

2.1. Glucocorticoid receptor cloning and identification of functional domain

The initial cloning of the GR revealed two GRs: alpha and beta. Oakley *et al* discovered that GR beta did not bind ligand(Oakley et al. 1996) -- biological functions of GR-beta are still in the early stages of discovery(Yudt & Cidlowski 2002). This review focuses on GR-alpha, which will be referred to as GR.

2.2. GR as a founding member of the nuclear receptor (NR) superfamily

The GR and the estrogen receptor (ER) are founding members of the NR superfamily, and were cloned within the same time period(Green et al 1986, Greene et al 1986). They are

highly homologous and are composed of domains that retain much of their function when dissociated from each other. Both GR and ER have three major domains, the NTD, DBD and LBD, as do all steroid receptors (Fig 1). The domains are dissociable and for certain functions are also interchangeable. For example, an ER chimera, which contains a GR binding domain, activates transcription in the presence of estradiol but does so by binding a GRE(Green & Chambon 1987).

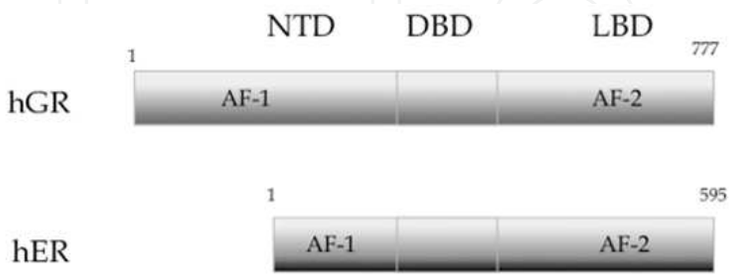


Figure 1. Domains of the GR and ER. (hGR) human glucocorticoid receptor, (hER) human estrogen receptor, (AF-1 and AF-2) activation functions 1 and 2, respectively

3. GR-regulated gene repression: response elements

Prior to the late 1980s, a prevailing view of GR-mediated repression was that it would require a palindromic DNA binding site. This assumption could not explain the fact that glucocorticoids repress a number of genes in the absence of a prototypic GRE. The discovery of composite elements and the discovery that elements for other transcription factors could sustain GR-mediated inhibition of gene activation were major advances in the understanding of GR-mediated repression.

3.1. Repression mediated by composite elements

Composite elements are hybrids (Lefstin & Yamamoto 1998). In the case of GR regulation they consist of half sites, one for a GR monomer and one for a monomer of a distinct transcription factor -- *e.g.* a monomer of an activator protein-1 (AP-1) family member. They are found in numerous genes, including those directly involved in regulating the HPA-axis -- *crh* and *pomc*.

Much of the initial molecular analysis of composite elements was performed using the proliferin gene (*proliferin*).The *proliferin* composite element consists of half sites for GRE and AP-1 binding and confers both activation and repression, dictated by the specific AP-1 family member bound (Diamond et al 1990, Pearce et al 1998). AP-1 family members include c-Jun, cFos and similar proteins. A GR monomer bound in the presence of a c-Jun monomer will stimulate activation from the *proliferin* element, whereas high levels of c-Fos inhibit activation(Diamond et al 1990). Like the *proliferin* element, the *crh* nGRE is composed of GRE and AP-1 half sites. In addition, the extent to which the nGRE directs repression is dependent on the AP-1 family member bound to the composite element (Malkoski & Dorin 1999).

3.2. Repression mediated through other transcription factors and components of the basal transcriptional activity

Three papers published simultaneously in 1990 reported that glucocorticoid-bound GR could down-regulate AP-1 stimulated gene expression (Jonat et al 1990, Schule et al 1990, Yang-Yen et al 1990). Mechanisms by which the GR down-regulates AP-1 activity and activity of other transcription factors are still being elucidated -- some interact with coregulators and others interact directly with components of the general transcription machinery. An example of the latter is GR down-regulation of nuclear factor-kappa B (NF-kB) activity. In the context of the interleukin-8 gene(*il-8*), GR is a physical and functional intermediary between the RelA (p65) component of NF-kB and the C'-terminal domain of polymerase II (pol II). GR alters the phosphorylation state of the C'-terminal domain of pol II and thus regulates its activity(Nissen & Yamamoto 2000). The GR also down-regulates *il-8* expression by interfering with the activity of the transcription elongation factor-b (Luecke & Yamamoto 2005). Thus, GR targets both initiation and elongation steps in the context of the *il-8* promoter. It is unknown whether or not GR works through either of these mechanisms in the context of *crh.*

4. Identification of NR coregulators

By the 1990s it was clear that NRs required additional factors to regulate gene expression. The discovery of NR co-regulators — coactivators and corepressors — permitted a quantum leap in the elucidation of NR mechanisms of gene regulation.

4.1. NR coactivators

Although there are numerous coactivators, this review focuses on the three members of the p160 family commonly referred to as SRCs-1, -2, and -3. In addition, a nomenclature group has codified the names of these coactivators as NCoA 1-3. Here they will be referred to collectively as the p160 family and individually the names first reported will be used with the agreed upon nomenclature indicated, *e.g.* SRC-1 (NCoA 1).

The first p160 was discovered by O'Malley as a coactivator for a progesterone receptor -- the steroid receptor co-activator-1, SRC-1 (NCoA 1)(Onate et al 1995). Subsequently, Stallcup reported a p160 coactivator for the mouse GR, Glucocorticoid Receptor Interacting Protein 1 GRIP1(Hong et al 1997, Hong et al 1996), also designated NCoA 2. The third p160, reported by several investigators, bears many names, including AIB1(Anzick et al 1997) and RAC3(Li et al 1997) but it is often referred to as SRC-3 (NCoA 3).

Each p160 contains two highly conserved regions. In the center of the protein is a cluster of three Leucine-X-X-Leucine-Leucine (LXXLL) motifs, in which X denotes any amino acid(Ding et al 1998, Heery et al 1997). These are also referred to as NR-boxes. The motifs are a requisite site of interaction with nuclear receptors; mutations of these sites abrogate NR activation functions (Feng et al 1998). p160s also contain a domain that binds to histone acetylases, *e.g.* the cAMP regulatory element binding protein (CREB)-binding protein (CBP), which remodel chromatin by acetylating specific lysines in histones(Marmorstein 2001).

Mechanisms by which p160s regulate *crh* expression are not well understood. The best studied of these is SRC-1. The SRC-1a isoform mRNA has been mapped to the PVH, and CRH mRNA levels have been evaluated in SRC-1 knockout mice (Lachize et al 2009). Paradoxically, SRC-1 is associated with *crh* repression(van der Laan et al 2008). There is precedent for this -- Rogatsky and colleagues reported that GRIP1 has a repressive function(Rogatsky et al 2001). The GRIP1 domain that supports this function, however, is unique to the GRIP1 p160. Thus, the mechanisms of SRC-1a down-regulation have yet to be identified.

4.1.1. Coactivator interaction with histone acetyl transferases (HATs)

The discovery of the p160s and the discovery of a coactivator for the cAMP regulatory element binding protein (CREB) - binding protein (CBP) and its homologue p300 occurred contemporaneously. In addition to a p160 binding domain the two coactivators contain a histone acetylase domain. CBP is a coactivator for numerous transcription factors that include a number of nuclear receptors and factors involved in inflammation, *e.g.* STAT1 (Horvai et al 1997). In addition to CBP and p300, other acetylases such as the p300/CBP-associated factor (p/CAF;(Yang et al 1996)play a role in nuclear receptor regulation; however, their role in GR regulated *crh* expression is poorly understood.

4.2. Nuclear receptor co-repressors

Some members of the NR family, such as the thyroid hormone receptor (TR), maintain a constitutively silent state of gene expression. The search for a co-repressor for TR led to the discovery of the Nuclear Receptor Corepressor (NCoR), whose homologue is known as the silencing mediator of retinoic acid receptor and the thyroid receptor (SMRT). The NR interaction site in NCoR is remarkably similar to the NR-boxes in the p160 coactivator family. The corepressor motif is L/I XXI/V-I, compared to the p160 coactivator motif, LXXLL. The corepressor motif is referred to as a CoRNR box. These features of coactivator and corepressor regulated gene expression are summarized in Table 1.

Coregulator	**Interaction Site**	**Associated Enzyme**	**Enzymatic Action**	**Effect on Chromatin**	**Effect on Transcription**
Coactivator	NR box LXXLL	HAT	Histone Acetylation	Decondensation	Activation
Corepressor	CoRNR box L/I XXI/V-I	HDAC	Histone Deacetylation	Condensation	Deactivation or Repression

Table 1. The chain of events for activation parallels that for repression.

The mechanisms by which co-repressors interact with GR to down-regulate *crh* expression are largely uncharacterized. Using transient transfection/reporter assays, van der Laan *et al.* reported that cotransfection of NCoR and SMRT did not accentuate glucocorticoid mediated *crh* repression. Instead, these repressors accentuated corticosterone inhibition of forskolin-stimulated expression(van der Laan et al 2008).

The corepressors NCoR and SMRT bind to histone deacetylases (HDACs). The specificity of an HDAC for a given receptor has been elucidated in some studies. An early report revealed that HDAC3 but not HDAC1 is involved in TR repression (Guenther et al 2001). Given the conserved nature of many functions across nuclear receptors one might predict that like the TR, GR- repressed transcription of *crh* expression would involve HDAC3 but not HDAC1. In the context of the *crh* promoter, however, the reverse is true(Miller et al 2011).

5. Structural analysis of GR

Structural analysis of GR permits identification not only of a single protein structure but also of protein interfaces involved in specific inter-molecular interactions.

5.1. The DNA binding domain

Crystallographic analysis of a GR dimer bound to its DNA recognition site revealed that GR zinc fingers intercalate with DNA (Freedman et al 1988, Luisi et al 1991). Subsequent NMR analysis of the DBD structure revealed inherent stability in the absence of DNA (Berglund et al 1992). The DBD is now known to have several functions in addition to binding DNA, and it may be that the inherent structure supports these functions.

5.2. The ligand binding domain

A characteristic of all NRs is that the LBD is longer and less structured than the DBD. This partially explains why the crystal structure of two smaller nuclear receptors, RXR-alpha and TR were the first to be solved (Bourguet et al 1995, Wagner et al 1995). The TR was the first ligand-bound NR to be crystallized; even so, it took years to optimize LBD purification in sufficient quantities to permit crystallization (Apriletti et al 1995, Apriletti et al 1988) (McGrath et al 1994). This process was facilitated by use of a radioactively labeled ligand (Apriletti et al 1995, Apriletti et al 1988), which allowed LBD to be tracked throughout purification. The discovery of NR boxes was taking place simultaneously with efforts to crystalize the TR LBD. Thus, crystallization of the ligand-bound TR bound to a GRIP1NR box followed shortly thereafter (Darimont et al 1998, Wagner et al 1995).

The next LBD structure to be solved was ER-alpha, again bound to ligand and an NR box. As a member of the steroid receptor branch of the NR superfamily it has a longer, more complex LBD than the TR. Thus, the protein is inherently more difficult to crystalize, and its crystal structure more difficult to solve. Coordinates used to solve the TR structure permitted ER modeling (Shiau et al 1998). Indeed, in the absence of TR crystal structure coordinates, solution of the ER crystal structure may have been intractable at the time.

The GR LBD is even less structured than either TR or ER-alpha. In fact, a mutation in the GR LBD was required to generate crystals. Co-crystallization of GR LBD bound to Dex and to an NR box revealed that the overall structure of the receptor LBD is the same as the TR and ER-alpha with three key differences: an additional dimerization function, a second set of charge

clamps, and an additional pocket (Bledsoe et al 2002, Bledsoe et al 2004). These distinctive features underscore the complexity of GR (Bledsoe et al 2004).

6. Epigenetics and chromatin modification

Strictly defined, the term epigenetics refers to an inheritable factor composed of something other than unmodified genomic DNA— this is distinct from chromatin modifications that regulate processes that are not inherited. Thus, most of the processes referred to here are not truly epigenetic, but rather consist of chromatin modifications that modulate gene expression.

6.1. Histone acetylation

Although typically associated with transcriptional activation, histone acetylation is also associated with repressed states of gene expression (Shahbazian & Grunstein 2007). The initial focus of study in this field was on chromatin acetylation via recruitment of histone acetyl transferases (HATs). The addition of an acetyl to a lysine (Lys) neutralizes the acid-base interaction with DNA. This neutralization, as well as the steric hindrance conferred by Lys acetylation, destabilizes histone:DNA interactions, and allows proteins such as transcription factors, transcription initiators, and elongation factors access to DNA binding sites.

6.2. Histone Deacetylation

Deacetylation is the counterpart to acetylation. Histone Deacetylases (HDACs) are comprised of a family of enzymes with three subdivisions. The nomenclature of mammalian HDACs is somewhat confounding, having arisen from sequential numbering as the enzymes were discovered. Class I includes HDAC 1-3, 8 and 11. They have one catalytic domain and for the most part are nuclear. Class II HDACs are larger than Class I and are divided into two subclasses, IIa and IIb. Class IIa HDACs include HDAC 4, 5, 7and have an N'-terminal domain unique to this class. In addition, Class IIa HDACs shuttle between the nucleus and cytoplasm. Class IIb HDACs, 6 and 10, have two HDAC domains instead of a unique N'-terminus, and are predominantly found in the cytoplasm (Verdin et al 2003). Class III HDACs are distinguished by their requirement for NAD^+. They are named sirtuins due to similarity to the yeast Sir2. Like Sir2, they are targets of intense study given their association with aging and neurodegenerative processes. Because HDACs are tightly correlated with repression they have been examined in the context of GR-repressed *crh* expression (Miller et al 2011).

7. Corticotropin Releasing Hormone (CRH)

Although CRH is widely expressed in the mammalian CNS, the focus here is on regulation of *crh* in the medial parvocellular region of the PVH (mpPVH).

7.1. CRH cloning

In 1983, a fragment of the human *crh* was cloned that contained the proximal promoter and coding region. The predicted amino acid sequence differs from the ovine by seven residues (Shibahara et al 1983). Cloning the rat cDNA and a portion of the promoter were reported in 1987(Thompson et al 1987). The rat cDNA has high sequence homology to human cDNA, and in fact the peptide sequences are identical. Rat and human proximal promoter sequences are also highly conserved (Thompson et al 1987).

7.2. *crh* regulation

A cAMP regulatory element (CRE) at approximately -200 in the proximal promoter plays a pivotal role in activating *crh* expression by recruitment of (CREB)(Seasholtz et al 1988, Thompson et al 1987). Interestingty this site not only mediates activation but also mediates repression by recruiting the inducible cAMP early repressor(Aguilera & Liu 2012). In addition, a negative GRE in the 200 base span contributes to *crh* down-regulation (Malkoski & Dorin 1999,(Malkoski et al 1997). Indeed the entire first 200 bases of the proximal promoter are highly conserved, underscoring the importance of this region to regulation of the stress response(Yao et al 2007).

Specific mechanisms of GR-mediated *crh* down-regulation have been difficult to parse, as is case for most glucocorticoid down-regulated genes. At the most basic level it is unclear whether glucocorticoids suppress *crh*-activated expression only, or if they also suppress basal levels of expression. This distinction is important in that recruitment of signal-specific co-activators would be required prior to GR inhibition. Repressionof basal activity, however, would entail recruiting a corepressor.

Most studies of inhibited *crh* regulation have used transient transfection assays or isolated DNA. In neither case is DNA in its natural state of chromatinization. More recent studies of *crh* expression underscore the importance of considering the chromatin environment.

8. Impact of chromatin modifications on analysis of GR-mediated *crh* down-regulation

A number of factors regulate the *crh* chromatin environment. Inhibition of activated *crh* expression involves both the CRE and the nGRE, and maintenance of basal activity involves histone acetylation and DNA methylation. Numerous steps in mechanisms of inhibition and repression have yet to be elucidated.

8.1.1. Repression of cAMP activated crh expression

CRE is required for regulation of *crh* expression through signal transduction. Phosphorylated CREB (pCREB) can interact with an inhibitory member of the CREB family, the inducible cAMP early repressor (ICER). ICER is a dominant negative of pCREB and

decreases cAMP activated expression. Further details on the role of pCREB and its family members in regulating *crh* expression can be found in the Aguilera and Liu review(Aguilera & Liu 2012).

8.1.2. Regulation through the nGRE

Repression mediated through the nGRE in the proximal *crh* promoter is similar to repression mediated through the *proliferin* nGRE. A monomer of GR and a monomer of the AP-1 family bind the composite element. The prototypic coactivator for pCREB is CBP, which is also a coactivator for AP-1(Bannister & Kouzarides 1995, Bannister et al 1995). Thus, in the context of *crh,* CBP might permit a functional interaction between the GR:AP-1 dimer and a dimer of CREB family members bound to the CRE.

8.2. Maintenance of basal levels of activation

Basal levels of activation involve a balance of activation and repression. There are numerous ways in which this balance may be maintained -- one is to maintain a constant state of chromatin modification. Two modification types that play a role in *crh* regulation are histone acetylation and methylation of CpG islands.

8.2.1. Histone acetylation

Activated *crh* expression involves CBP recruitment to the proximal promoter. CBP is a HAT coactivator for both pCREB and c-Jun, so activation of either the pKA pathway or the pKC pathway could be involved in CBP recruitment.

Analysis of global histone 3 and 4 (H3 and H4) acetylation in the context of estradiol-regulated *crh* expression has been reported(Lalmansingh & Uht 2008). As is the case in estradiol regulation, Dex regulates H3 and H4 acetylation differentially, as measured by chromatin immunoprecipitation followed by PCR amplification(Miller et al 2011). Dex increases H4 acetylation, a finding that underscores the fact that acetylation may be associated a state of repression as well as activation.

The level of histone acetylation is a function of the presence of enzymatically active HATs and HDACs. In the case of Dex-regulated *crh* expression, the amount of ligand bound GR is increased in the region of the promoter(Miller et al 2011). When measured at the same time, HDAC levels are also increased. Furthermore, GR binds HDAC1 in a Dex-dependent manner, suggesting the possibility that GR recruits HDAC1 to the *crh* promoter (Figure 2).

Like the differential acetylation of H3 and H4, the Dex associated increase in HDACs displays a degree of specificity — although HDAC1 is increased at the promoter, HDAC3 is not (Miller et al 2011). The mechanisms by which these enzymes leave and are recruited to chromatin are poorly understood and merit further study.

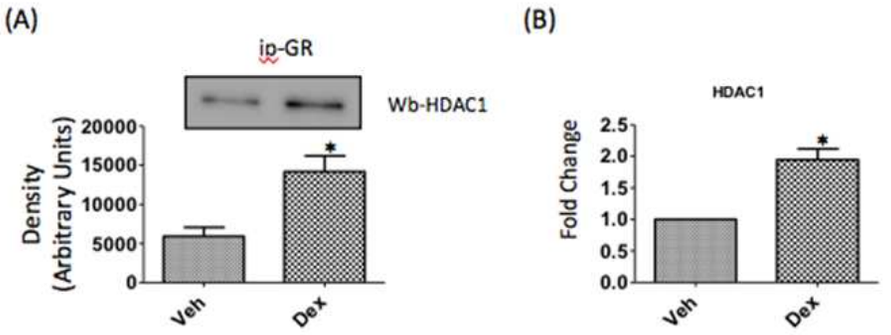


Figure 2. Dex treatment leads to increased HDAC1:GR complexes and increased HDAC1 at the *crh* promoter. (A) Co-immunoprecipitation analysis; nuclear extract was immunoprecipitated with a polyclonal antibody against GR. Western blot analysis of the immune-precipitate revealed an increase in the co-immunoprecipitation of HDAC1. n=3; Bars represent the mean ± SEM and are represented as the fold difference of the Veh *, $P < 0.05$. (B) ChIP analysis of the CRH promoter; cells were treated with Dex and chromatin was immune-precipitated with an anti HDAC1 antibody. Quantitative RT-PCR analysis of the immune-precipitated DNA indicates enrichment of HDAC1 at the promoter. n=3; Bars represent the mean ± SEM and are represented as the fold difference of the Veh *, $P < 0.05$.

8.2.2. DNA methylation

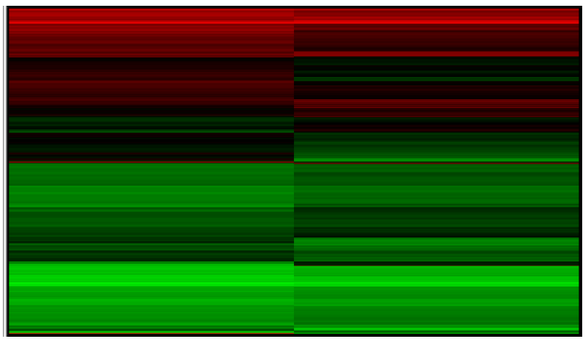
Methylation of CpG islands of the GR promoter was one of the earliest reported true epigenetic phenomena in that it was associated with inheritance -- in this case a behavioral phenotype(Weaver et al 2004). More recently, CpG island methylated *crh* has been described in the context of social defeat(Elliott et al 2010).

The first report of CpG island methylation in the context of *crh* regulation was an offshoot from a study of a mouse model of Rett Syndrome. This syndrome occurs in girls and manifests as diminished intelligence, repetitive motor movements, and anxiety — all of which have variable penetrance. The genetic lesion in Rett Syndrome is a mutation in the methyl CpG (meCpG) binding protein 2 (MeCP2). This protein binds to meCpG islands and represses the expression of bound genes. McGill and colleagues found that one of these genes is *crh.* Remarkably, mice bearing the MeCP2 mutation have a hyperactive HPA axis associated with elevated levels of CRH mRNA in the PVH, central amygdala, and BNST—all regions that express GRs and which are associated with HPA axis regulation. In addition, meCpG sites have been mapped in the *crh* proximal promoter region and were found to be present in the same region as the CRE and nGRE(McGill et al 2006). These findings underscore the importance of this region in *crh* regulation.

9. A role for bioinformatics in GR-regulated *crh* expression

Dalwadi and Uht recently investigated expression patterns of two neuronal cell lines, which were derived from embryonic PVH and amygdala. Paradoxically, even though neurons in these populations express CRH and contain GRs, they differ in the response to glucocorticoid treatment. In the mpPVH, glucocorticoids down-regulate *crh* expression whereas in the amygdala they up-regulate it. Expression microarrays are currently being

analyzed using the expression pattern of two neuronal cell lines, amygdalar AR-5 and hypothalamic IVB - both differentially express *crh* in response to Dex treatment. The number of genes associated with development of projections is similar between the two cell lines, whereas the number of genes involved in steroid hormone responsiveness is two-fold greater in the hypothalamic line compared to the amygdalar line. Given the importance of the hypothalamus relative to amygdala in regulation of steroid hormone physiology, these results are not unexpected. However, the two lines also differ in the relative expression of genes associated with response to oxidative stress and to DNA binding, as categorized in the Gene Ontology database (GO; Figure 3). These differences are intriguing and have spurred further investigation.



GO Term	AR-5	IVB
DNA Binding	36	21
Neuron Projection Development	9	8
Response to Oxidative Stress	10	6
Response to Steroid Hormone Stimulus	6	12

Figure 3. Hierarchal cluster showing relative abundance of genes between the AR-5 and IVB cell lines (red - abundant, green - less abundant). (GO) Gene Ontology as defined by the DAVID analysis program. (AR-5) Amygdalar cell line, (IVB) Hypothalamic cell line.

More refined techniques are now available for bioinformatics analysis of gene expression. One of those is the combination of chromatin immunoprecipitation and microarray assays (ChIP-chip). In this approach, DNA isolated from ChIPs is used to probe a genomic microarray. ChIP-chip has been used to analyze GR binding sites. So and colleagues used a combination of conventional expression array analysis followed by ChIP-chip. When glucocorticoid-induced genes were compared to glucocorticoid-repressed genes, analysis revealed that the GR-holoreceptor induced all genes that were regulated via a conventional palindrome. In distinction, none of the genes repressed contained such an element (So et al 2007). Such a clear-cut distinction is rare in biology.

A second example of a bioinformatics approach useful in the analysis of NR mediated gene regulation is global run-on and sequencing (GRO-seq)(Core et al 2008). This technique permits unbiased analysis of all RNA transcripts, which allows detection of both mRNA and

non-coding RNAs. It has been used in a number of biological systems, including use of this technique to determine the nature of transcripts induced by 17estradiol treatment of MCF-7 cells, a prototypic breast cancer cell line. To date, however, there are no reports of GRO-seq analysis of glucocorticoid regulated genes.

10. Summary

In the last fifteen years an explosion of new information has facilitated novel ways of looking at GR mediated gene expression. The seminal findings by Yamamoto in the early 1980s —that GRs bind to specific palindromic glucocorticoid response elements(Payvar et al 1983)— is now frequently referred to as the classic mechanism of gene regulation. At present, numerous alternate mechanisms of gene regulation are being elucidated. Many of these involve interactions with coregulatory factors. Such interactions have helped bridge the gap between transcription and chromatin remodeling, which in turn has resulted in the intersection of the NR field with the field of epigenetics.

This review has focused on glucocorticoid regulation of genomic effects via the GR. Other areas currently being investigated include the actions of GR splice variants, and the role of glucocorticoid regulation of heat shock proteins. Lastly, the effects of glucocorticoids at the cell membrane (non-genomic events), mechanisms of cell membrane transport of glucocorticoids, and nuclear import of the GR holoreceptors are all steps in regulation that merit further analysis.

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Dedicated to the Memory of Wylie Vale 1941 - 2012.

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