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Topic 1.5

Genetic dissection of gluco- and mineralocorticoid receptor function in mice*

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Abstract: Nuclear hormone receptors function to transduce hormonal signals into transcriptional responses by controlling the activity of specific target genes. These target genes comprise a genetic network whose coordinate activity defines the physiological responses to hormonal signals. Dissecting nuclear hormone receptor functions in vivo by gene inactivation and transgenic strategies represents an invaluable and powerful approach to increase our knowledge of these genetic networks and their physiological functions. Glucocorticoids and mineralocorticoids are involved in numerous physiological processes important to maintain metabolic, cardiovascular, central nervous, and immune system homeostasis. Germline and somatic gene targeting as well as an increased dosage of the glucocorticoid receptor (GR) allows the characterization of the various functions and molecular modes of action of this receptor. Most of the effects of the GR are mediated via activation and repression of gene expression. To separate activating from repressing functions of the GR, a point mutation was introduced which allowed us to characterize and distinguish functions dependent on GR binding to DNA from those mediated by protein/protein interaction. Cell/tissue-specific mutations of the gluco- and mineralocorticoid receptor is the basis for the evaluation of their cell-specific functions, including the characterization of target genes of the receptors in order to describe their specific effects on different targets.

INTRODUCTION

Glucocorticoids and mineralocorticoids regulate diverse functions important to maintain central nervous system, cardiovascular, metabolic, and immune homeostasis [1]. The corticosteroid hormones produced in the adrenal cortex are ideal candidates for integrating such a complex array of physiological functions. According to the lipophilic nature of their steroid structure, they diffuse from their source, permeate to their targets, and exert their functions by binding two closely related corticosteroid receptors: the mineralocorticoid receptor (MR, type-I receptor) and the glucocorticoid receptor (GR, type-II receptor) [2]. Under physiological conditions, glucocorticoids are able to activate both receptors, whereas mineralocorticoids are specific for the MR. Mineralocorticoid-responsive cells (e.g., the principal cells of the collecting duct in kidney) express the enzyme 11-β-hydroxysteroiddehydrogenase, type 2. Conversion of cortisol to cortisone by 11-β-hydroxysteroiddehydrogenase type 2 prevents glucocorticoids from binding to the MR, and thus this pre-receptor specificity mechanism ensures mineralocorticoid-specific effects.

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Mineralocorticoids control genes involved in transepithelial sodium transport and thereby regulate sodium and potassium homeostasis and water balance [3]. Aldosterone, the main circulating mineralocorticoid, has a major role in blood-pressure volume regulation in normal subjects, and is thought to be involved in the pathogenesis of hypertension and target-organ damage.

Glucocorticoids regulate genes involved in the production and mobilization of glucose. Glucocorticoids increase the breakdown of fat and proteins and induce the synthesis of gluconeogenic enzymes to generate blood glucose [3]. In addition, glucocorticoids promote maturation of the lung and are highly beneficial in the treatment of the acute respiratory distress syndrome in newborn infants [4]. Glucocorticoids and their receptor also play a role in maturation of erythroid progenitors [5]. Furthermore, glucocorticoids can induce apoptosis of thymocytes; they are important anti-inflammatory and immunosuppressive agents [1]. Their immunomodulatory activity has been exploited for the treatment of diverse inflammatory and allergic disorders including asthma [1].

Levels of mineralocorticoids and glucocorticoids are controlled by tight regulatory mechanisms [3]. The renin-angiotensin-aldosterone system (RAAS) controls the mineralocorticoid release, while the hypothalamic-pituitary-adrenal (HPA) axis regulates glucocorticoid secretion. The hypothalamus secretes corticotropin-releasing hormone (CRH), which acts on the anterior pituitary gland to secrete the adrenocorticotropin hormone (ACTH). ACTH stimulates the release of glucocorticoids from the cortex of the adrenal gland. Increased blood glucocorticoid levels in turn repress the transcription and inhibit the release of both, CRH and ACTH. Glucocorticoids are released in response to various stressors (physical, emotional, chemical, physiological) [6]. Stress is considered a protective mechanism that prepares the organism to react to threatening stimuli in an appropriate way. These stimuli activate the HPA axis, which in conjunction with other physiological adaptations coordinate the behavioral responses of the organism. In addition, the levels of glucocorticoids show a diurnal rhythm reaching peak levels at the onset of the active phase of the organism. Chronic changes in control of the HPA axis may have pathological consequences, since it is now well established that depression- and anxiety-related disorders are associated with dysregulation of the HPA axis.

Mouse molecular genetic approaches led the foundation for the enormous progress toward understanding the various functions of steroid hormone receptors. Conventional knock-out technology was supplemented by more sophisticated gene targeting strategies. Exploiting the Cre/loxP recombination system from bacteriophage λ in mice allowed the generation of conditional mutations, cell- and tissue-specific mutations, and function-selective mutations [7,8]. Genetic manipulations of the corticosteroid receptors in mice provided important information on both, the molecular mechanism of receptor function and their role in development and physiology. Often, these analyses have resulted in unexpected findings, that is, the liver-specific function of the glucocorticoid receptor (GR) in growth control (see below). Figure 1 summarizes the various types of mutations ranging from null and hypomorphic mutations to cell/tissue-specific and function-selective mutations, which have been generated with the help of the Cre/loxP recombination system.

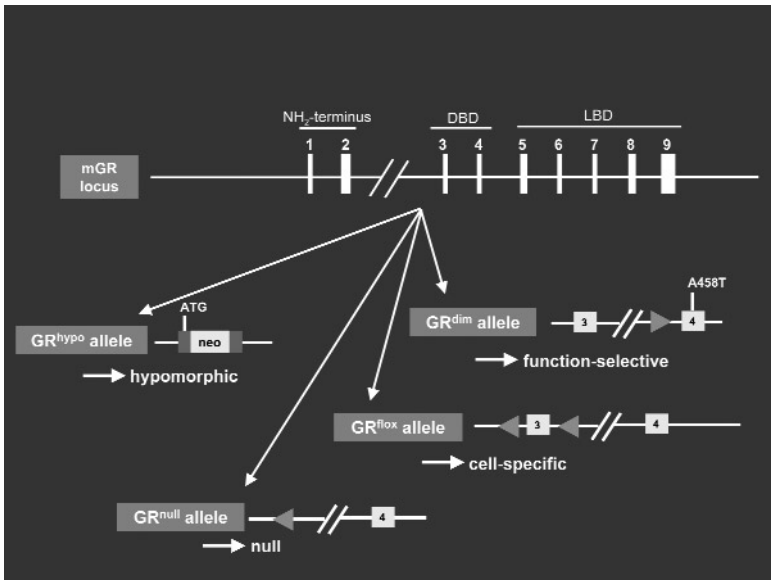

Fig. 1 Analysis of GR gene function by null, cell-specific, and function-selective mutations. The organization of the GR gene is shown at the top. The four types of mutations of the GR gene are shown in relation to the endogenous GR gene.

LOSS-OF-FUNCTION MOUSE MUTANTS FOR THE GLUCOCORTICOID AND THE MINERALOCORTICOID RECEPTOR

Two GR-inactivating mutations have been generated: a hypomorphic allele that resulted from insertion of a PGK-neomycin-resistance cassette right after the ATG of the receptor coding sequence [9] and a null allele that has been generated by Cre-loxP mediated excision of exon 3 (F. Tronche and G. Schütz, unpublished). In the hypomorphic allele, alternative splicing results in a truncated mRNA, which leads to the synthesis of a shortened protein lacking most of the NH_2-terminal part of the protein before the DNA-binding domain causing 20 % survival of the homozygous mutant animals. The allele with deletion of exon 3 represents a loss-of-function mutation with a complete penetrance of the phenotype. Mice without functional GR die from atelectasis of the lungs shortly after birth. GR function in lung development and perinatal survival is poorly understood, but dimeric DNA binding of the receptor is not required (see below). Primary GR target genes essential for lung development remain to be established. Analysis of these mice with regard to receptor function revealed, among other things, that the GR is required for rapid expansion of erythroid progenitors under hypoxic conditions in vivo [5]. Table 1 lists a summary of the phenotypic consequences of the GR null/hypomorphic mutation [9,10].

Mineralocorticoid receptor (MR)-deficient mice have a normal prenatal development. During the first week of life, MR-deficient mice develop symptoms of pseudohypoaldosteronism with hyponatremia, hyperkalemia, high renal sodium loss, and a strongly activated renin-angiotensin-aldosterone system (RAAS) [11]. MR knockout mice die in the second week after birth but daily subcutaneous injections of isotonic NaCl solution until weaning and continued oral NaCl supply lead to survival [11]. The NaCl-rescued MR knockout mice display a strongly enhanced fractional renal sodium excretion, hyperkalemia, and persistently high levels of renin and aldosterone [12]. The activity of the amiloride-sensitive epithelial sodium channel (ENaC) is strongly reduced in colon and kidney, but at day 8 after birth there is no down-regulation of the mRNA abundance of the three ENaC subunits. Therefore, mineralocorticoids do not regulate the sodium reabsorption by transcriptional control of ENaC but of other yet unidentified MR target genes [11].

Table 1 Alterations of physiological functions found in mice carrying a targeted disruption of the GR gene.

Lung	Perinatal death due to respiratory failure in newborn mice
Liver	Reduced expression of genes encoding gluconeogenic enzymes
Adrenals	Hypertrophy and hyperplasia of the cortex with increased expression of steroidogenic enzymes; impaired chromaffin cell differentiation in the medulla, and absence of epinephrine synthesis
HPA-axis	Elevated serum levels for ACTH and CORT; increased expression of POMC and CRH
Bone marrow	Impaired proliferation of erythoid precursor cells
Thymus	Loss of glucocorticoid-dependent apoptosis in thymocytes

FUNCTION-SELECTIVE MOUSE MUTANTS: THE DNA-BINDING DEFICIENT GR REVEALS THE IMPORTANCE OF GR CROSS-TALK WITH OTHER TRANSCRIPTION FACTORS

The GR mediates the effects of glucocorticoids by positive and negative regulation of gene transcription in two modes of action, DNA binding-dependent and DNA binding-independent. Binding of GR to a glucocorticoid response element (GRE) activates transcription [4], binding to so-called negative GREs (nGRE) represses transcription [13]. To study activities of the receptor that might be independent of DNA-binding, a function-selective mouse mutant was generated by a knock-in strategy. By exploiting the Cre/loxP system a point-mutation was introduced into the mouse genome resulting in the amino acid exchange A458T within the D-loop, one of the important dimerization interfaces of the receptor [14]. This function-selective mutation abolishes homodimerization of the receptor and thereby impairs binding of GR to DNA. However, regulation of gene activity by protein/protein interaction remains intact.

The dimerization-defective mutant referred to as GR^{dim} represents a powerful tool for studying the molecular mechanisms of glucocorticoid action in vivo. Surprisingly, homozygous GR^{dim} mutant mice are viable, indicating that dimeric DNA-binding of the receptor is not essential for survival. Activating and inhibitory functions of the GR could be demonstrated (Table 2).

Table 2 GR^{dim} mice allow to decipher processes which require DNA binding of GR and those which are mediated by protein-protein interactions of GR with other transcription factors.

DNA binding-dependent processes (impaired in GR^{dim} mice)	**DNA binding-independent processes (not impaired in GR^{dim} mice)**
Induction of GRE-dependent reporters in embryonic fibroblasts and gluconeogenic enzymes in liver	Repression of TPA-induced expression of collagenase-3 and gelatinase B in embryonic fibroblasts and skin
Glucocorticoid-dependent apoptosis of thymocytes	Lung maturation, survival at birth
Expression of pro-opiomelanocortin (POMC) in the anterior pituitary	Immunoreactivity of corticotropin releasing hormone (CRH) in the median eminence
Long-term proliferation of erythroid progenitor cells in vitro, stress erythropoiesis in vivo	Repression of proinflammatory cytokines in thymocytes and macrophages, repression of inflammatory responses

Animal studies supported by the analysis of cells derived from GR^{dim} mice demonstrated that important anti-inflammatory activities of the receptor are maintained in this GR dimerization-deficient mouse model [15,16]. Inflammatory responses including the induced ear edema following TPA treatment and phorbolester-induced local inflammation of the skin could be repressed by glucocorticoids in GR^{dim} mice. DNA binding-independent GR functions are demonstrated to be sufficient for antagonizing AP-1-dependent gene activation in skin [17]. Intraperitoneal injection with lipopolysaccharide from gramnegative bacteria (LPS from E. coli) stimulates cytokine synthesis and release from thymocytes and macrophages. Interestingly, glucocorticoids suppress the cytokine response in both wild-type and GR^{dim} mice. Glucocorticoids also repress the proinflammatory potential of NFκB activity even when the receptor is impaired for DNA-binding [15]. Findings based on GR^{dim} mice promise that selective glucocorticoid receptor mediators (SGRMs) with potent anti-inflammatory activity can be found. Glucocorticoids are among the most potent immunosuppressive and anti-inflammatory drugs, used both locally and systemically in the treatment of inflammation, leukemia and autoimmune diseases [18]. However, long-term therapies are usually accompanied by severe side effects such as osteoporosis, atrophy of the skin, myopathy, and psychosis. One approach to improve the treatment would be to develop new GR ligands that show highly potent immunosuppressive and anti-inflammatory activity but reduced side effects. Given that many immunoregulatory genes, such as cytokines, are controlled by the AP-1 and NFκB families of transcription factors, it can be assumed that part of the immunosuppressive and anti-inflammatory activity of GR is mediated via its DNA binding-independent cross-talk function. It is thus conceivable that at least some of the side effects require the DNA binding-dependent function of GR.

To provide the proof-of-concept for this model and at the same time to elucidate the molecular mode of action of GR in more detail, regulation of cytokine expression and inflammation was studied in GR^{dim} mice. Analysis of some important pro-inflammatory cytokines (IL-1, IL-6, TNFα) in thymocytes and macrophages showed that suppression is largely independent of the DNA binding function of GR. Taken together, this shows that by using GR^{dim} mice it is possible to discriminate the molecular mode of action of GR in the immune system and to exploit this information for the development of improved therapeutic approaches.

Synthesis and release of glucocorticoids from the adrenal cortex are regulated by negative feedback loops in the HPA axis. Increase in the secretion of glucocorticoids cause a decrease in synthesis and secretion of its regulating substances CRH and ACTH [1,3]. GR^{dim} mice enabled us to investigate the molecular mechanisms underlying this negative feedback loop. Immunohistochemical analysis revealed that CRH levels in the hypothalamus of GR^{dim} mice do not differ significantly from those of wild-type mice. Therefore, glucocorticoids control hypothalamic CRH synthesis by protein/protein interactions between the GR and other factors [14]. The transcriptional mechanism of this control is however not understood. Expression of the gene encoding POMC, the precursor of ACTH, is elevated by almost an order of magnitude demonstrating loss of negative control of POMC transcription. These findings illustrate the different modes of action of GR in HPA-axis control and strongly support the hypothesis of negative GREs to which multimers of the receptor have to bind in order to exert this feedback control [13].

CELL/TISSUE-SPECIFIC MUTATIONS OF THE GR AND MR GENES

Mice without a functional GR or MR die after birth. Therefore, in order to define the function of these two receptors in the adult organism we had to develop cell/tissue-specific mutations. We anticipated that the generation of somatic mutations would not only avoid the lethality of germline mutant mice, but that it would also allow us to define the function of this receptor in a particular cell or tissue. This analysis would then also allow to estimate to which extent this function would contribute to the physiology of the entire organism. To analyze the function of these two receptors in specific cells and tissues, exon 3

of the GR and exon 3 of the MR gene were flanked by loxP sites using homologous recombination in embryonic stem cells. To attain a specific mutation, it is required that the Cre recombinase is expressed in the desired cells or tissues. Since we have observed that the penetrance of the mutation depends to a large extent on the expression pattern of the recombinase we use either bacterial artificial chromosomes or yeast artificial chromosomes for the expression of the Cre recombinase. In this way, a high degree of specificity and selectivity and copy number-dependent expression could be achieved. Figure 2 gives an overview of the specific GR mutations that we have generated or that are in the process of their development.

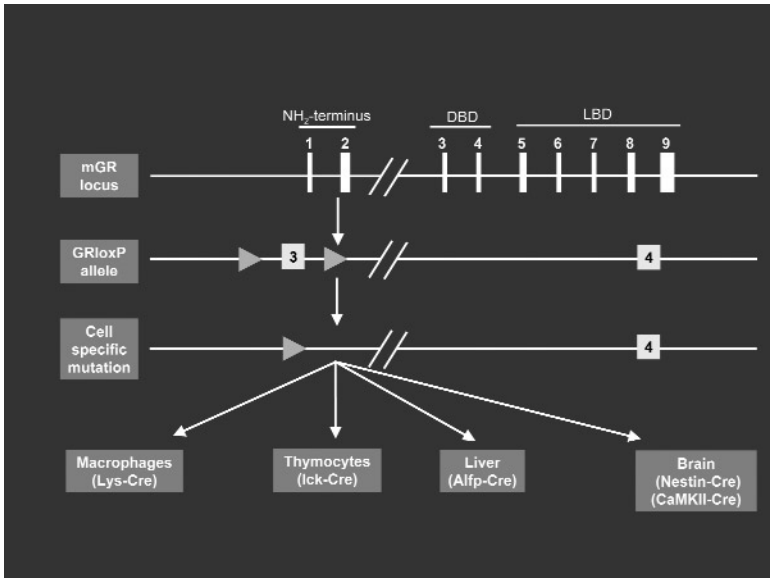

Fig. 2 Summary of cell- and tissue-specific mutations of the GR gene. Specific mutations in the GR locus were obtained by expressing the Cre recombinase in monocytes/macrophages under control of the regulatory elements of the lysozyme gene, in thymocytes under control of the lck gene, in hepatocytes under control of the regulatory elements of the albumin gene, and in brain with regulatory sequences of the nestin and CaMKIIα gene, respectively.

The GR and MR are both highly expressed in the central nervous system, in particular in the hippocampus. The pre-receptor specificity mechanism that prevents activation of the MR by corticosteroids in mineralocorticoid target tissues is not operative in the CNS. It is an intriguing question what the function of these two receptors might be. The GRs and MRs are thought to regulate an overlapping set of target genes since DNA-binding specificity of the two receptors is identical in vitro. We therefore think that specific inactivation of the MR and GR gene in the central nervous system might allow us to define their specific functions in greater detail.

In order to define GR function in the nervous system, we used the nestin gene promoter and enhancer to drive Cre recombinase expression [19]. The nestin gene is active in neuronal and glial cell precursors and leads to the deletion of the GR gene in the entire nervous system. In contrast with the germline mutation, lack of the GR in the central nervous system is not lethal. The animals have a Cushing-like syndrome with elevated glucocorticoids, altered fat distribution, and reduced bone density. Inactivation of the GR in the central nervous system reduces anxiety-related behavior. These mice are less anxious in tasks that exploit the behavioral conflict between exploring and avoiding an aversive compartment, the elevated zero maze, and the dark light box. These findings indicate an important role of GR signaling in emotional behavior [10,19]. To generate mutations lacking both receptors in the fore-

brain region, we used a bacterial artificial chromosome carrying the CaMKIIα gene to drive Cre recombinase expression. Mice with loss of the MR as far as analyzed show no alteration in HPA axis activity. Using the same Cre-expressing mice, we have generated a novel brain-specific GR mutation.

To study the role of GR in liver functions of the adult, we wished to inactivate the GR selectively in parenchymal cells of the liver. This was achieved by choosing gene regulatory sequences of the albumin gene for expression of the Cre recombinase [20]. Mice with a hepatocyte-specific alteration displayed after four weeks a severe growth deficit. Since no alterations in the serum levels of growth hormone and glucocorticoids could be found in these animals, we reasoned that the growth deficit resides in growth hormone signaling. Growth hormone is known to affect growth through stimulation of synthesis of insulin-like growth factor 1 (IGF-1) [21]. Interestingly, when we determined the mRNA levels of IGF-1 and growth hormone-regulated genes in the liver we found that the level was drastically reduced in the mutant. The level of Stat5α and β, which are thought to be crucial for mediation of the growth hormone signaling was not altered nor was their phosphorylation status. Mice with the dimerization-defective mutation are of normal size, and the expression of IGF-I and other growth hormone-regulated genes is unaltered. These results demonstrate that GR function in hepatocytes is crucial for body growth. The different response in GR^{dim} mice in comparison to mice with a hepatocyte-specific mutation provides strong evidence that the growth-promoting activity of the receptor does not require binding to a glucocorticoid-responsive element. It rather appears to function as a coactivating molecule thus synergizing with Stat5 activity, supporting previous in vivo experiments, which demonstrated a requirement of GR for Stat5 activation by prolactin [22].

To study the function of GR in the immune system, we inactivated the GR in thymocytes. Inactivation of GR function in thymocytes was achieved by using the lck gene to drive the Cre recombinase expression. The lck gene is activated during the early differentiation of thymocytes. Thymocytes of these mutant animals are entirely resistant to glucocorticoid-induced apoptosis. To generate mice lacking the receptor in monocytes/macrophages, we used the lysozyme gene promoter for expression of Cre recombinase. When these mice were challenged by treatment with a given dose of LPS, it was observed that all mutant mice died within 36 hours. Wild-type mice survived this challenge. Thymocytes and macrophages from these two mutants are now being analyzed with regard to their gene expression profiles. It will be of interest to define a gene or genes which are required for the glucocorticoid-induced apoptosis of thymocytes. Furthermore, the basis for the protective effect of glucocorticoids with regard to the LPS challenge will certainly be much better understood once LPS-induced genes which are affected by glucocorticoid treatment, have been defined.

To investigate the effects of an increased gene dosage of the GR, we have generated transgenic mice carrying two additional copies of the GR gene by using a yeast artificial chromosome (YAC) carrying the mouse GR gene [23]. Expression of plasmid transgenes in mice is often variable and does not necessarily reflect the expression pattern of the endogenous gene, disadvantages which can be circumvented by YAC transgenesis. Interestingly, overexpression of the GR from a 290 kb YAC alters the basal regulation of the HPA, resulting in reduced expression of C2H and ACTH and a fourfold reduction in the level of circulating glucocorticoids. Animals with an increased gene dosage of GR are more resistant to stress and endotoxic shock. In addition, primary thymocytes obtained from these transgenic mice show an enhanced sensitivity to glucocorticoid-induced apoptosis. These results underscore the importance of tight regulation of GR expression for the control of physiological and pathological processes. Furthermore, they may explain differences in the susceptibility of humans to inflammatory diseases and stress, depending on individual prenatal and postnatal experiences known to influence the expression of the GR.

CONCLUSIONS

Exploiting the Cre/loxP recombination system in the mouse a series of alleles of the GR has been generated. These mutants have been instrumental in the molecular genetic analysis of GR functions in vivo.

They allow us to define four different modes of action of the receptor as illustrated in Fig. 3. The receptor is able to activate gene transcription by binding as a dimer to a GR element in the control region of a regulated gene. The growth-promoting activity of the receptor does not require binding of the receptor to a GRE, but is mediated by interaction of the receptor with Stat5. The receptor functions rather as a coactivator in Stat5 signaling. The receptor inhibits expression of genes such as the POMC gene and the prolactin gene in the anterior pituitary by binding to so-called negative GREs in the control regions of these genes. This hypothesis is strongly supported by our observations of elevated levels of the mRNAs encoding these proteins in mice with the dimerization-defective and thus DNA binding-deficient GR. The receptor is also able to modulate target gene activity by a DNA binding-independent mechanism. It thus is able to enhance the transcriptional activity of the Stat5 proteins and inhibit the activity of AP-1- and NFκB-dependent transcription. It is a fascinating challenge to define the multiple activities of GR in molecular detail in the future.

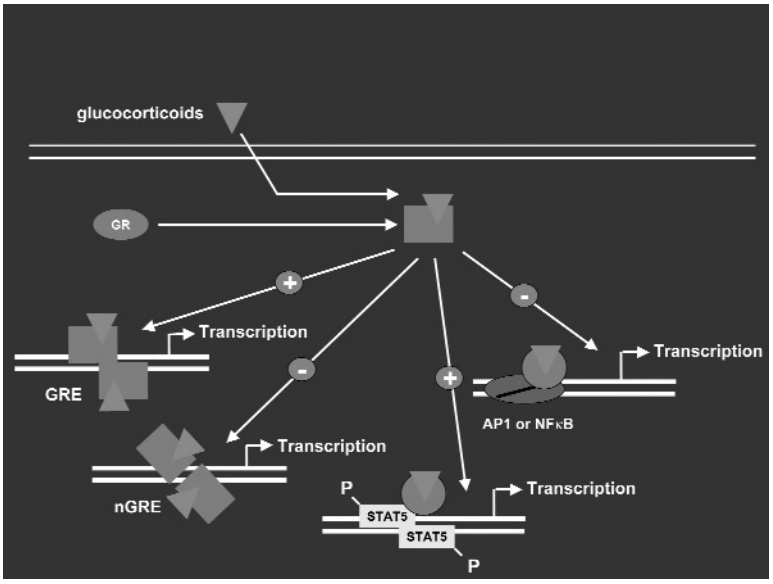

Fig. 3 The GR modulates transcription by different modes of action. The GR is able to influence transcription by binding to a GRE or nGRE. It is assumed that binding of GR to a nGRE leads to an altered GR conformation. The GR can also activate or inhibit transcription by protein/protein interaction as shown on the right.

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