Glucocorticoid Signaling in the Cell: Expanding Clinical Implications to Complex Human Disorders

The Impact of New Biology

George P. Chrousos, MD,
University of Athens

Glucocorticoid Signaling

- New Biology
- Molecular Actions of Glucocorticoids
- Pervasiveness of Glucocorticoid Actions
- Expanding Clinical Implications

Glucocorticoid Signaling

- New Biology
- Molecular Actions of Glucocorticoids
- Pervasiveness of Glucocorticoid Actions
- Expanding Clinical Implications

NEW BIOLOGY

Units: Genes, molecules, cells
Modules: Functionally inter-linked collections of units such as genes, molecules, neurons, or other cells
Networks: Functionally inter-linked collections of modules
Systems: Functionally inter-linked collections of networks
Programs: Functional temporal connections between systems

Complex Systems

- Multiple interactants
- Self-organizing
- Adapting through feedback loops
- Resilient to perturbations
- Emergent properties

Complex Systems

- Systems nested within systems
- Networks and programs nested within systems

SOME KEY NUMBERS

~ 3 billion bases ($3x10^{12}$)

~ 100 billion neurons ($100x10^{12}$) X 10000 synapses per neuron (10^{18} synapses)

NEW BIOLOGY- POSTGENOMIC ERA

Human genome:

- **About 20-30 thousand genes**
- **100-140 thousand transcripts (mRNA, ncRNA)**
- **200-260 thousand proteins**

Single nucleotide polymorphisms (snp's), microsatellites or copy number variants (cnvs):

- **About 3 million snp's**
- **About 20 million microsatellites**
- **>700 cnv's (many million bases)**
- **Over 10 k disease-related mutations**

GENOMIC VARIANCE

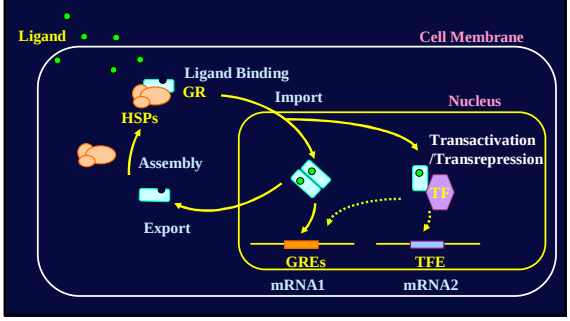
Human to Human:
~ 10 per thousand
(with CNVs 10-12 percent)

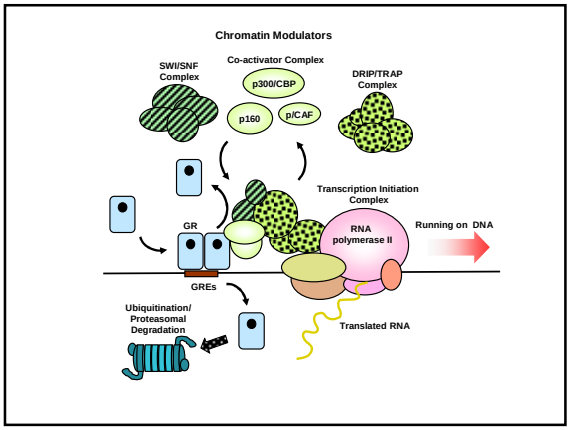
Human to Chimpanzee:
~ 1-2 percent

Glucocorticoid Signaling

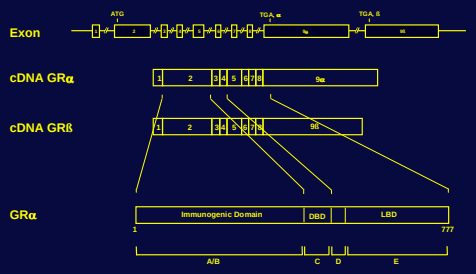
- New Biology
- Molecular Actions of Glucocorticoids
- Pervasiveness of Glucocorticoid Actions
- Expanding Clinical Implications

Glucocorticoid Receptor Signaling

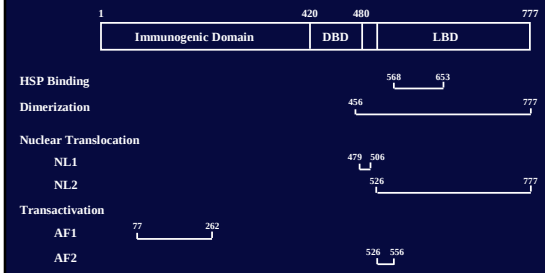


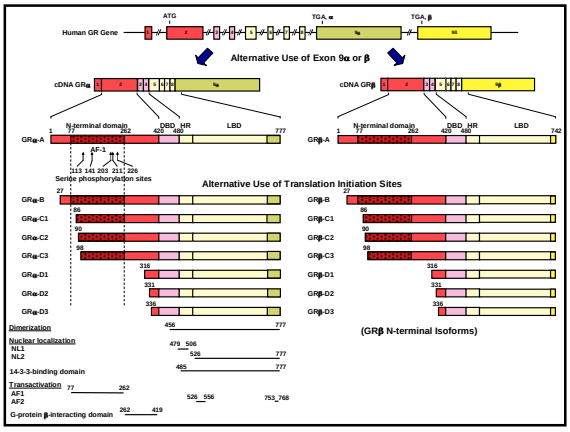


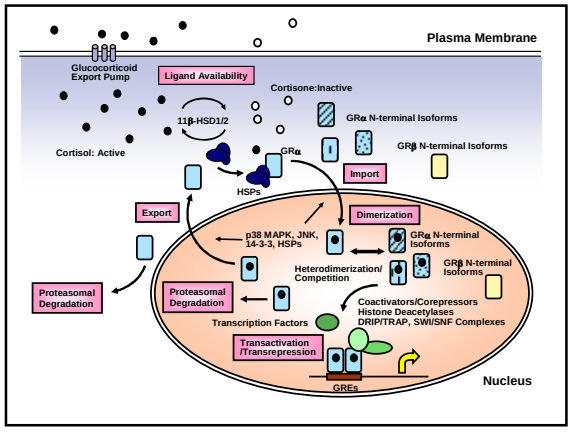
Human Glucocorticoid Receptor



Functional Domains of Human Glucocorticoid Receptor α



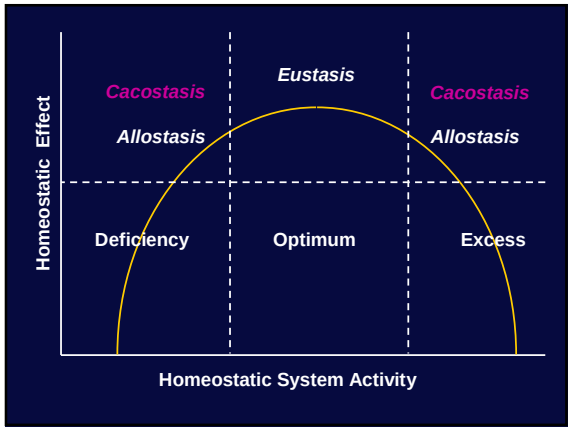


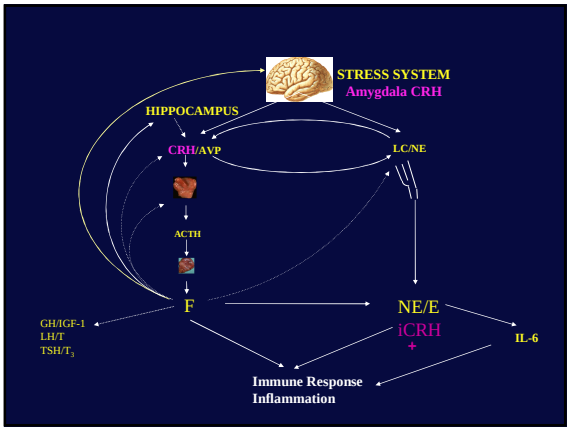


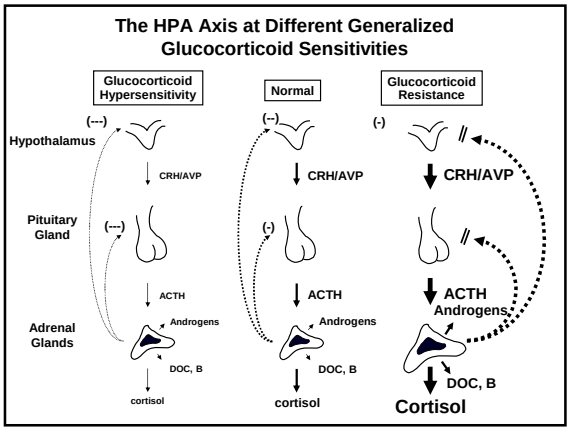
«Μηδέν άγαν»

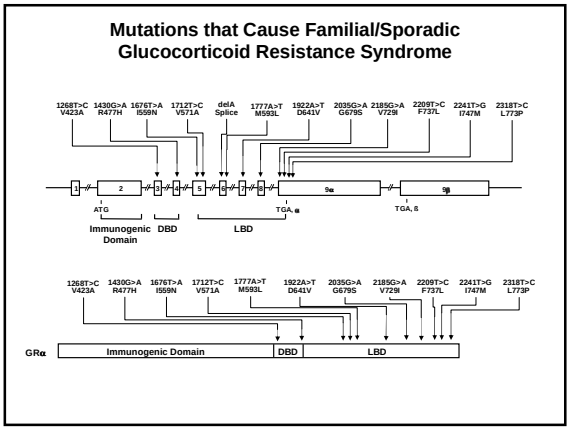
‘Everything in moderation’

Inscription at the Oracle of Delphi



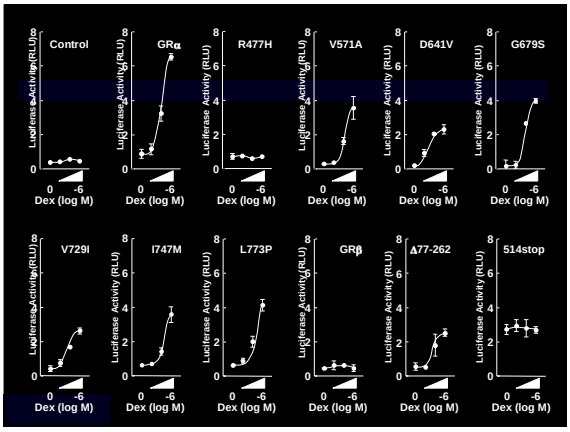


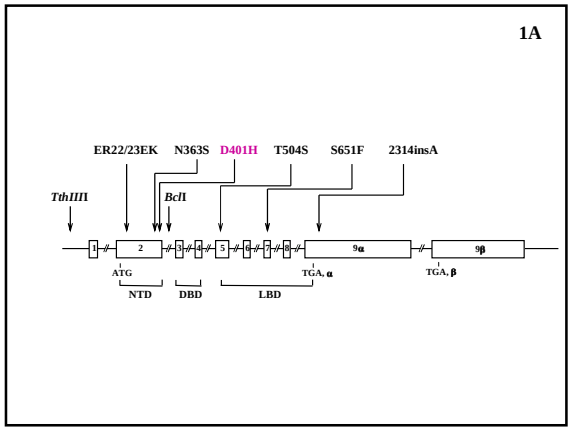


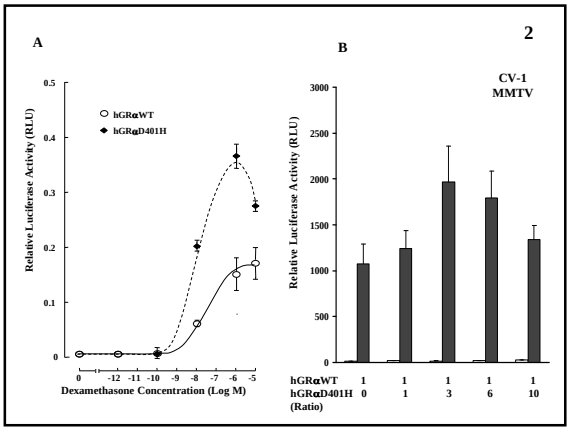


Characterization of GR Mutants

- GRE-binding Defective: GRαR477H
- Ligand-binding Defective: GRαV571A, D641V, G679I, V729I
- AF-1 Defective: GRα(Δ77-262)
- AF-2 Defective: GRαI747M, L773P
- Entire LBD Deletion: GR514stop



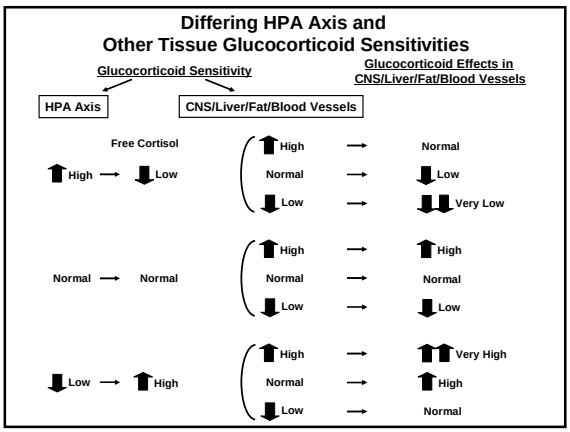


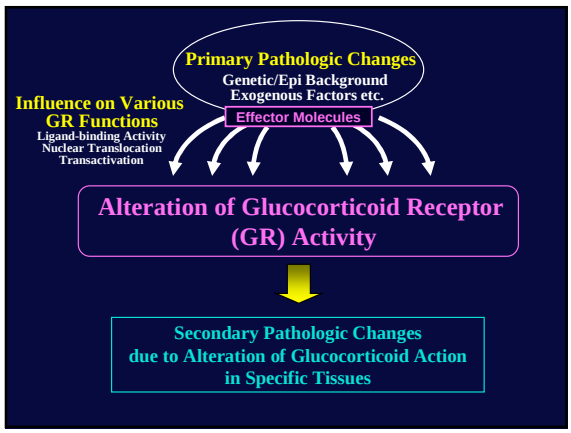


Clinical Manifestations in Tissue Hypersensitivity or Resistance to Glucocorticoids

	Glucocorticoid Excess	*Glucocorticoid Deficiency*
AFFECTED AREA	**GLUCOCORTICOID HYPERSENSITIVITY**	**GLUCOCORTICOID RESISTANCE**
Central Nervous System	Insomnia, anxiety, depression, defective cognition	Fatigue, somnolence, Malaise, defective cognition
Liver	Increased gluconeogenesis* and liposynthesis, insulin resistance	Hypoglycemia, increased insulin sensitivity
Fat	Accumulation of visceral* fat	Loss of weight
Muscles	Insulin resistance*	Increased insulin sensitivity
Blood Vessels	Hypertension*	Hypotension
Bone	Stunted growth, osteoporosis	
Inflammation/ Immunity	Immune suppression, suppressed inflammation	Increased inflammation/ autoimmunity

*dysmetabolic syndrome





Politically Correct 1980's

- Hypothesis-driven Research

ANATHEMA

- "Shotgun Research"
- "Fishing expedition"

Politically Correct 2000's

- Discovery-driven Research

NO LONGER ANATHEMA

- "Shotgun Research"
- "Fishing Expedition"

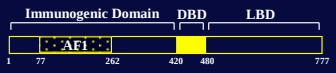
Finding Molecules that Potentially Alter GR Action

Yeast Two-hybrid Screening

Using GR Fragments as Baits

Yeast Two-hybrid Screening Using GR LBD as Bait

Human GR



Bait Fragment

LexA System/Human Jurkat Cell cDNA Library

CLOCK transcription factor

Circadian Rhythm Transcription Factor CLOCK/BMAL1 Regulates the Transcriptional Activity of the Glucocorticoid Receptor through Acetylation

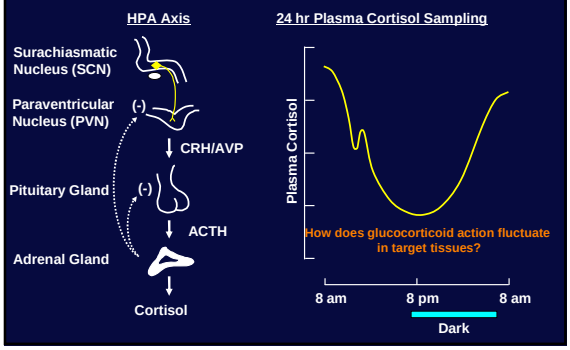
Nancy Nader[1] George P. Chrousos[2] and Tomoshige Kino[1]

1: Program In Reproductive and Adult Endocrinology, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

2: First Department of Pediatrics, Athens University Medical School, Athens 11527, Greece

FASEB J 2009

Circadian Fluctuation of Plasma Cortisol



- **3-10 % of the mammalian transcriptome oscillates with a 24 h rhythm.**
- **CLOCK System-controlled genes and downstream output genes are involved.**

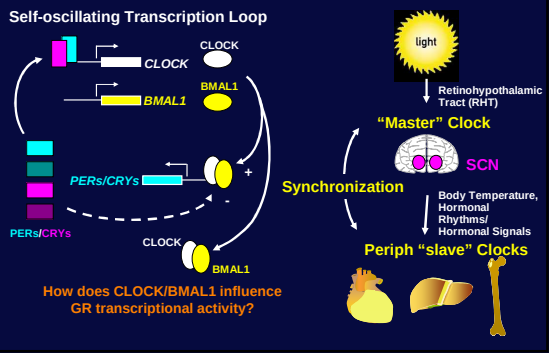
CLOCK/BMAL1: Circadian Rhythm Transcription Factors

- Master regulators of the circadian rhythms both in the central nervous system and peripheral tissues/organs.
- Basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) superfamily of transcription factors
- CLOCK is a histone acetyltransferase (HAT) with homology to the p160s.

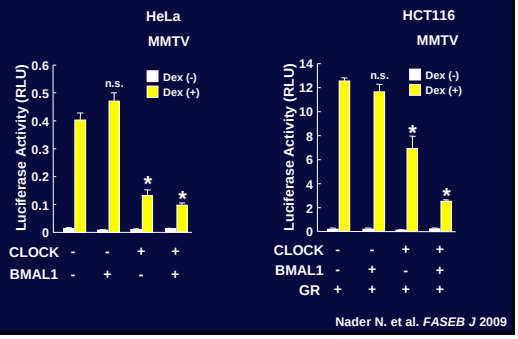
CLOCK/BMAL1: Circadian Rhythm Transcription Factors

The CLOCK/BMAL1 heterodimer forms a self-oscillating, negatively regulated feed-back loop system through mutual regulation of expression/activity with their downstream transcription factors, such as the *Period* (*PER1*, *PER2* and *PER3*) and *Cryptochrome* (*CRY1* and *CRY2*) genes.

CLOCK/BMAL1: Circadian Rhythm Transcription Factors

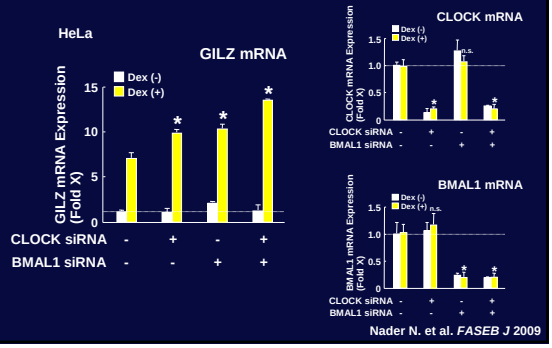


CLOCK/BMAL1 Represses GR-induced Transcriptional Activity

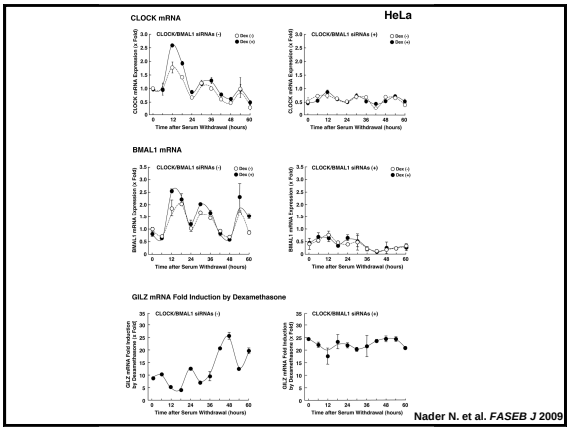


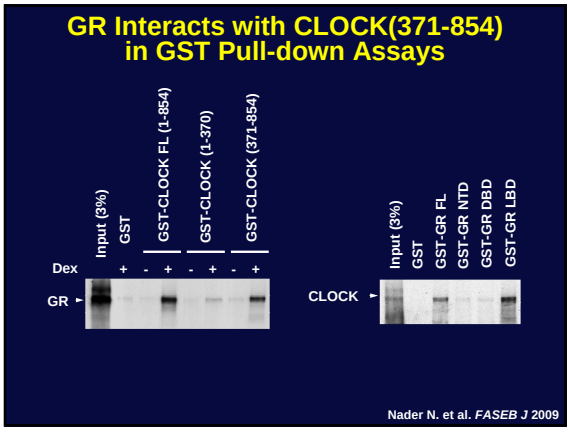
Nader N. et al. *FASEB J* 2009

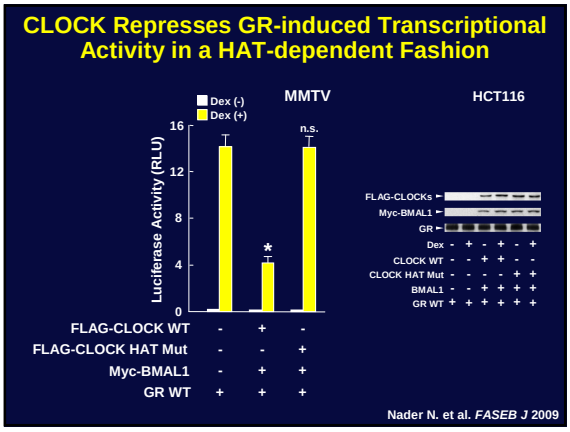
CLOCK/BMAL1 Knockdown Enhances GR-induced Transcriptional Activity

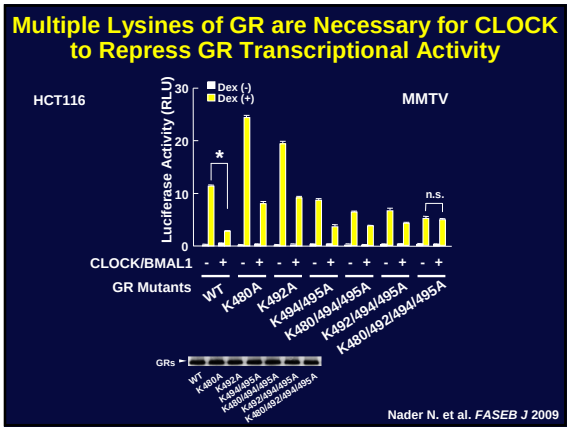


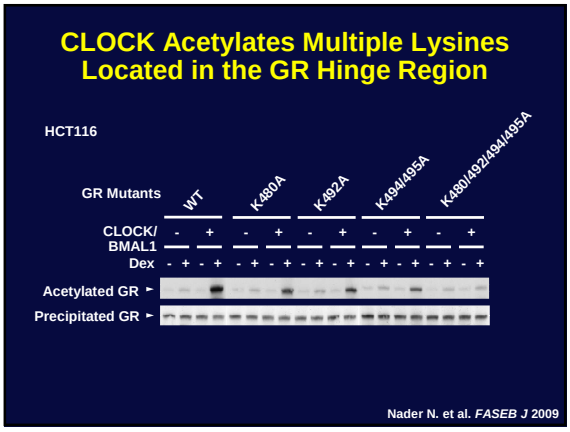
Nader N. et al. *FASEB J* 2009

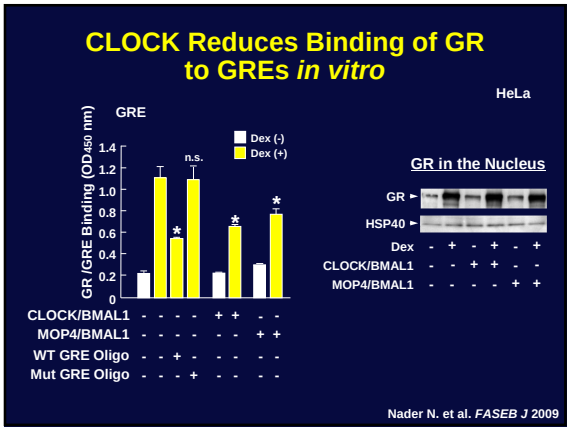

HeLa
CLOCK mRNA
BMAL1 mRNA
GILZ mRNA Fold Induction by Dexamethasone
Time after Serum Withdrawal (hours)
Nader N. et al. FASEB J 2009


GR Interacts with CLOCK(371-854) in GST Pull-down Assays
Input (3%)
GST
GST-CLOCK FL (1-854)
GST-CLOCK (1-370)
GST-CLOCK (371-854)
Dex
GR
Input (3%)
GST
GST-GR FL
GST-GR NTD
GST-GR DBD
GST-GR LBD
CLOCK
Nader N. et al. FASEB J 2009

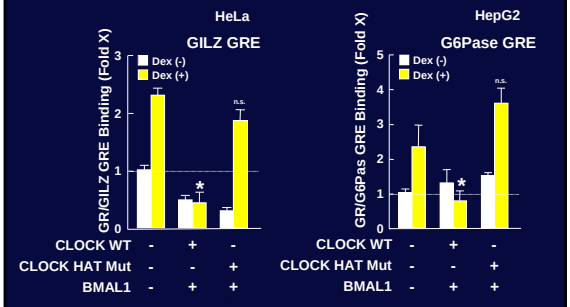

CLOCK Represses GR-induced Transcriptional Activity in a HAT-dependent Fashion
Dex (-)
Dex (+)
MMTV
HCT116
Luciferase Activity (RLU)
n.s.
FLAG-CLOCK WT
FLAG-CLOCK HAT Mut
Myc-BMAL1
GR WT
FLAG-CLOCKs
Myc-BMAL1
GR
CLOCK WT
CLOCK HAT Mut
BMAL1
Nader N. et al. FASEB J 2009


Multiple Lysines of GR are Necessary for CLOCK to Repress GR Transcriptional Activity
HCT116
MMTV
Dex (-)
Dex (+)
Luciferase Activity (RLU)
n.s.
CLOCK/BMAL1
GR Mutants
WT
K480A
K492A
K494/495A
K480/494/495A
K492/494/495A
K480/492/494/495A
GRs
Nader N. et al. FASEB J 2009


CLOCK Acetylates Multiple Lysines Located in the GR Hinge Region
HCT116
GR Mutants
WT
K480A
K492A
K494/495A
K480/492/494/495A
CLOCK/ BMAL1
Dex
Acetylated GR
Precipitated GR
Nader N. et al. FASEB J 2009


CLOCK Reduces Binding of GR to GREs in vitro
HeLa
GRE
GR /GRE Binding (OD450 nm)
Dex (-)
Dex (+)
n.s.
GR in the Nucleus
GR
HSP40
Dex
CLOCK/BMAL1
MOP4/BMAL1
CLOCK/BMAL1
MOP4/BMAL1
WT GRE Oligo
Mut GRE Oligo
Nader N. et al. FASEB J 2009

CLOCK/BMAL1 Reduces the GR/GRE Binding *in vivo* (ChIP Assays)

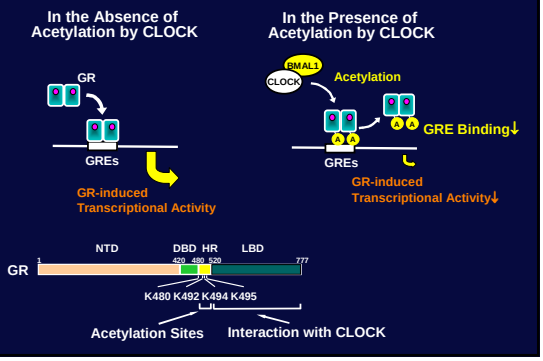


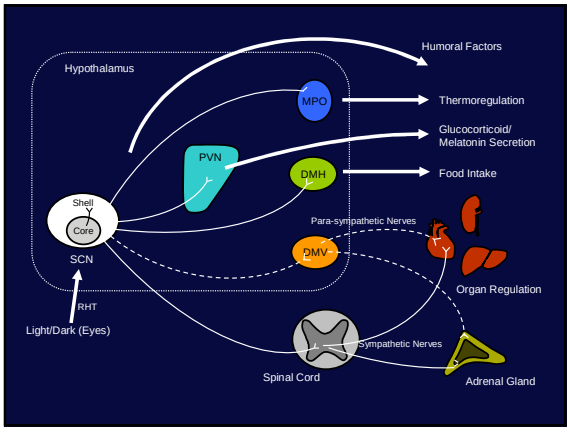
Nader N. et al. *FASEB J* 2009

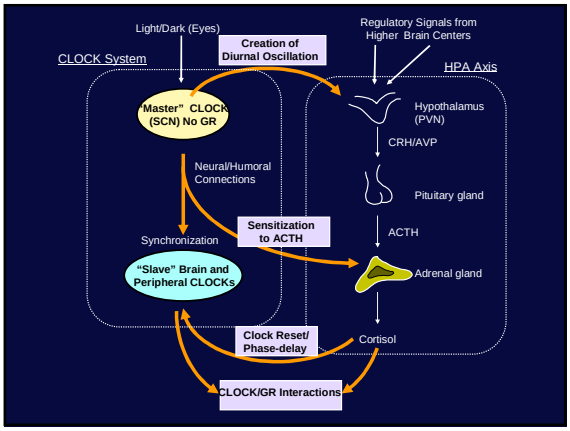
Results

- CLOCK/BMAL1 repressed GR-induced transcriptional activity.
- CLOCK acetylated multiple lysines located in the GR hinge region though its HAT domain.
- CLOCK-induced acetylation suppressed the GR/GRE binding *in vitro* and *in vivo*.

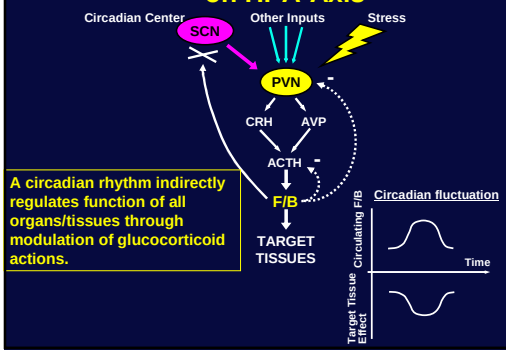
CLOCK/BMAL1 Represses GR-induced Transcriptional Activity through Acetylation

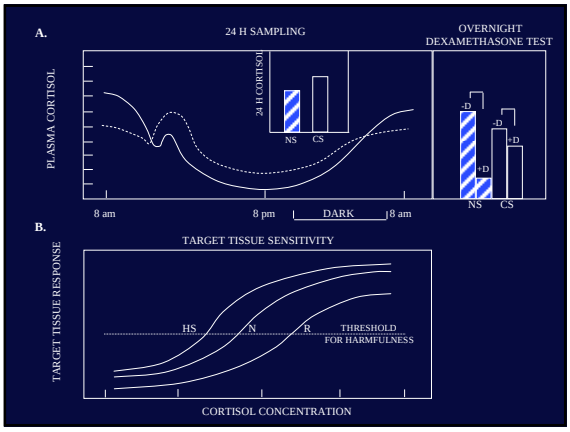






Influence of Circadian Clock on HPA-Axis





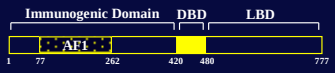
Glucocorticoids reset the "slave" CLOCKs but not the "master" CLOCK, and phase-shift their circadian rhythm by modulating expression of several clock-related genes.

Perturbation of either the CLOCK SYSTEM or the HPA axis leads to similar metabolic and immune pathologies:

- Obesity and Metabolic Syndrome
- Immune Dysfunction, as if due to a hyperactive stress system

Yeast Two-hybrid Screening Using GR 263-419 as Bait

Human GR

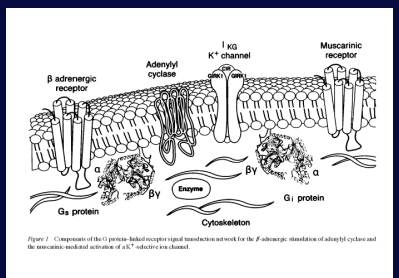


Bait Fragment

LexA System/Human Jurkat Cell cDNA Library

β Subunit of Trimeric Guanine Nucleotide-binding Proteins (Gβ)

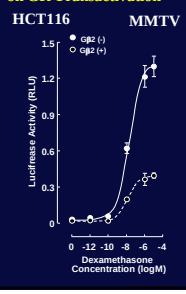
Trimeric G Protein Complex



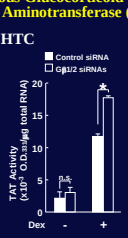
(from Clapham DE & Neer EJ *Ann Rev. Pharmacol. Toxicol.* 1997 37: 167-203)

Gβ as a Negative Regulator of GR Transactivation

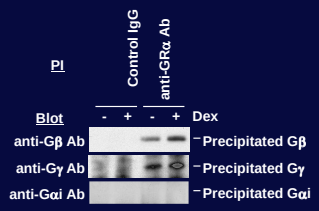
Effect of Gβ2 Overexpression on GR Transactivation



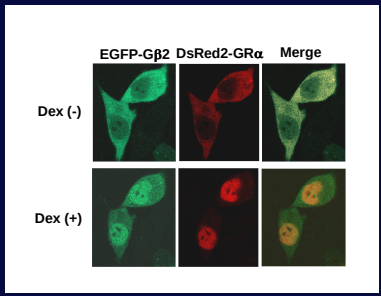
Effect of Gβ1/2 siRNAs on Endogenous Glucocorticoid-responsive Tyrosine Aminotransferase (TAT) Activity



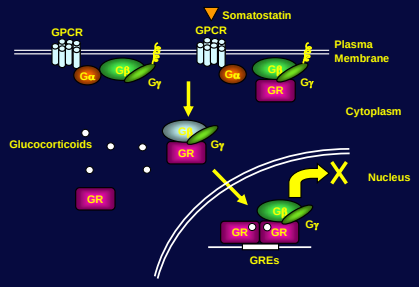
Gβ and Gγ, but not Gα, are Co-precipitated with GR



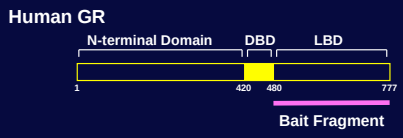
Gβ Co-migrates with GR from the Cytoplasm into the Nucleus



Regulation of the Glucocorticoid Receptor Activity by GPCR Ligands through G Proteins



Cyto-trap Yeast Two-hybrid Screening using the human GR LBD as bait



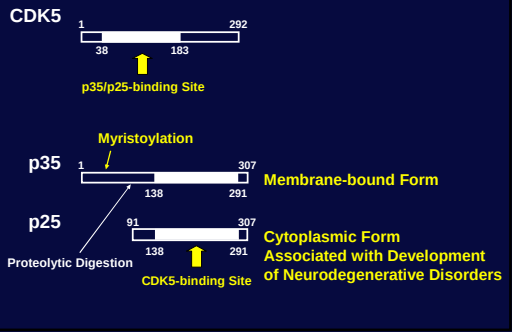
Cyto-trap System/Human Fetal Brain cDNA Library

Cyclin-dependent Kinase 5 (CDK5) Activator p35

CDK5/p35 (p25)

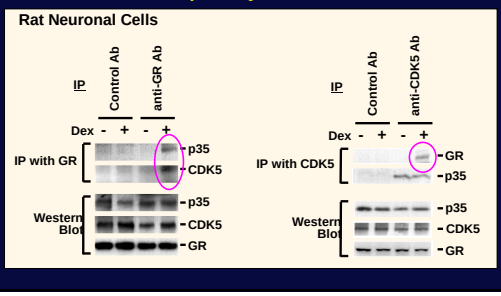
1. Member of the Cyclin-dependent serine/threonine kinase family.
2. Activated by forming a heterodimer with partner p35.
3. Functions specifically in the CNS and is essential for the development of the fetal brain (formation of layer structure of the cerebral neocortex) and for dendritogenesis (sprouting) and synaptogenesis at any age.
5. Aberrant activation of CDK5 by proteolytically produced p25 is associated with development of neurodegenerative disorders, such as Alzheimer's disease and amyotrophic lateral sclerosis.

CDK5/p35 (p25)

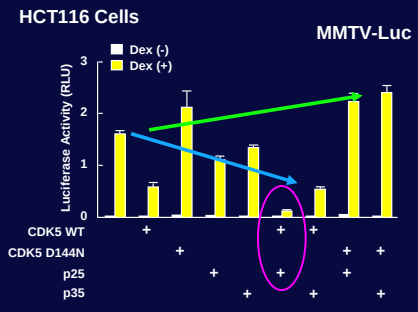


Interaction of GR and CDK5/p35 in Rat Primary Neurons

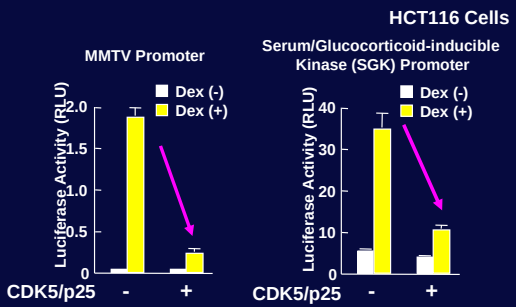
GR Interacts with CDK5/p35 in a ligand-dependent fashion in rat primary neuronal cells



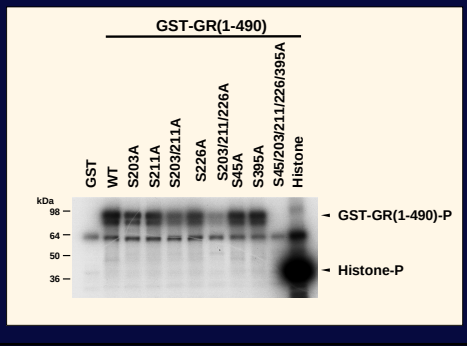
CDK5/p35/p25 Suppresses GR-induced Transcriptional Activity in HCT116



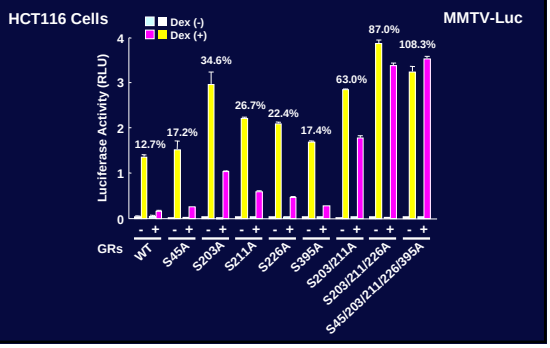
CDK5/p35/p25 Suppresses GR Transcriptional Activity on the SGK Promoter



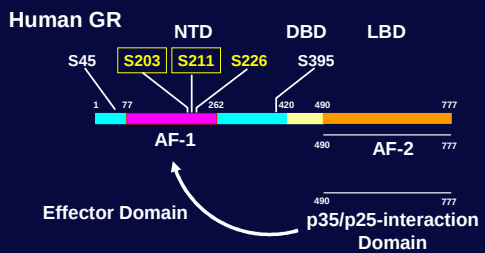
CDK5/p25 Phosphorylates GR at Serines 45, 203, 211, 226 and 395 *in vitro*



GR Serines 45, 203, 211, 226 and 395 Mediate CDK5/p25-induced Suppression of GR Transcriptional Activity



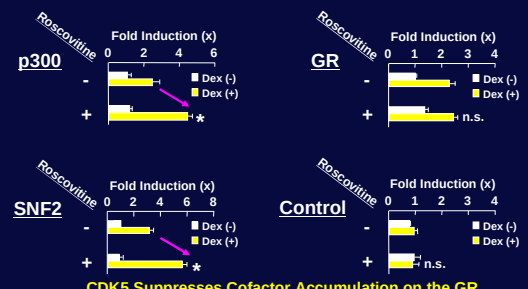
CDK5/p35 Phosphorylates Multiple Serines of the GR



LBD: Binding Domain, NTD: Effector Domain

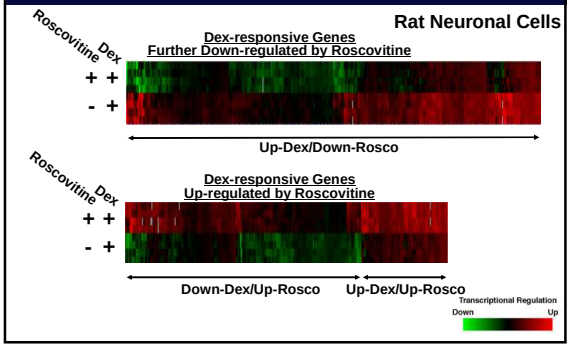
CDK5 Inhibitor Roscovitine Facilitates Cofactor Accumulation on the Glucocorticoid-responsive SGK Promoter in ChIP Analyses

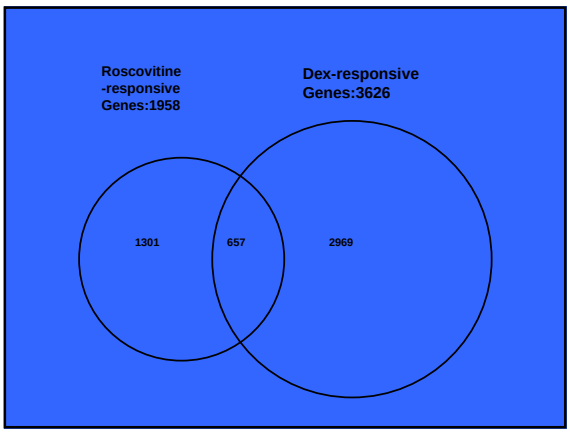
Rat Neuronal Cells — SGK/GREs



CDK5 Suppresses Cofactor Accumulation on the GR through Phosphorylation

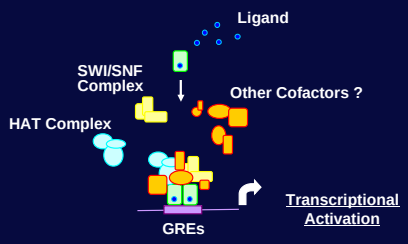
CDK5 Modulates GR Transcriptional Activity in a Gene-specific Fashion in Microarray Analyses





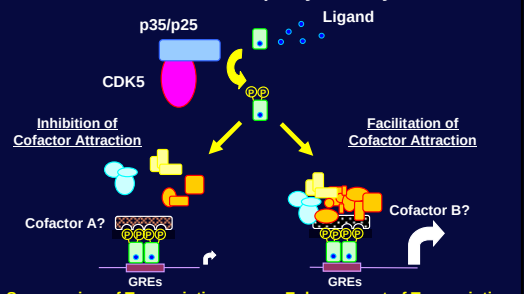
Regulation of GR Transcriptional Activity by CDK5

In the Absence of GR Phosphorylation by CDK5



Regulation of GR Transcriptional Activity by CDK5

In the Presence of GR Phosphorylation by CDK5



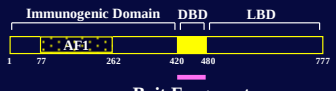
Suppression of Transcription — Enhancement of Transcription

Summary

1. CNS-specific CDK5 physically interacts with GR at its LBD through the activator p35/p25.
2. CDK5 phosphorylates GR at multiple serines (45, 203, 211, 226, 395) located in the N-terminal domain.
3. CDK5/p35 modulates GR transcriptional activity by changing attraction of cofactors to a promoter in a promoter- and, probably, CNS region- specific fashion through phosphorylation.

Yeast Two-hybrid Screening Using GR 420-489 as Bait

Human GR



Bait Fragment

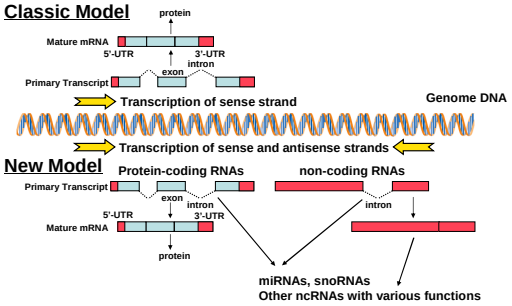
LexA System/Human Jurkat Cell cDNA Library

Growth Arrest-specific 5 (Gas5)

What is Non-coding (nc) RNA?

1. RNAs that do not code proteins.
2. tRNAs and miRNAs are examples of ncRNAs.
3. ncRNAs have distinct biologic activities.
4. ~60% of the mouse genome is transcribed, producing ~200,000 independent transcripts, of which ~half consist of ncRNA.
5. Interestingly, about ~20% of coding and non-coding genes express both sense and anti-sense RNAs, forming double stranded RNAs through hybridization of the sense and antisense strands.

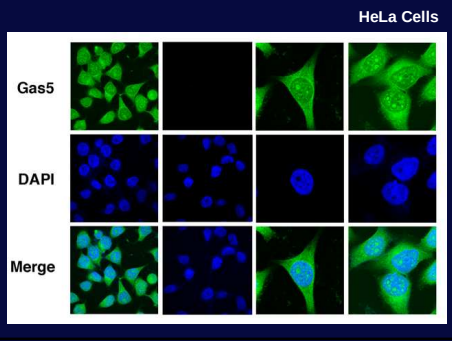
What is Non-coding (nc) RNA?



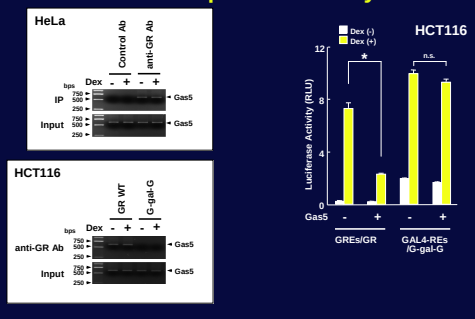
Growth Arrest-specific 5 (Gas5)

- Non-protein-coding RNA (Human Gas5: 598 bases).
- Originally isolated as a gene specifically expressed in growth-arrested cells (or in G_0 phase of the cell cycle).
- Physiologic functions are not known as yet.

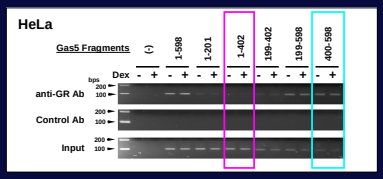
Subcellular Localization of Gas5 in RNA-FISH

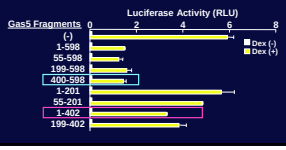


Gas5 is Associated with the DNA-binding Domain of the GR and Suppresses its Transcriptional Activity



Gas5 Interacts with the GR DBD via its 3' Portion



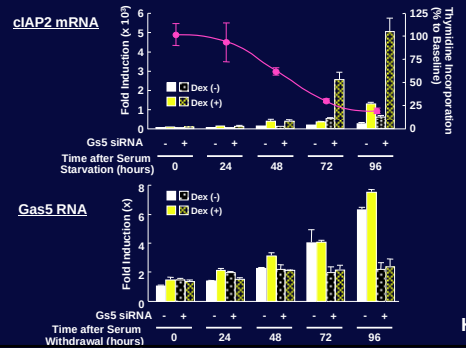


Monitoring endogenous GR-induced Transcriptional Activity

cIAP2 (Cellular Inhibitor of Apoptosis Protein 2)

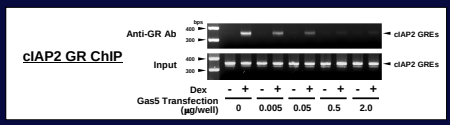
- An ubiquitously expressed protein
- Inhibits apoptosis by directly binding to and inhibiting the cell death proteases caspase-3, -7, and -9
- Glucocorticoids stimulate cIAP2 gene expression through GREs located ~500 bps upstream of the transcription initiation site

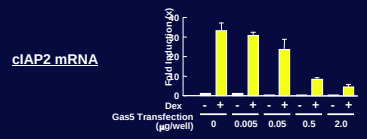
Gas5 Induced by Serum Starvation/Growth Arrest Inhibits GR-stimulated cIAP2 mRNA Expression



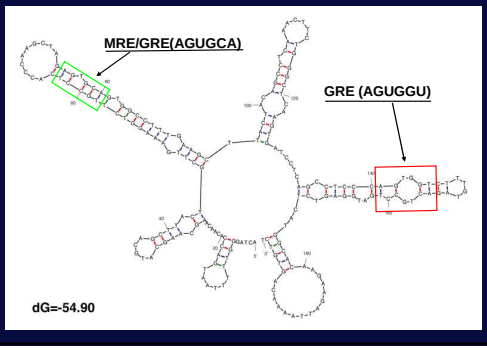
HeLa

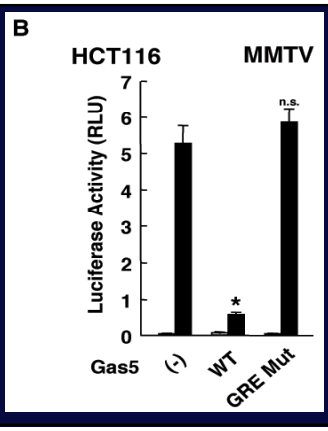
Gas5 Inhibits Binding of GR to the cIAP2 GREs in ChIP Assays



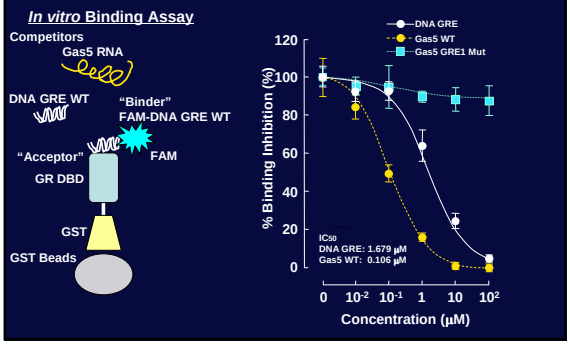


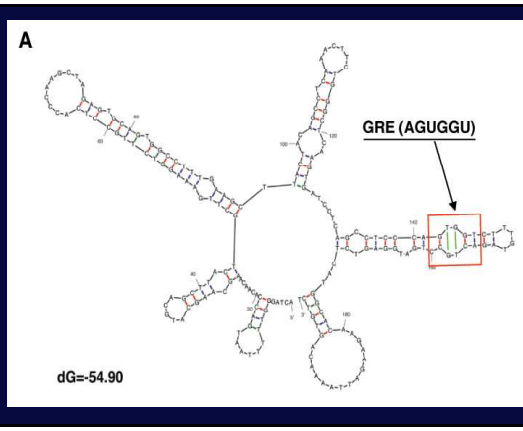
2-D Structure of 3'-Terminal Gas5





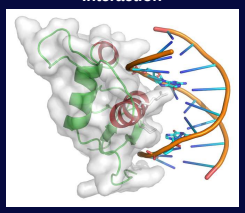
Gas5 RNA Competes with DNA GRE for Binding to GR DBD *in vitro*



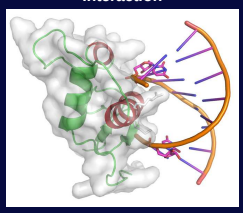


Interaction of GR DBD and Gas5 through its RNA "GRE"

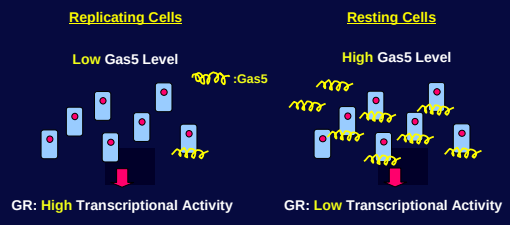
GR DBD-DNA GRE Interaction



GR DBD-RNA "GRE" Interaction



Gas5 is a Growth-related Inhibitor of GR Transcriptional Activity



Gas5 Silences GR Transcriptional Activity in Growth-arrested Cells

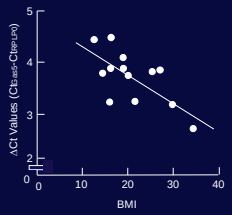
Speculation

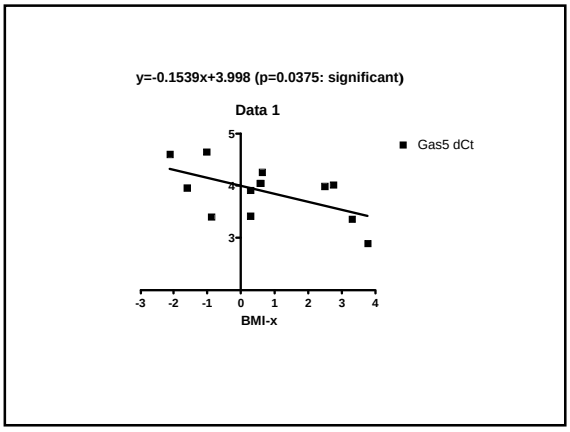
Gas5 is a growth arrest-related, RNA "GRE"-harboring co-repressor of the GR in resting cells, restricting the expression of glucocorticoid-responsive genes.

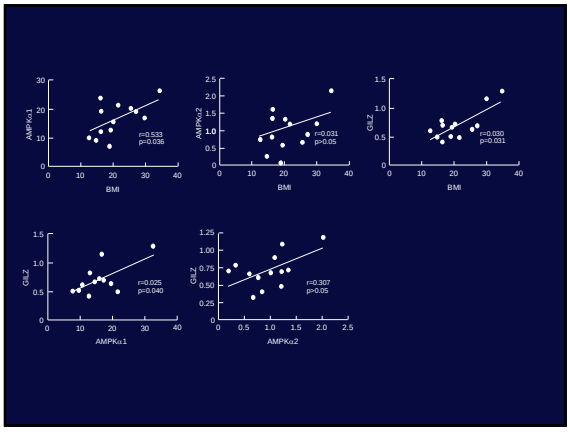
This might be an adaptive response to starvation, saving energy resources.

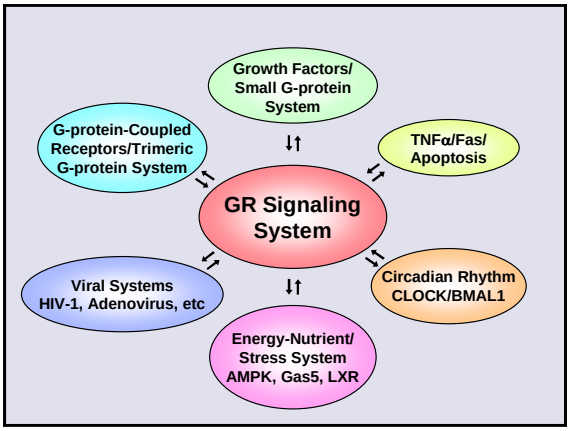
Gas5 ncRNA Levels Correlate with BMI in Peripheral Mononuclear Cells

y=-0.05307x+5.005 (p=0.0103)



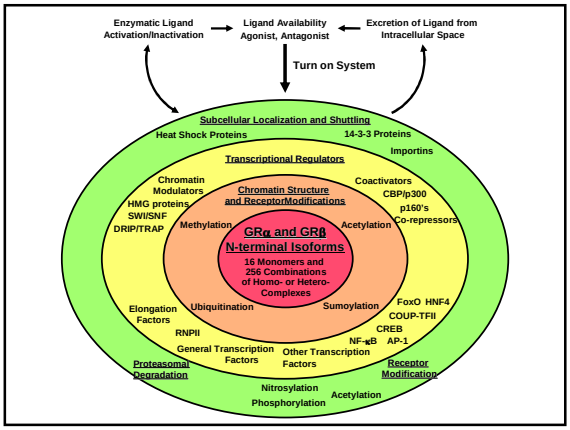


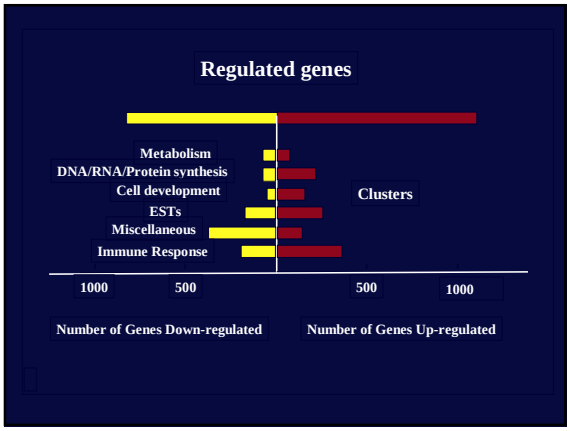


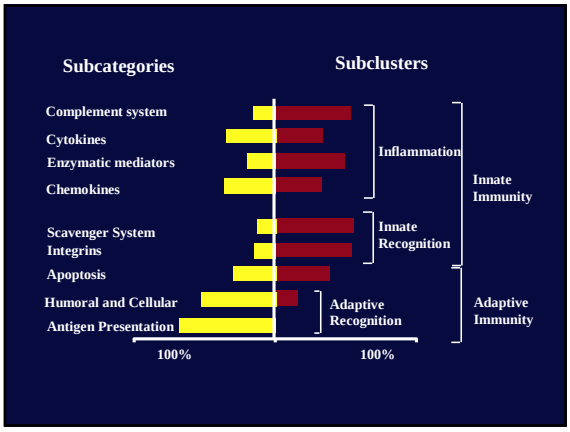


Glucocorticoid Signaling

- New Biology
- Molecular Actions of Glucocorticoids
- Pervasiveness of Glucocorticoid Actions
- Expanding Clinical Implications







Glucocorticoid Signaling

- New Biology
- Molecular Actions of Glucocorticoids
- Pervasiveness of Glucocorticoid Actions
- Expanding Clinical Implications

Change of Tissue Sensitivity to Glucocorticoids in Pathologic Conditions

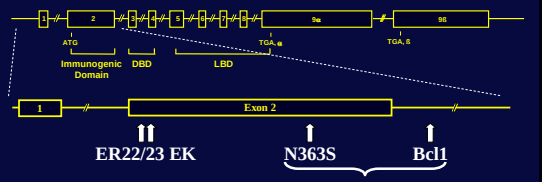
Resistance

- Familial/Sporadic Glucocorticoid Resistance Syndrome
- Bronchial Asthma, Rheumatoid Arthritis, Systemic Lupus Erythematosus, Ulcerative Colitis
- Acute Respiratory Distress Syndrome/Sepsis
- Depression

Hypersensitivity

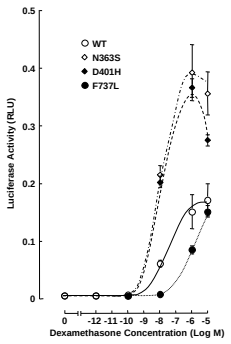
- Generalized Glucocorticoid Hypersensitivity Syndrome
- Visceral-type Obesity-related Dyslipidemia, Insulin Resistance, Carbohydrate Intolerance and Hypertension
- AIDS-related Insulin Resistance and Lipodystrophy Syndrome
- Depression

Alteration of Tissue Glucocorticoid Sensitivity in Pathologic States: Glucocorticoid Receptor Polymorphisms

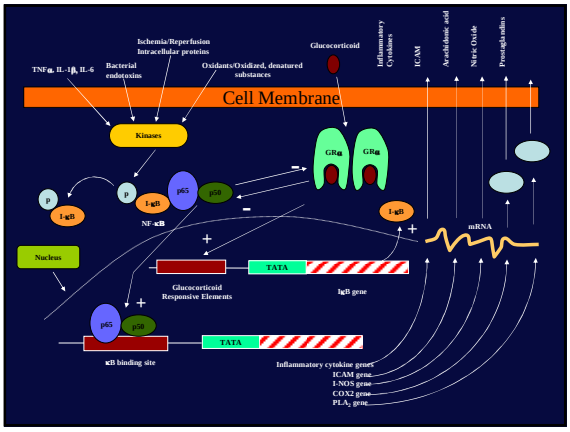


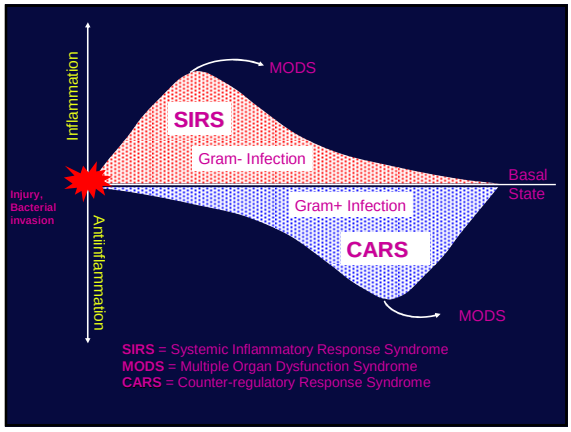
"Glucocorticoid Resistant"
- Better Body Composition
- Healthier Metabolic Profile
- Better Survival
- Lower Risk of Dementia
- Depression

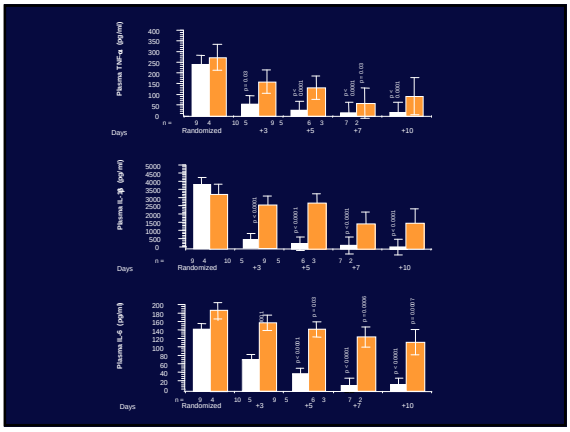
"Glucocorticoid Hypersensitive"
- More Body Fat, Less Lean Body Mass
- Insulin Resistance
- Metabolic s. manifestations
- Depression

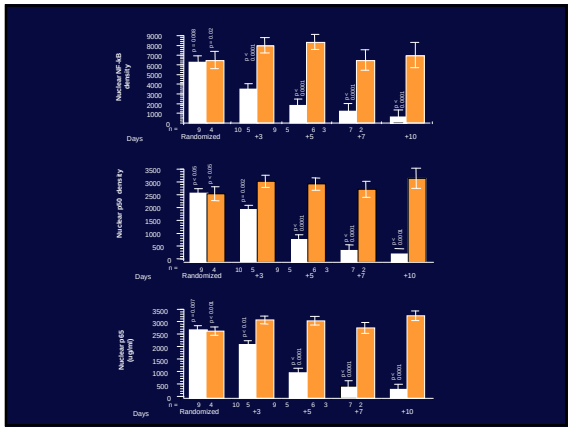


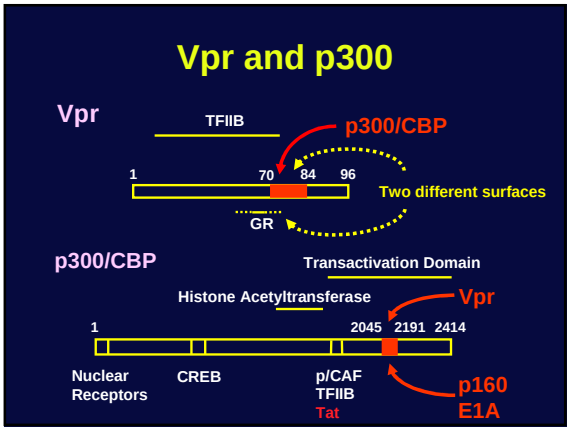
Interactions of the Stress and Immune Systems

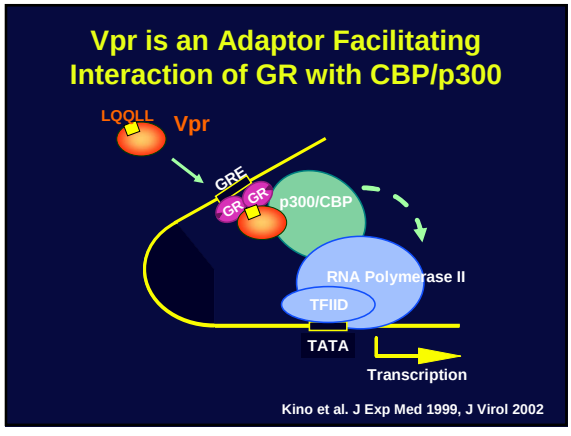

Cell Membrane
Kinases
Glucocorticoid
Bacterial endotoxins
Oxidants/Oxidized, denatured substances
Ischemia/Reperfusion Intracellular proteins
Inflammatory Cytokines
ICAM
Arachidonic acid
Nitric Oxide
Prostaglandins
GRα
GRα
p65
p50
I-κB
NF-κB
mRNA
Nucleus
TATA
Glucocorticoid Responsive Elements
IκB gene
κB binding site
Inflammatory cytokine genes
ICAM gene
i-NOS gene
COX2 gene
PLA2 gene

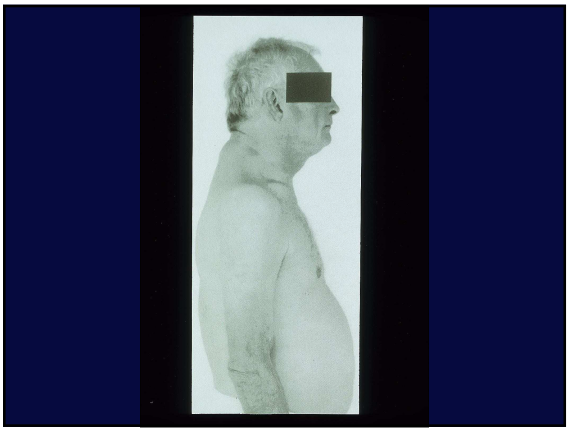

Inflammation
MODS
SIRS
Gram- Infection
Basal State
Injury, Bacterial invasion
Gram+ Infection
CARS
Antiinflammation
MODS
SIRS = Systemic Inflammatory Response Syndrome
MODS = Multiple Organ Dysfunction Syndrome
CARS = Counter-regulatory Response Syndrome

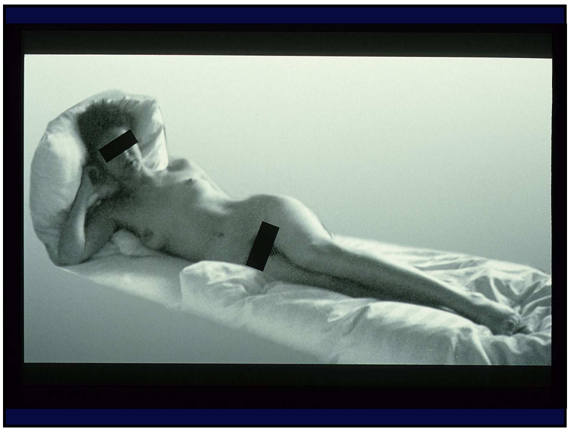

Plasma TNF-α (pg/ml)
Plasma IL-1β (pg/ml)
Plasma IL-6 (pg/ml)
Days
Randomized
+3
+5
+7
+10

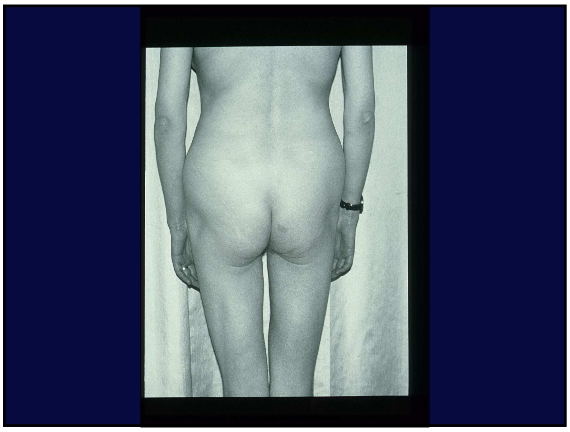

Nuclear NF-kB density
Nuclear p65 density
Nuclear p65 (pg/ml)
Days
Randomized
+3
+5
+7
+10

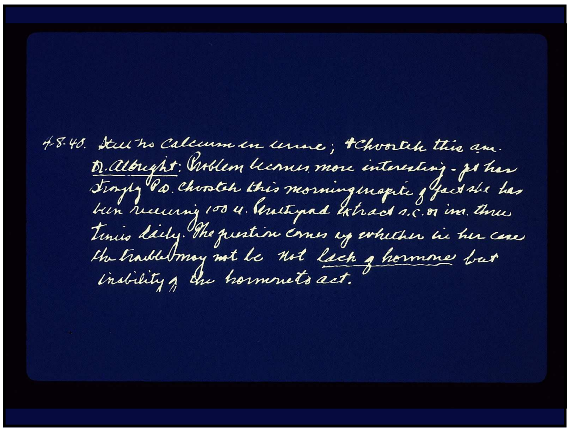

Vpr and p300
Vpr
TFIIB
p300/CBP
1
70
84
96
Two different surfaces
GR
p300/CBP
Transactivation Domain
Histone Acetyltransferase
Vpr
1
2045
2191
2414
Nuclear Receptors
CREB
p/CAF
TFIIB
Tat
p160
E1A

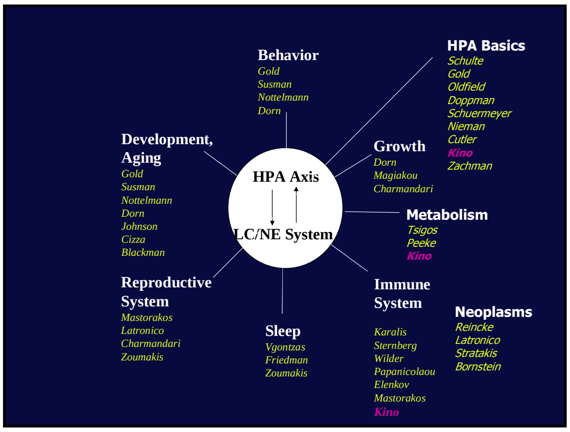

Vpr is an Adaptor Facilitating Interaction of GR with CBP/p300
LQQLL
Vpr
GRE
GR
GR
p300/CBP
RNA Polymerase II
TFIID
TATA
Transcription
Kino et al. J Exp Med 1999, J Virol 2002








4-8-40. Still no Calcium in urine; +Chvostek this a.m.
Dr. Albright: Problem becomes more interesting - pt has strongly P.O. Chvostek this morning inspite of fact she has been receiving 100 u. Parathyroid extract s.c. or im. three times daily. The question comes up whether in her case the trouble may not be not lack of hormone but inability of the hormone to act.


Behavior
Gold
Susman
Nottelmann
Dorn
HPA Basics
Schulte
Gold
Oldfield
Doppman
Schuermeyer
Nieman
Cutler
Kino
Zachman
Development, Aging
Gold
Susman
Nottelmann
Dorn
Johnson
Cizza
Blackman
HPA Axis
LC/NE System
Growth
Dorn
Magiakou
Charmandari
Metabolism
Tsigos
Peeke
Kino
Reproductive System
Mastorakos
Latronico
Charmandari
Zoumakis
Sleep
Vgontzas
Friedman
Zoumakis
Immune System
Karalis
Sternberg
Wilder
Papanicolaou
Elenkov
Mastorakos
Kino
Neoplasms
Reincke
Latronico
Stratakis
Bornstein

'Positive health requires a knowledge of man's primary constitution (which today we call genetics) and of the powers of various foods, both those natural to them and those resulting from human skill (today's processed food). But eating alone is not enough for health. There must also be exercise, of which the effects must likewise be known. The combination of these two things makes regimen, when proper attention is given to the season of the year, the changes of the winds, the age of the individual and the situation of his home. If there is any deficiency in food or exercise the body will fall sick.'

Hippocrates 480 BC

Change of Tissue Sensitivity to Glucocorticoids in Physiologic/Pathologic Conditions

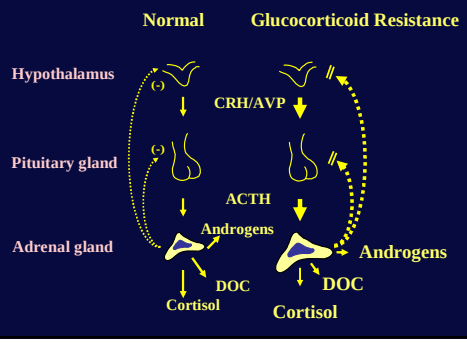
Resistance

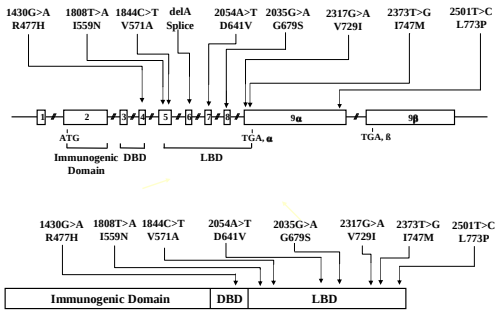
- Familial/Sporadic Glucocorticoid Resistance Syndrome
- Bronchial Asthma, Rheumatoid Arthritis, Osteoarthritis, Systemic Lupus Erythematosus, Ulcerative Colitis
- Acute Respiratory Distress Syndrome/Sepsis
  Adenoviral Infection (E1A), Anthrax Infection

Hypersensitivity

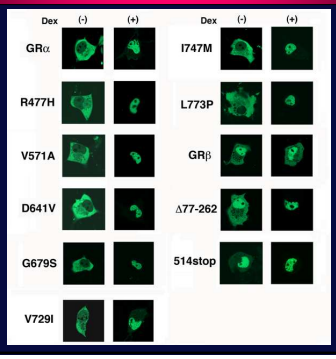
- Visceral-type Obesity-related Hyperlipidemia, Insulin Resistance and Hypertension
- AIDS (Vpr, Tat)
  Rare Generalized Glucocorticoid Hypersensitivity Syndrome

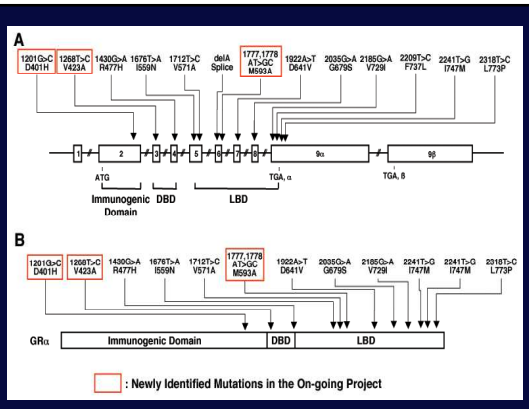
Alterations in HPA Axis Activity

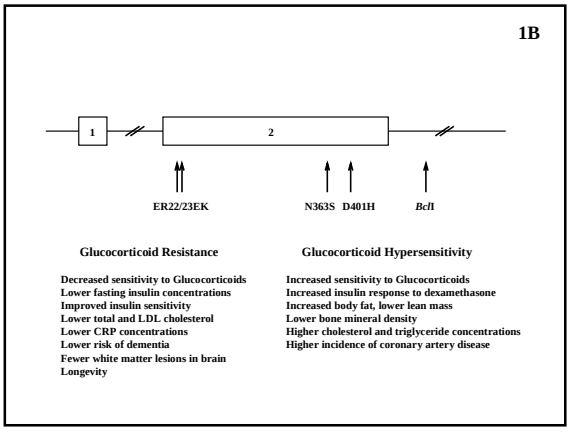


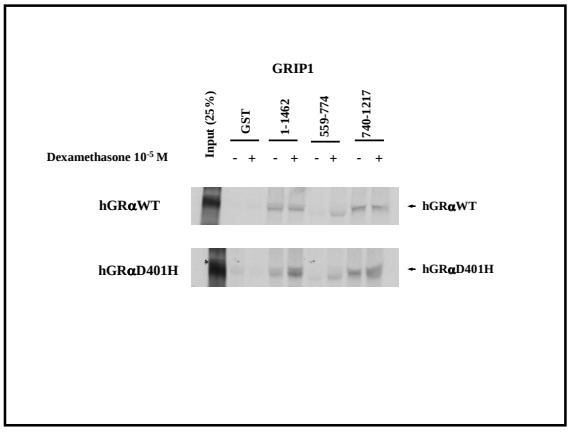


Subcellular Localization of GFP-GRs Employed





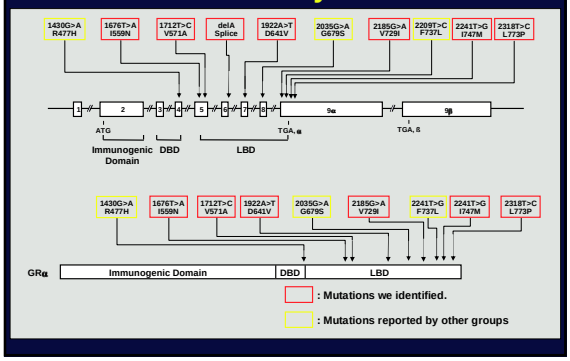


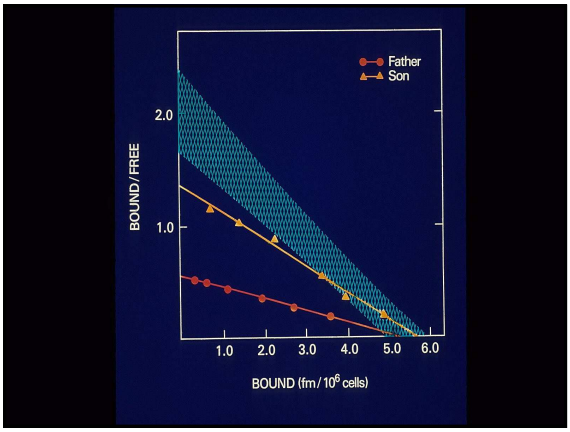


Clinical Manifestations Associated with Changes in Tissue Sensitivity to Glucocorticoids

	Glucocorticoid Excess	Glucocorticoid Deficiency
AFFECTED AREA	GLUCOCORTICOID HYPERSENSITIVITY	GLUCOCORTICOID RESISTANCE
Central Nervous System	Insomnia, Anxiety, Depression, Defective Cognition	Fatigue, Somnolence, Malaise, Depression, Defective Cognition
Liver	Increased Gluconeogenesis	Hypoglycemia, Increase Insulin Sensitivity
Fat	Accumulation of Visceral Fat (Metabolic Syndrome)	Loss of Weight
Muscle	Insulin Resistance	Increased Insulin Sensitivity
Blood Vessels	Hypertension	Hypotension
Bone	Osteoporosis	
Inflammation/Immunity	Immune Suppression, Suppressed Inflammation	Increased Inflammation/ Autoimmunity

Familial/Sporadic Glucocorticoid Resistance Syndrome





The “master” CLOCK adjusts circadian rhythm of the “slave” CLOCKs through still incompletely understood neuronal and humoral connections.

- Perturbation of either the CLOCK system or the HPA axis means perturbation of the other.
- Perturbation of either system leads to similar metabolic and immune pathologies