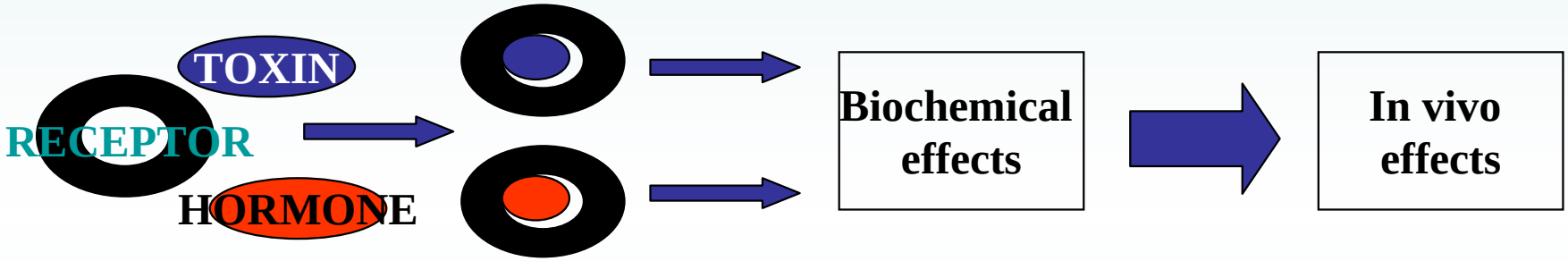
INTRACELLULAR RECEPTORS

MECHANISMS of chronic toxicity

- **Various chronic effects have uniform biochemical basis**



2 Types of Receptors

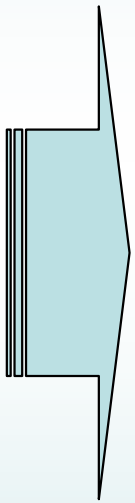
- Intracellular
- Cell Surface

INTRACELLULAR RECEPTORS
(for lipid soluble messengers) function in the nucleus as transcription factors to alter the rate of transcription of particular genes

- ligand-activated transcription factors
- crucial role in cell signaling
- activation of different responsive elements (genes)

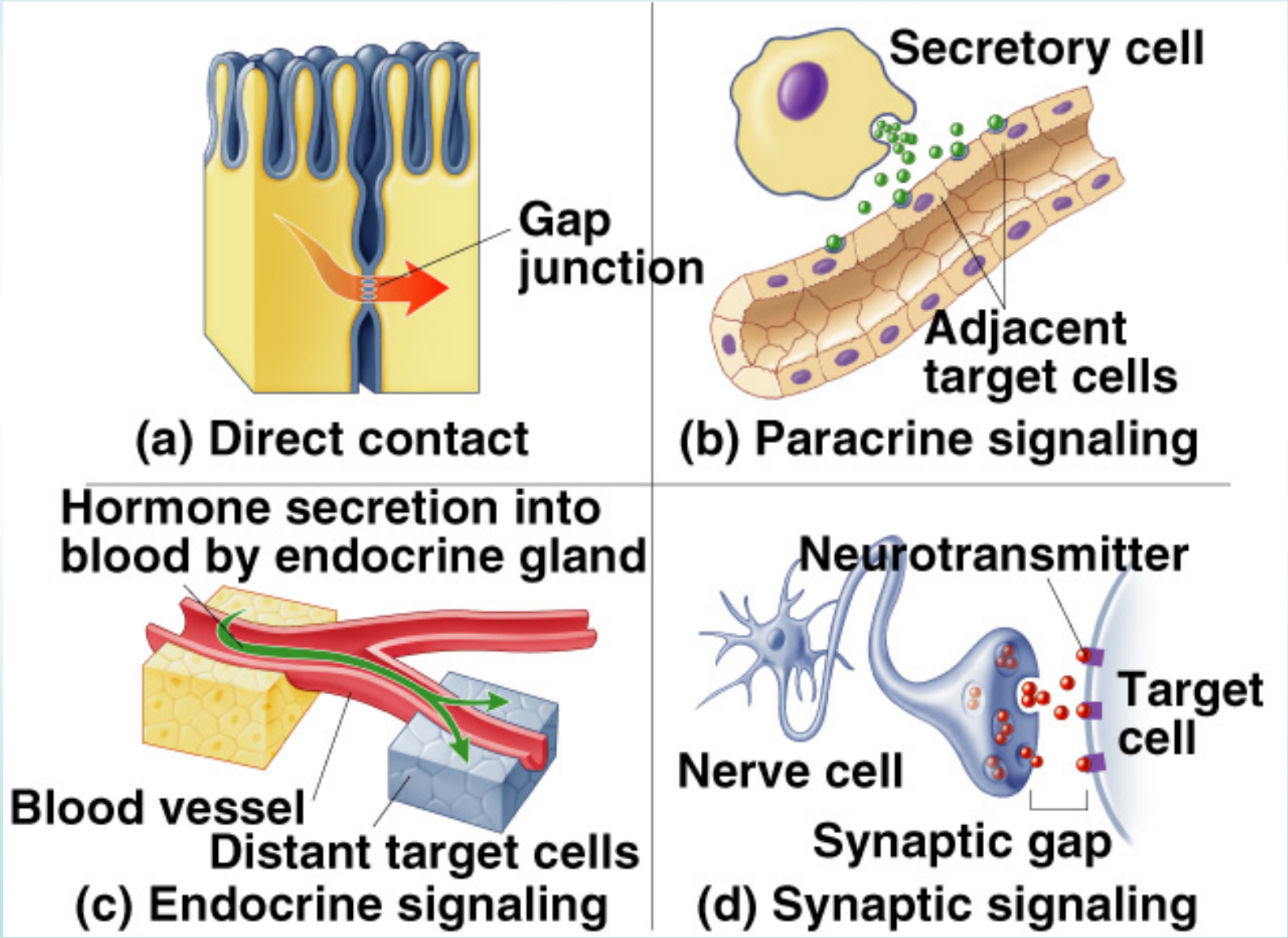
SINGLE mechanism -> SEVERAL effects
=> understanding to mechanisms may predict effects

Estrogen receptor activation



1) **female reproduction disorders**
2) **male feminisation**
3) **tumor promotion**
4) **immunomodulations**
5) **developmental toxicity**

Types of signaling in multicellular organisms



Modes of cell-cell signaling

1. **Direct** cell-cell or cell-matrix

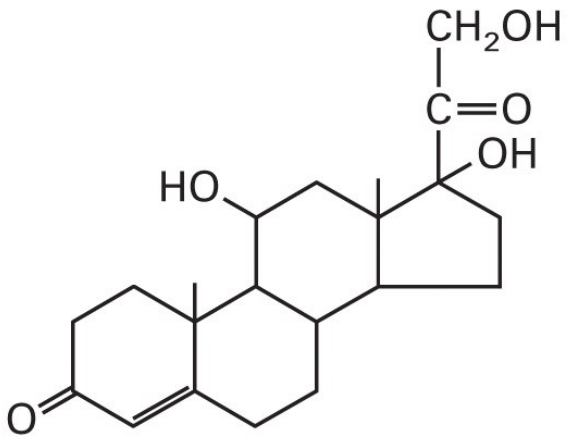
2. **Secreted molecules**.

A. Endocrine signaling. The signaling molecules are hormones secreted by endocrine cells and carried through the circulation system to act on target cells at distant body sites.

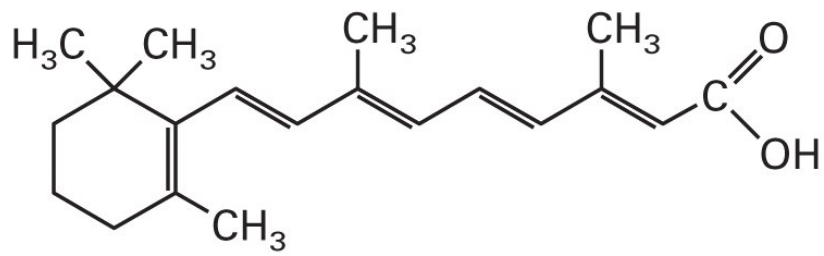
B. Paracrine signaling. The signaling molecules released by one cell act on neighboring target cells.

C. Autocrine signaling. Cells respond to signaling molecules that they themselves produce (response of the immune system to foreign antigens, and cancer cells).

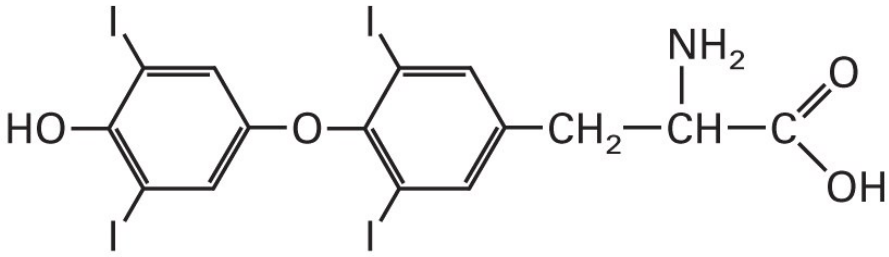
- Intracellular signal molecules are small, lipid-soluble molecules such as steroid hormones, retinoids, thyroid hormones, Vitamin D. (made from cholesterol)
- These molecules diffuse through plasma and nuclear membranes and interact directly with the transcription factors they control.



Cortisol



Retinoic acid



Thyroxine

The intracellular (nuclear) receptor superfamily

Steroid hormones, thyroid hormones, retinoids and vitamin D

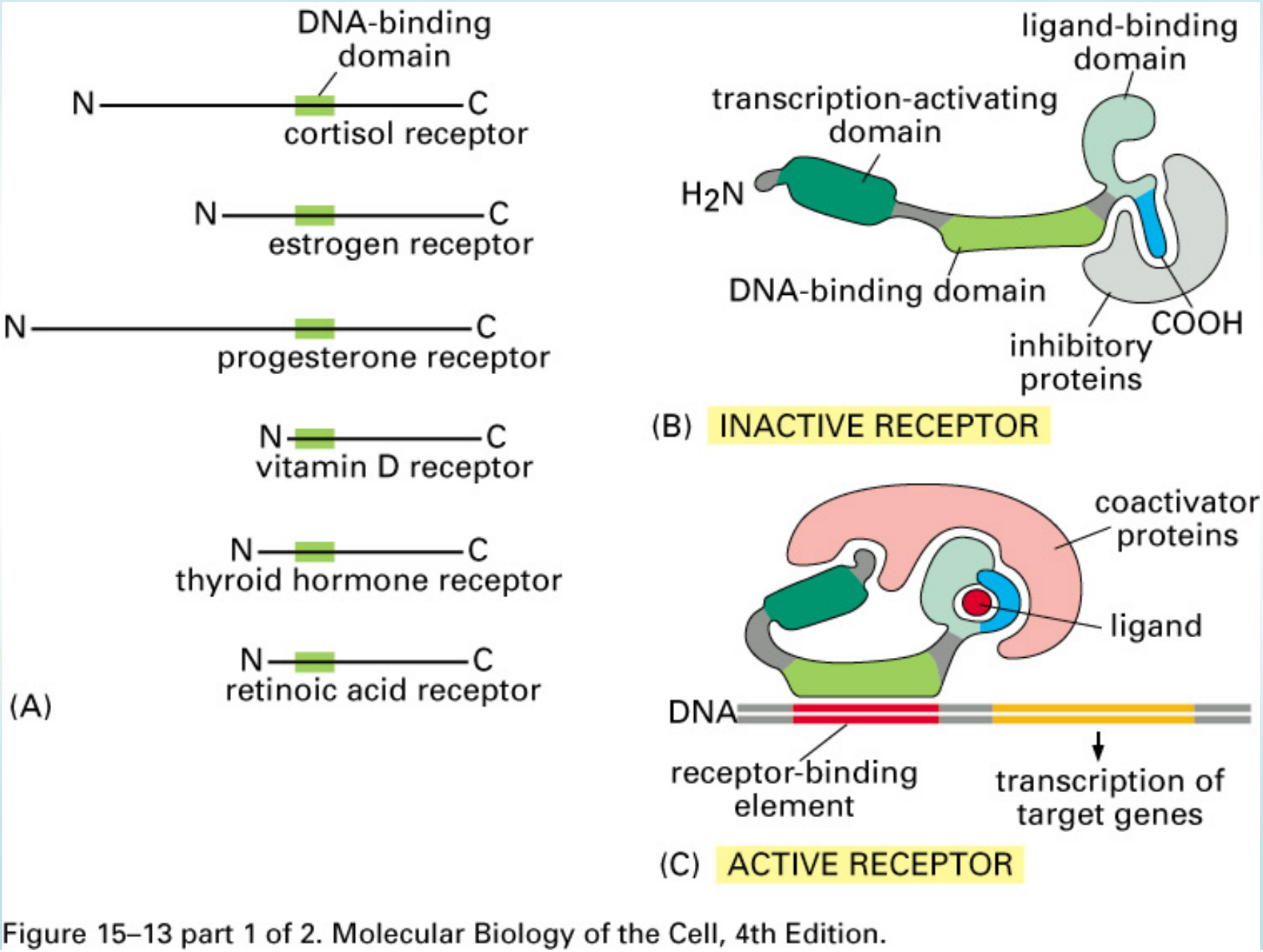
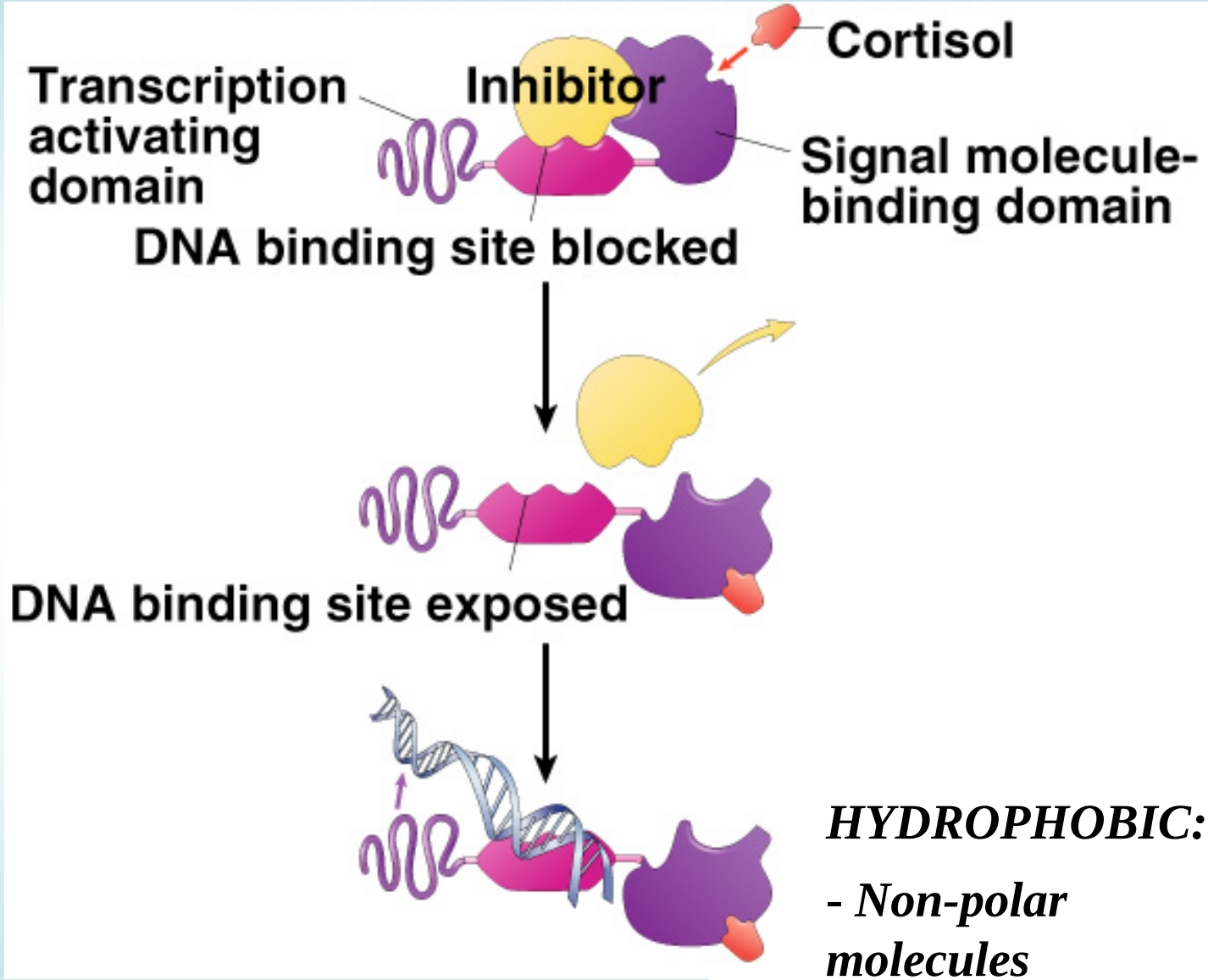


Figure 15–13 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Intracellular receptor

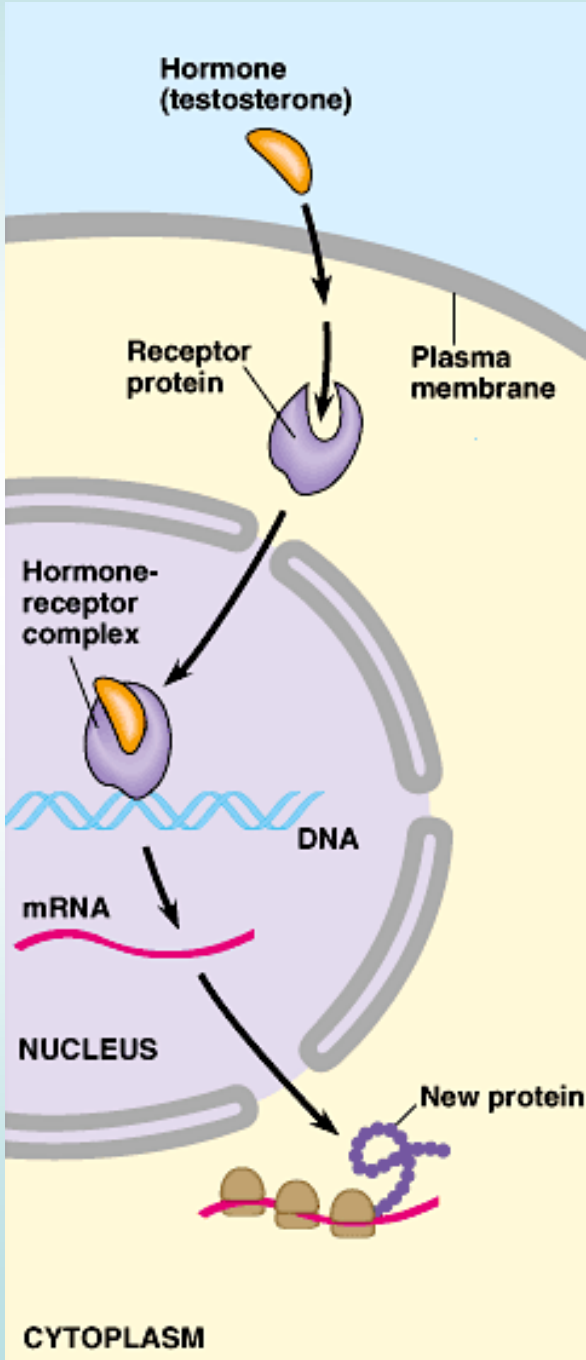


HYDROPHOBIC:

- Non-polar molecules

- Gases

- Steroids



Intracellular Receptors

- alter gene expression

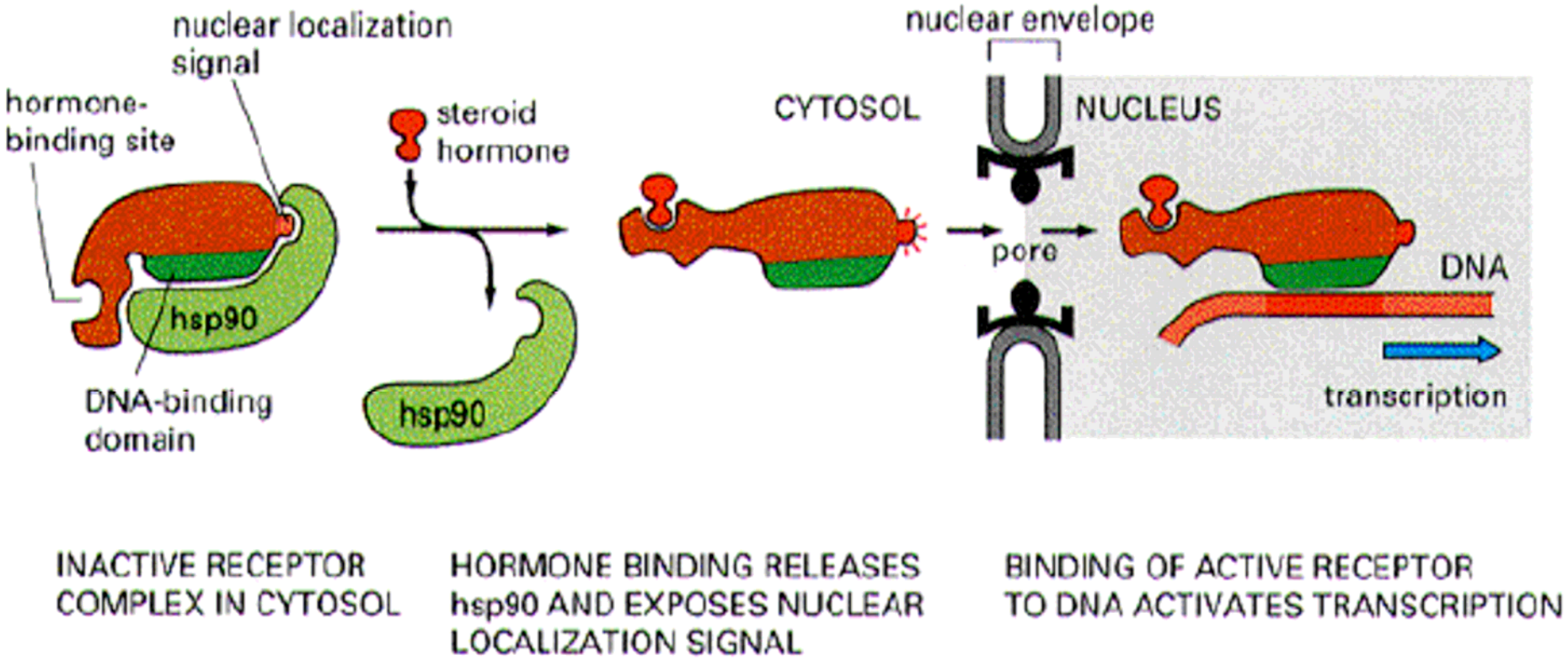
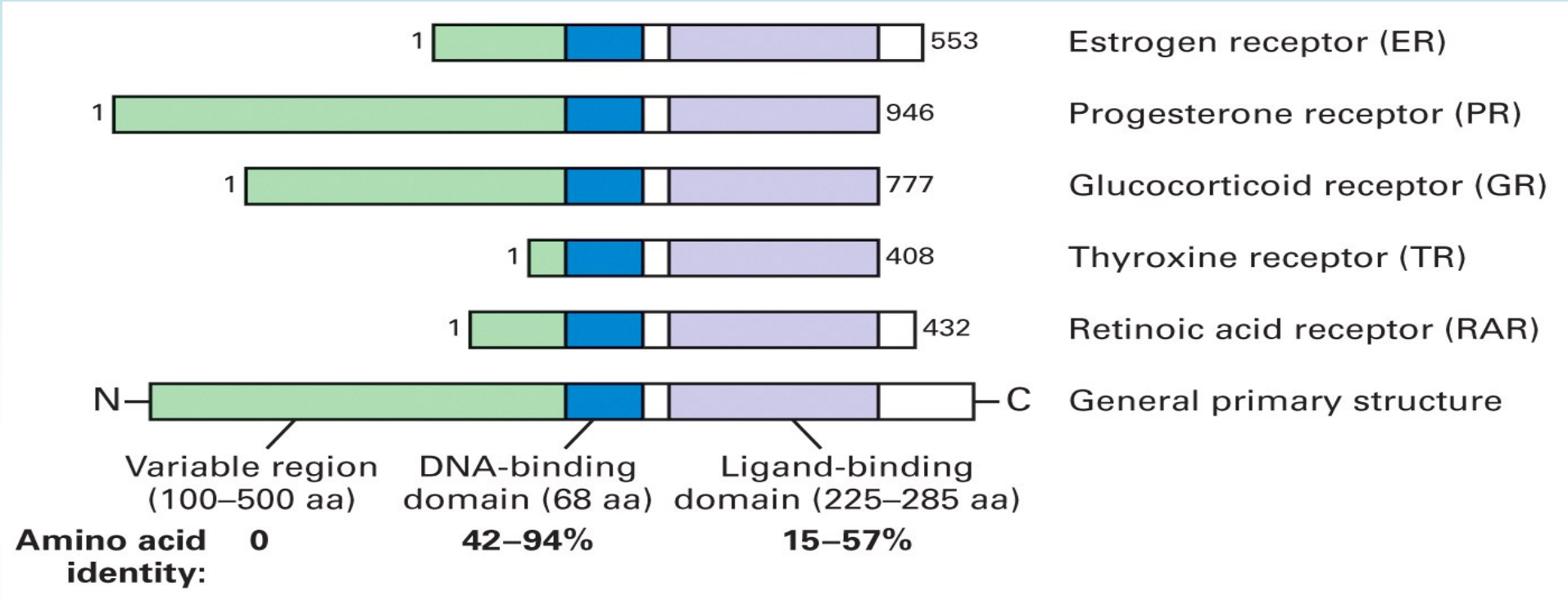


Figure 12-19, Page 462 from: *Molecular Biology of the Cell* by Alberts et al. 1994, Garland Publishing Inc. New York, NY

Sequence similarities and three functional regions



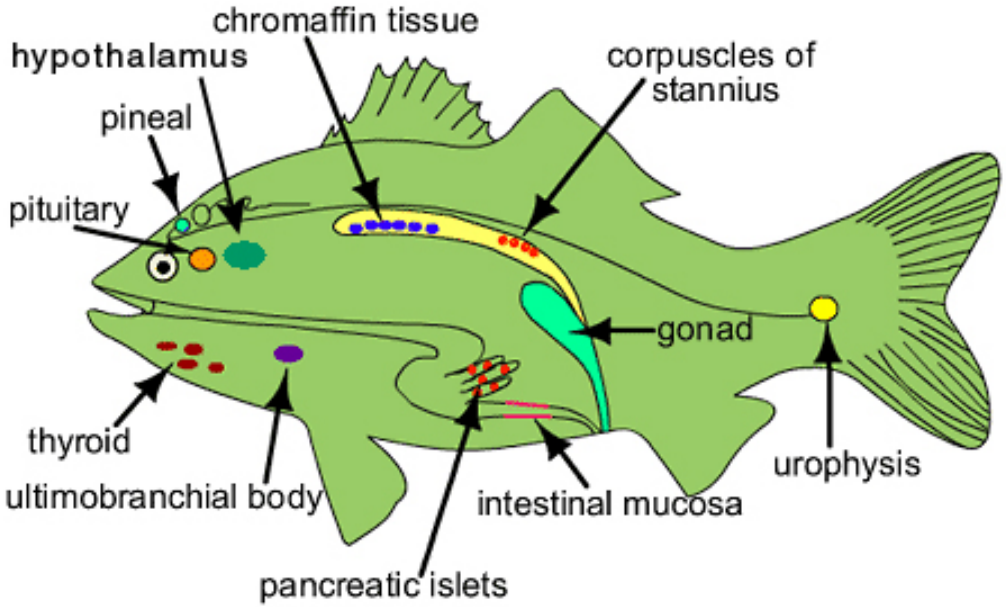
- N-terminal region of variable length; in some receptors portions of this region act as activation domain
- At the center, **DNA binding domain**, made of a repeat of C_4-zinc finger motif
- Near the C-terminal end, **hormone binding domain**, which may act as an activation or repression domain.

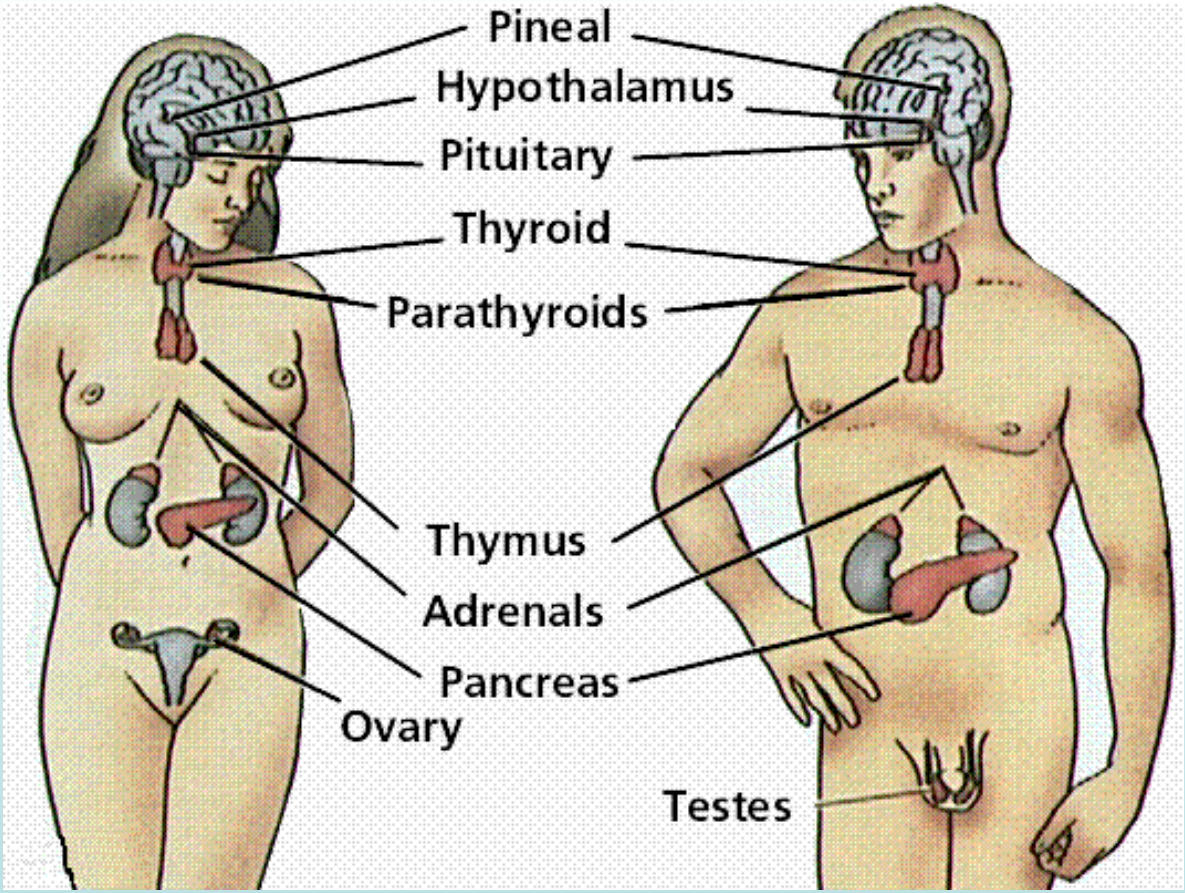
- Steroid hormones are often required to dimerize with a partner to activate gene transcription
- Receptors for vitamin D, retinoic acid and thyroid hormone bind to responsive elements as heterodimers
- Second component of the heterdimer is RXR monomer (i.e, RXR-RAR; RXR-VDR)

Regulation of transcription activity

- Regulatory mechanisms differ for hetero-dimeric and homodimeric receptors
- Heterodimeric receptors are exclusively nuclear; without ligand, they repress transcription by binding to their cognate sites in DNA
- Homodimeric receptors are mostly cytoplasmic in the absence of ligands
- Hormone binding leads to nuclear translocation of receptors
- Absence of hormone causes the aggregation of receptor as a complex with inhibitor proteins, such as Hsp90

Endocrine System





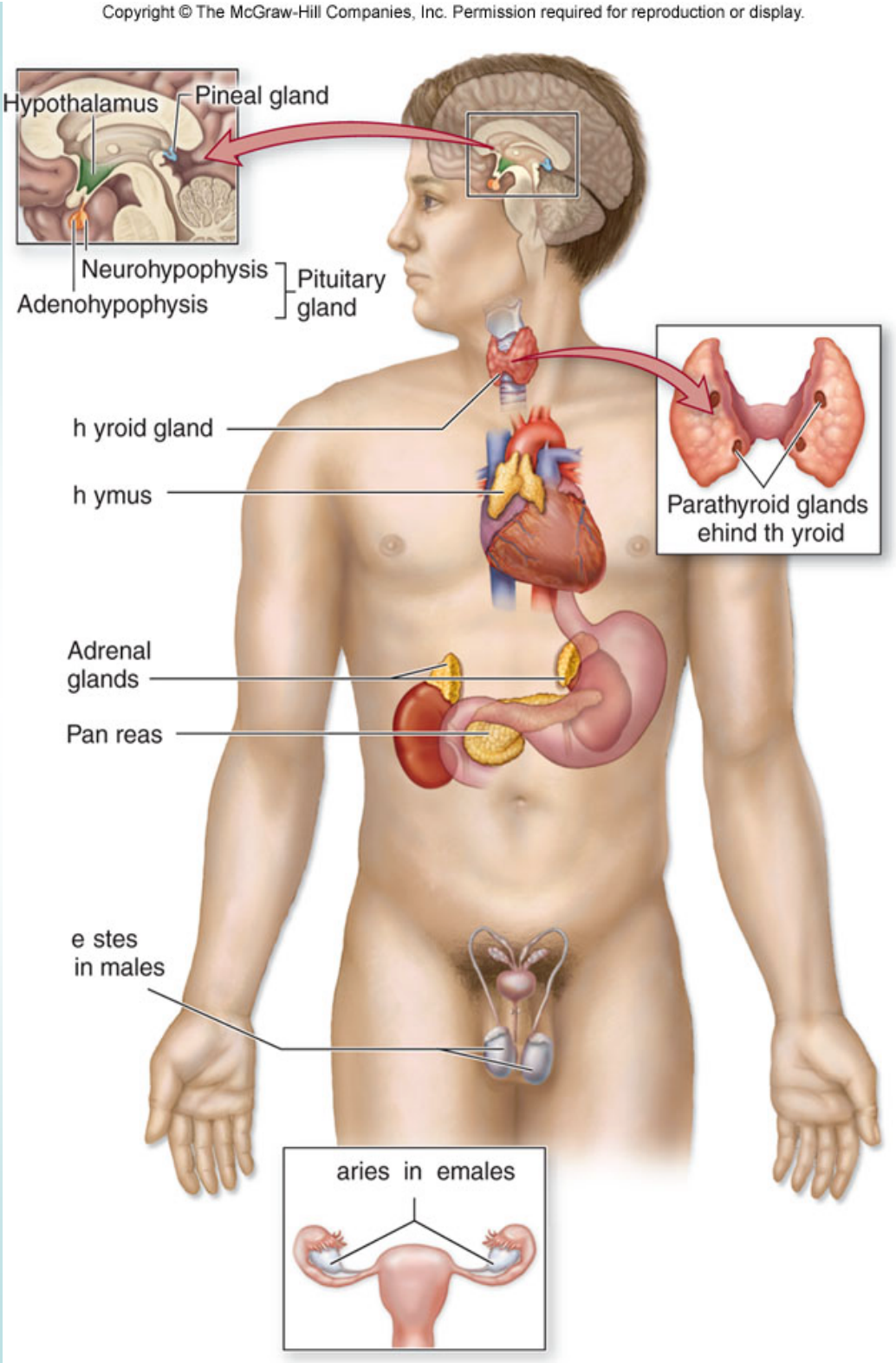
Endocrine System

The **endocrine system** includes all the organs and tissues that produce hormones

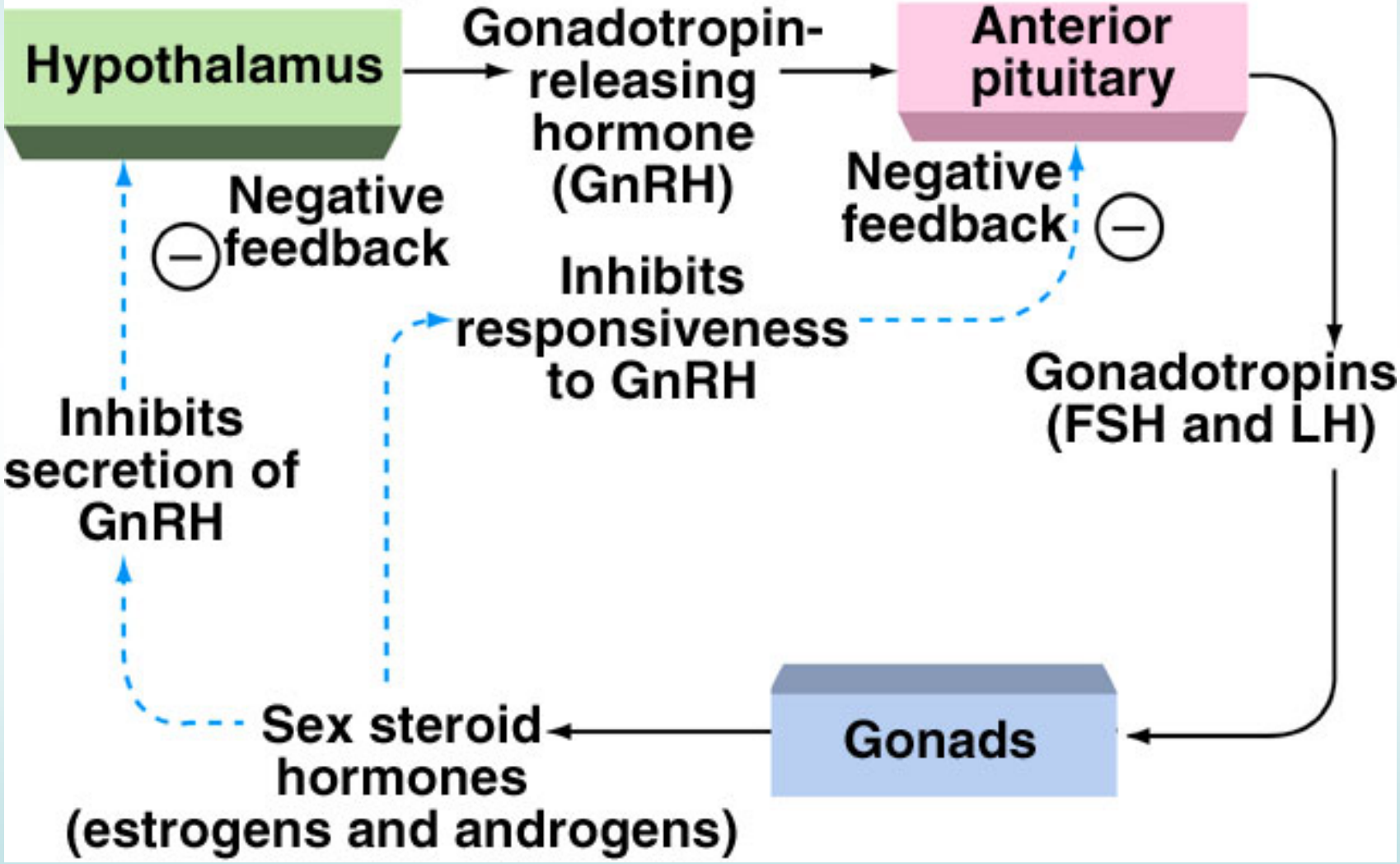
- Includes **endocrine glands**, which are specialized to secrete hormones
- Also organs, like the liver, that secrete hormones in addition to other functions

A **hormone** is a chemical that is secreted into extracellular fluid and carried by the blood

- can therefore act at a distance from source
- only targets with receptor can respond



Hypothalamus-pituitary-gonad Axis (Control System)



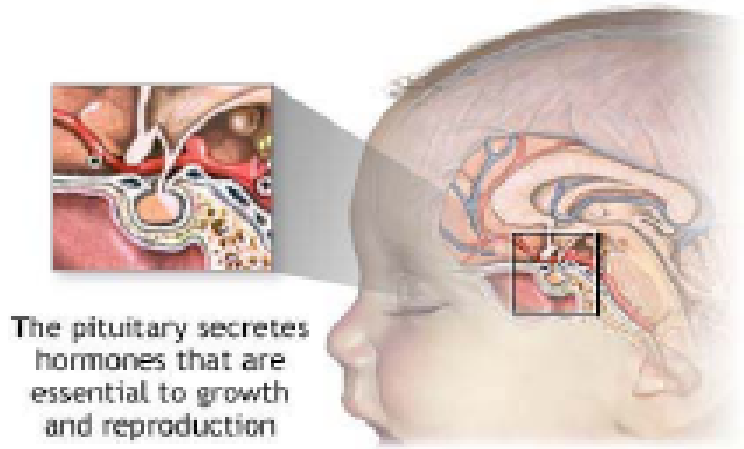
Steroid hormones synthesis - upstream signals

Luteinizing Hormone (LH) - stimulates progesterone and testosterone

Adrenocorticotropic hormone (ACTH) - stimulates cortisol

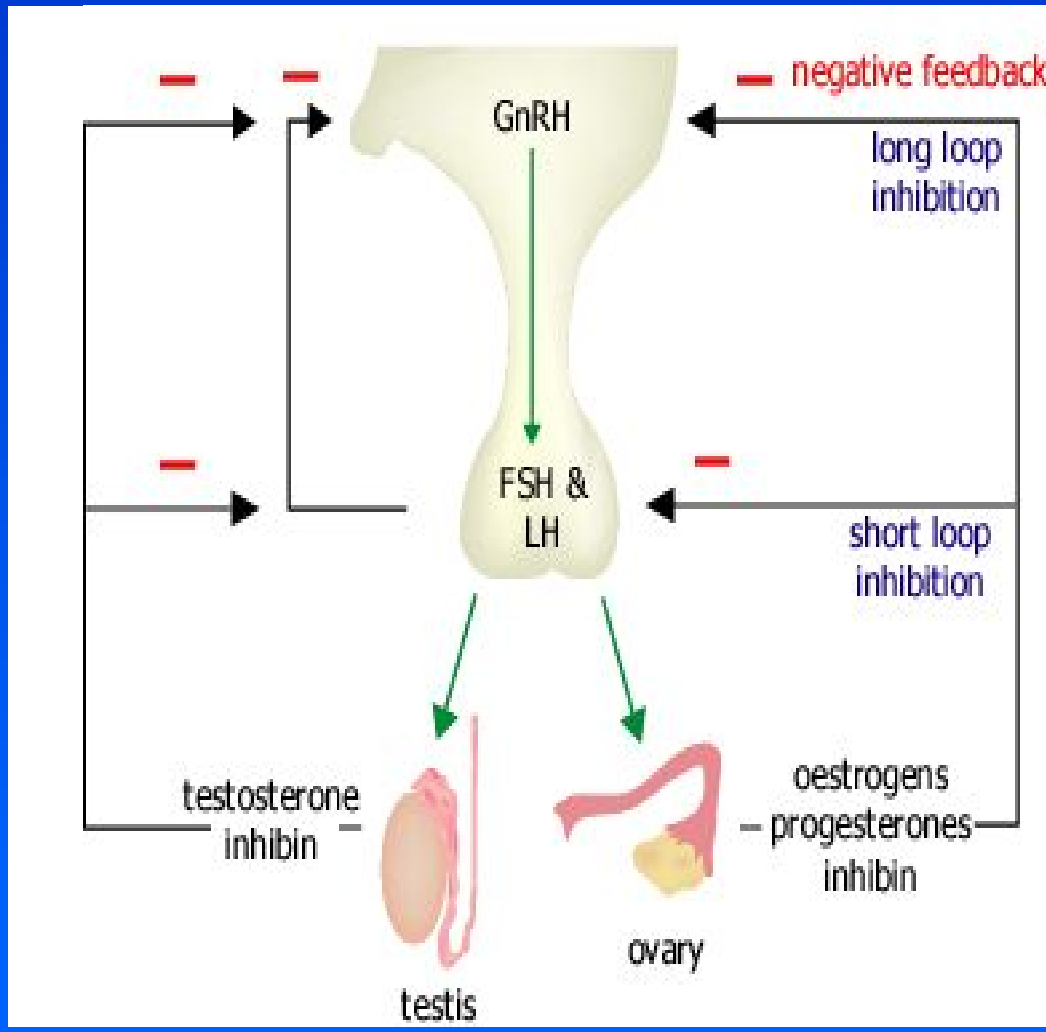
Follicle Stimulating Hormone (FSH) - stimulates estradiol

Angiotensin - stimulates aldosterone



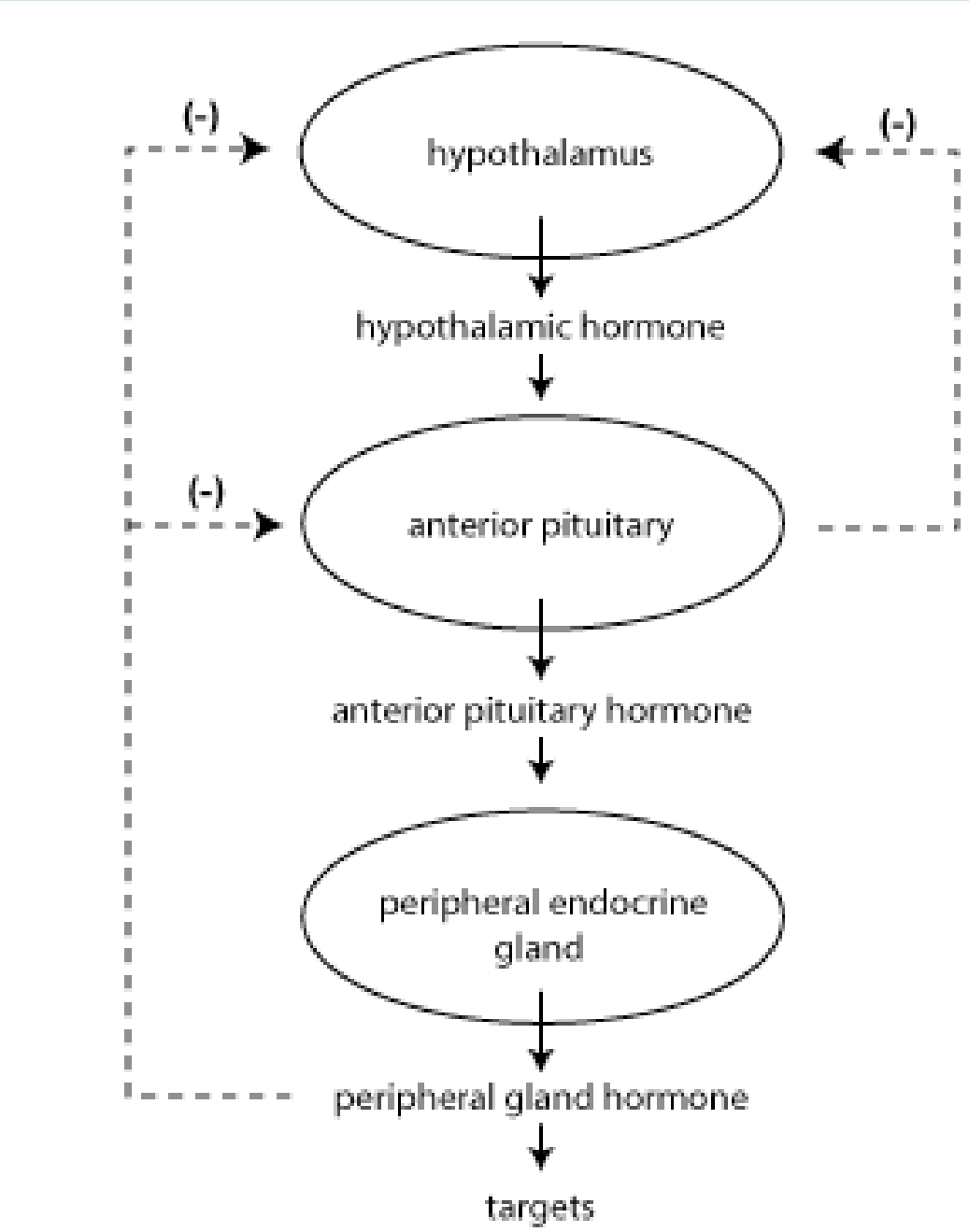
Hypothalamo-pituitary axis

- Regulation of hormone synthesis
- Hypothalamus – Gonadotropin releasing hormone (GnRH)
- Pituitary – folicle stimulating (FSH) and luteineising hormone (LH)



Feedback Mechanisms

- For hormone secretion regulated by the negative feedback loop: when gland X releases hormone X, this stimulates target cells to release hormone Y. When there is an excess of hormone Y, gland X "senses" this and inhibits its release of hormone X.

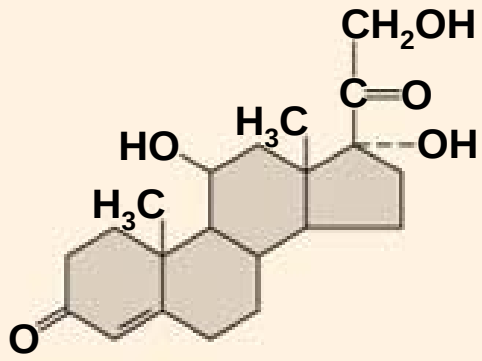


Lipophilic Hormones

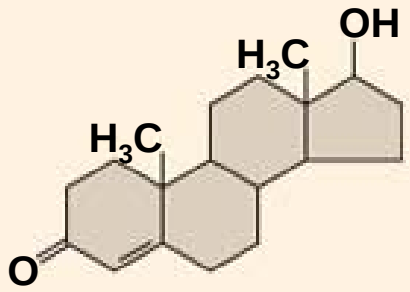
Lipophilic hormones include the steroid hormones (derived from cholesterol) and the thyroid hormones (tyrosine + iodine)

-As well as the retinoids, or vitamin A

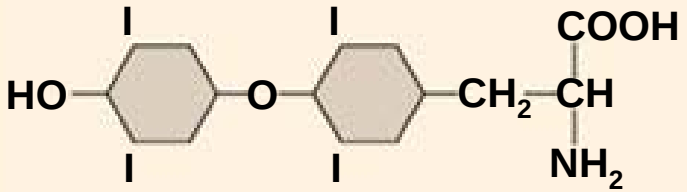
Cortisol (Hydrocortisone)



Testosterone



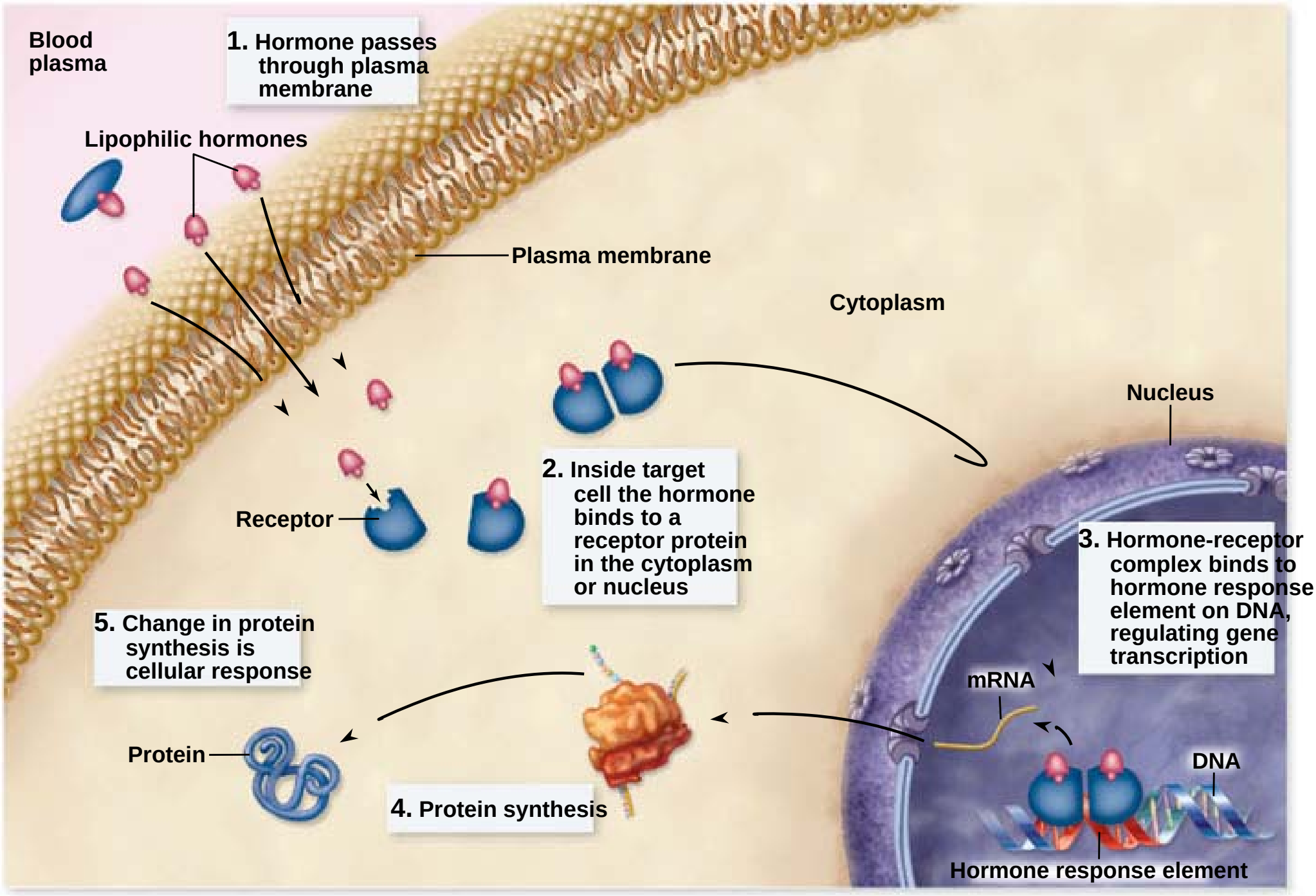
Thyroxine



Lipophilic Hormones

These hormones circulate in the blood bound to transport proteins

- Dissociate from carrier at target cells
- Pass through the cell membrane and bind to an intracellular receptor, either in the cytoplasm or the nucleus
- Hormone-receptor complex binds to **hormone response elements** in DNA
- Regulate gene expression

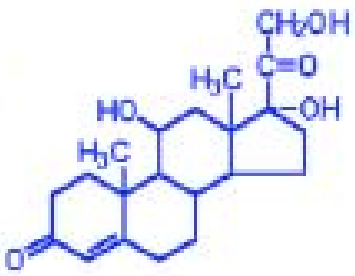


Steroid hormones - a review

Steroid hormones are derived from cholesterol metabolism in mitochondria

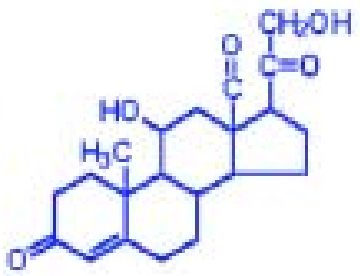
Cortisol

The dominant glucocorticoid in humans. Synthesized from progesterone in the *zona fasciculata* of the adrenal cortex. Involved in stress adaptation, elevates blood pressure and Na^+ uptake. Immunomodulation.



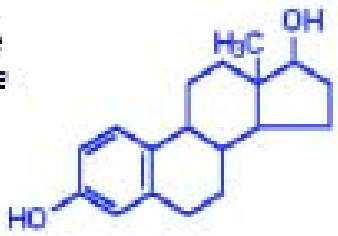
Aldosterone

Principal mineralocorticoid. Produced from progesterone in the *zona glomerulosa* of adrenal cortex, raises blood pressure and fluid volume, increases Na^+ uptake.



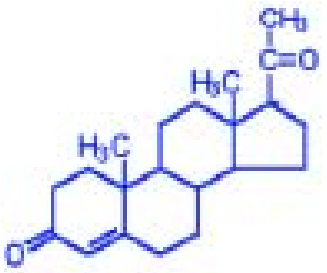
Estradiol

An estrogen, principal female sex hormone, produced in the ovary, responsible for secondary female sex characteristics. After menopause estrogen is produced from testosterone in the adrenal glands.



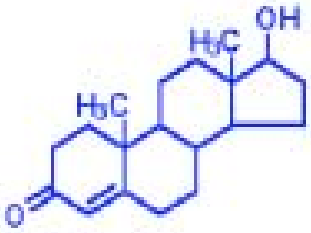
Progesterone

Produced from pregnenolone and secreted from the corpus luteum. Responsible for changes associated with luteral phase of the menstrual cycle, differentiation factor for mammary glands



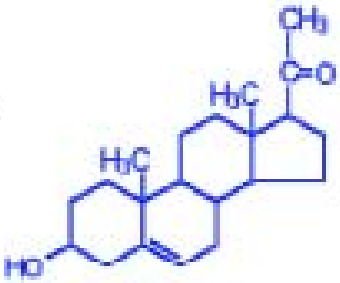
Testosterone

An androgen, male sex hormone synthesized in the testes from progesterone. Responsible for secondary male sex characteristics.



Pregnenolone

Made directly from cholesterol, the precusor molecule for all C_{18}, C_{19} and C_{21} steroids

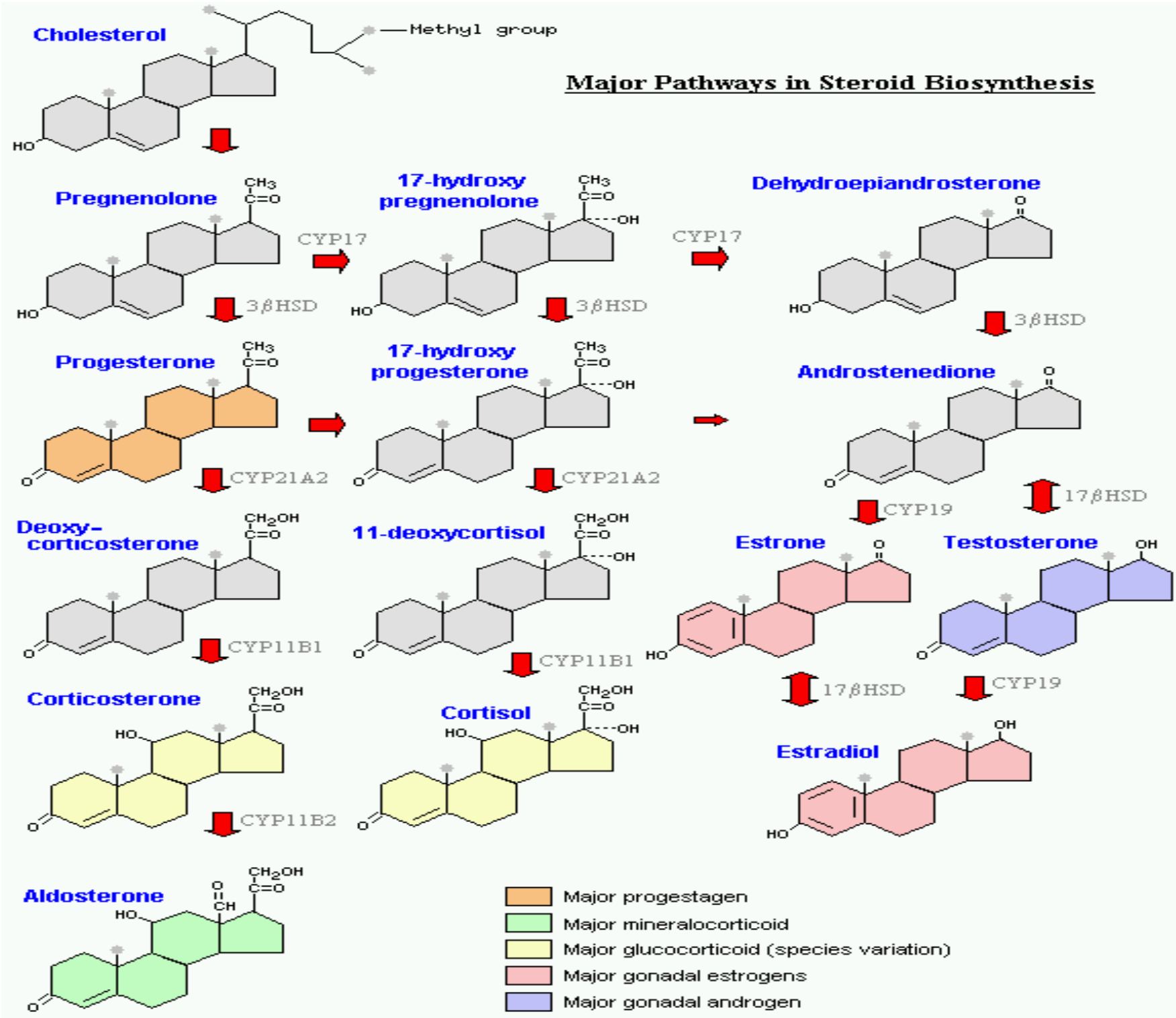


Steroid Hormones

They include sex steroids (estrogen, progesterone, testosterone)
corticosteroids (glucocorticoids and mineralcorticoids)

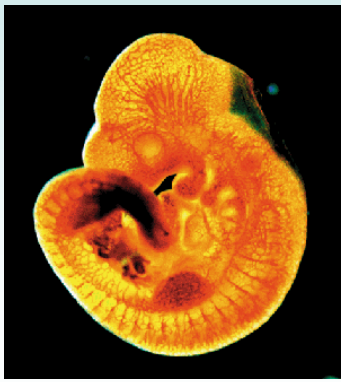
Thyroid hormone, vitamin D3, and retinoic acid have different structure and function but share the same mechanism of action with the other steroids.

- Steroid hormones and thyroid hormone diffuse easily into their target cells
- Once inside, they bind and activate a specific intracellular receptor
- The hormone-receptor complex travels to the nucleus and binds a DNA-associated receptor protein
- This interaction prompts DNA transcription to produce mRNA
- The mRNA is translated into proteins, which bring about a cellular effect


Cholesterol
Methyl group
Major Pathways in Steroid Biosynthesis
Pregnenolone
CYP17
17-hydroxy pregnenolone
CYP17
Dehydroepiandrosterone
3βHSD
3βHSD
3βHSD
Progesterone
17-hydroxy progesterone
Androstenedione
CYP21A2
CYP21A2
17βHSD
CYP19
Deoxy-corticosterone
11-deoxycortisol
Estrone
Testosterone
CYP11B1
CYP11B1
17βHSD
CYP19
Corticosterone
Cortisol
Estradiol
CYP11B2
Aldosterone
Major progestagen
Major mineralocorticoid
Major glucocorticoid (species variation)
Major gonadal estrogens
Major gonadal androgen

Endocrine disruption

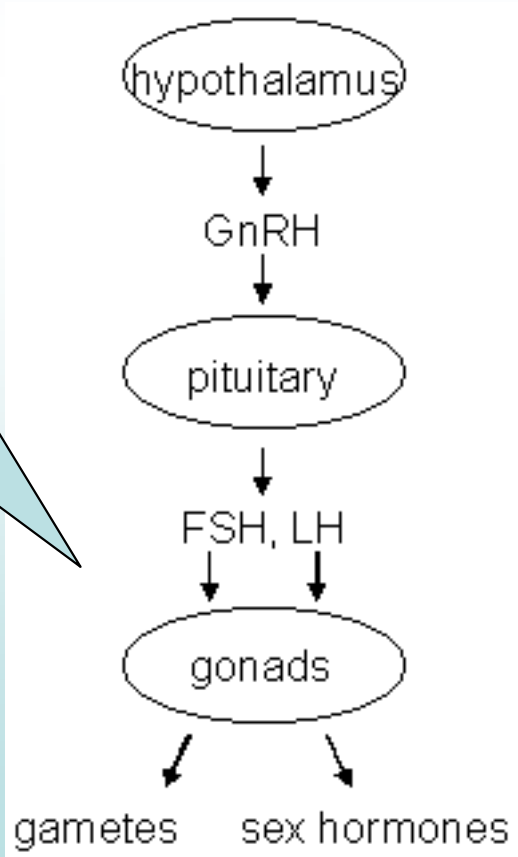
- Interference of xenobiotics with normal function of hormonal system



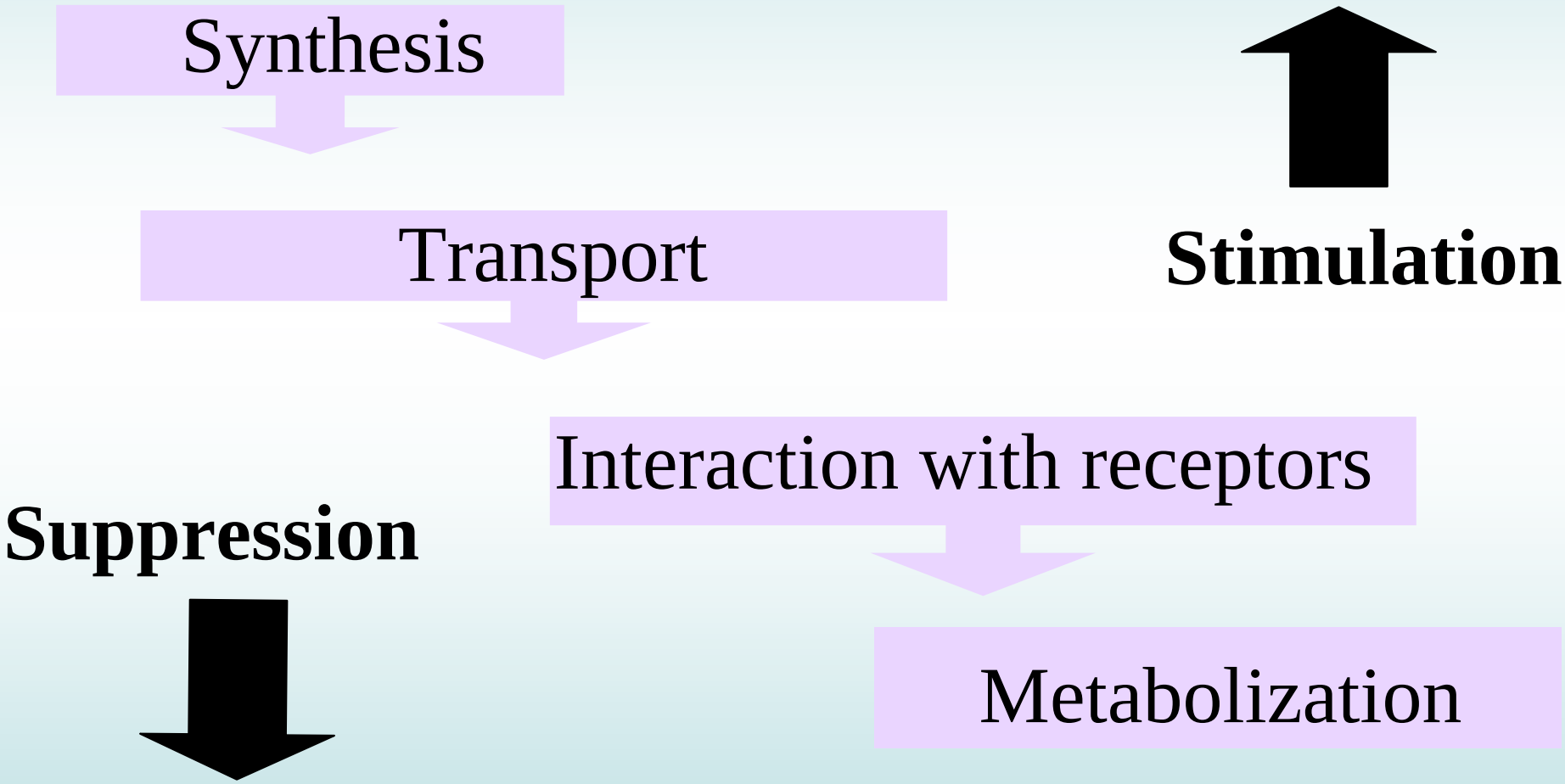
Possible consequences:

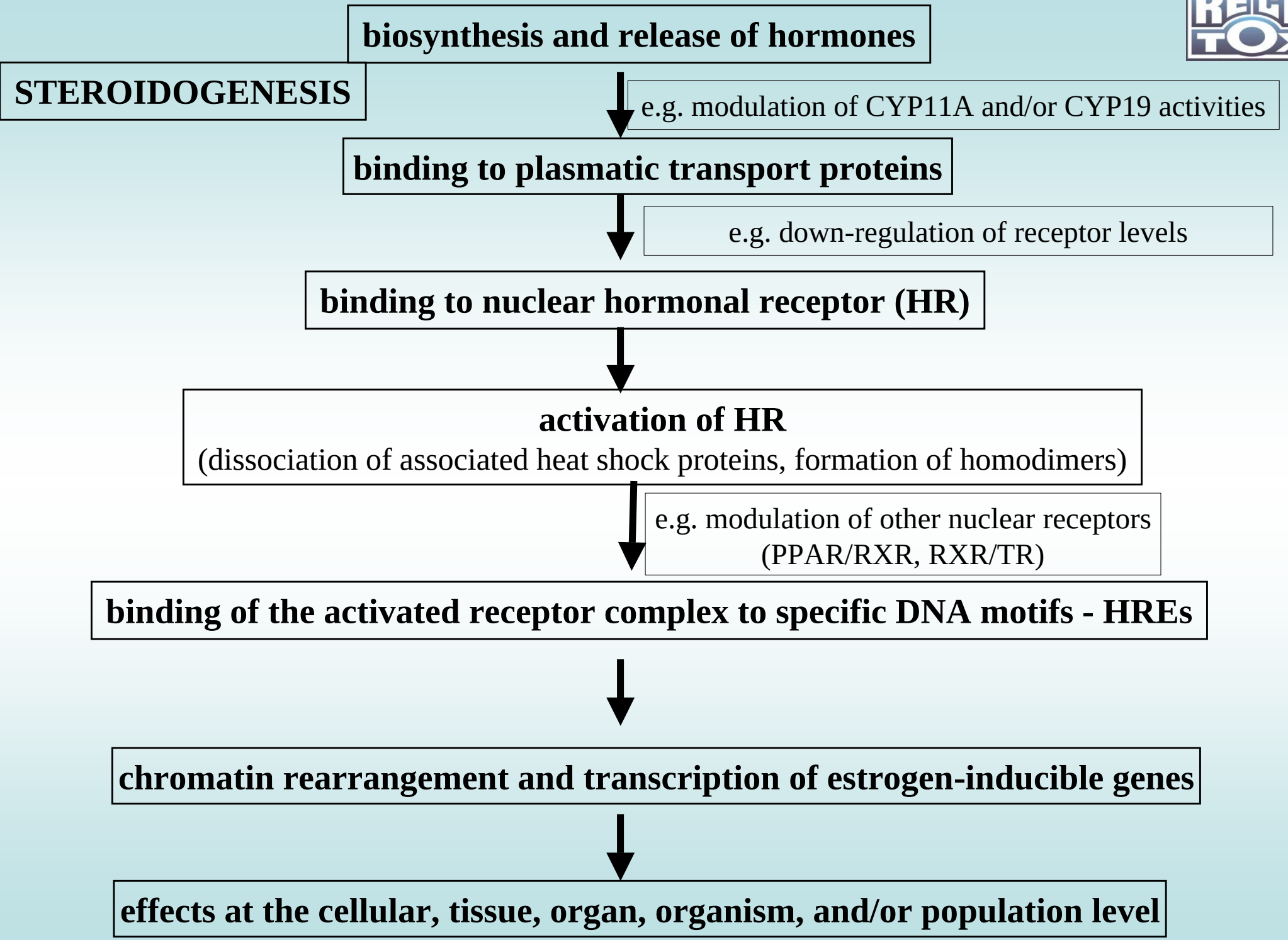
Disruption of homeostasis, reproduction, development, and/or behavior.

- Shift in sex ratio, defective sexual development
- Low fecundity/fertility
- Hypo-immunity, carcinogenesis
- Malformations



Interaction with hormone system




biosynthesis and release of hormones
STEROIDOGENESIS
e.g. modulation of CYP11A and/or CYP19 activities
binding to plasmatic transport proteins
e.g. down-regulation of receptor levels
binding to nuclear hormonal receptor (HR)
activation of HR
(dissociation of associated heat shock proteins, formation of homodimers)
e.g. modulation of other nuclear receptors
(PPAR/RXR, RXR/TR)
binding of the activated receptor complex to specific DNA motifs - HREs
chromatin rearrangement and transcription of estrogen-inducible genes
effects at the cellular, tissue, organ, organism, and/or population level

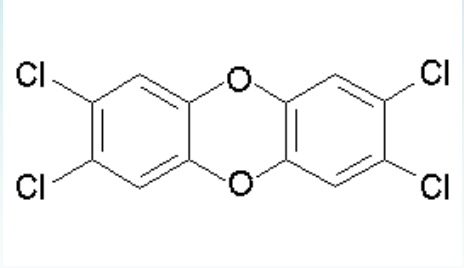
Mechanisms of **steroid hormones signalling disruption**

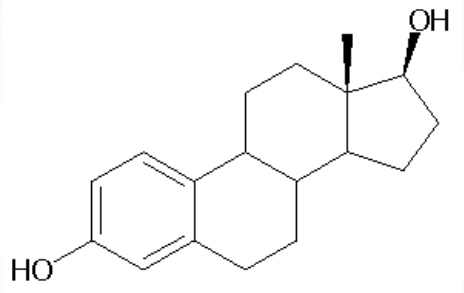
- Illegitimate activation of hormonal receptor (HR)
- Binding to HR without activation
- Decrease of HR cellular levels
- FSH/LH signalling disruption
- Changes in hormone metabolism

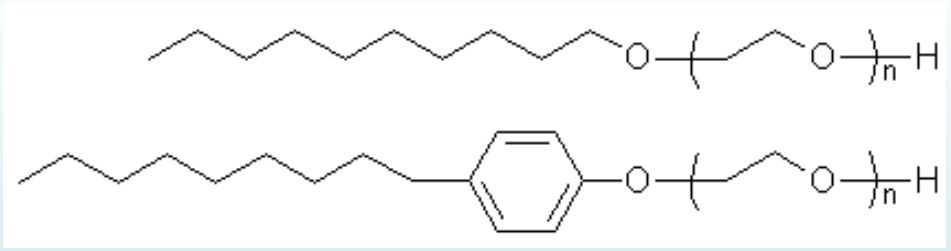
Endocrine disrupters in the environment?

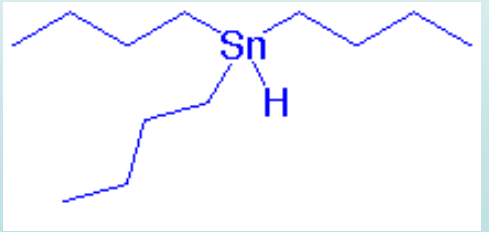
EDCs…

- POPs and their metabolites
- steroid hormones and their derivatives from contraception pills
- alkylphenols
- organometallics (butyltins)
- pharmaceuticals
- pesticides

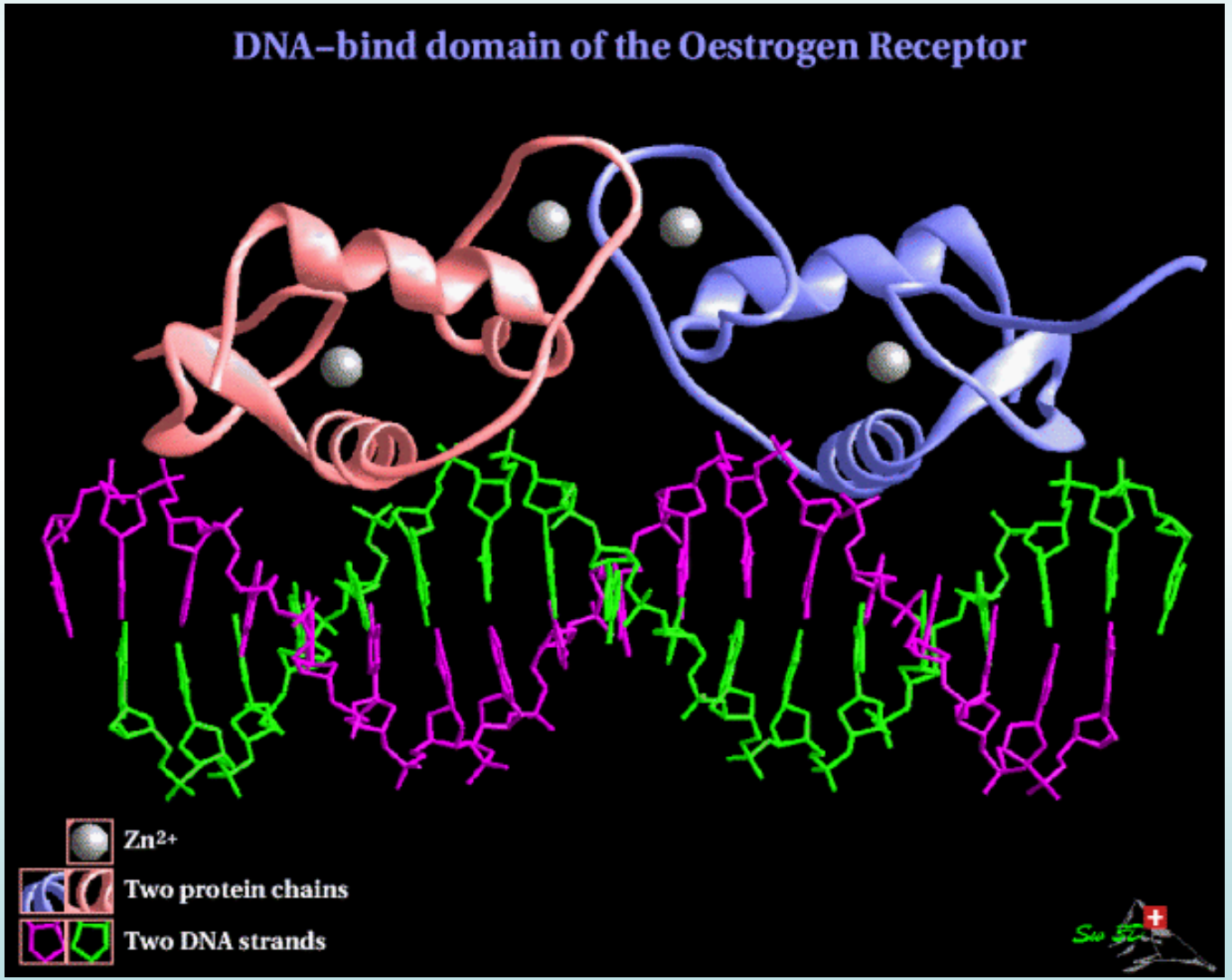




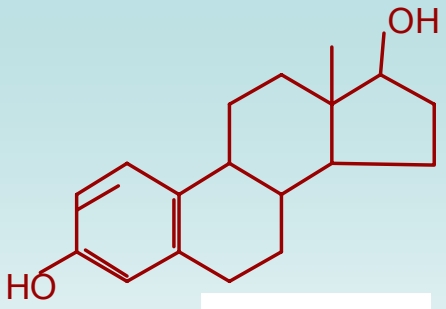




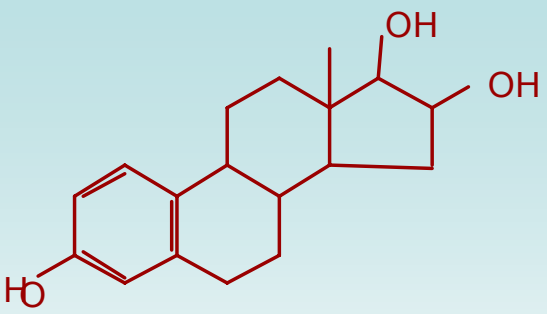
ESTROGEN RECEPTOR - ER



Estrogens:



17-β-estradiol



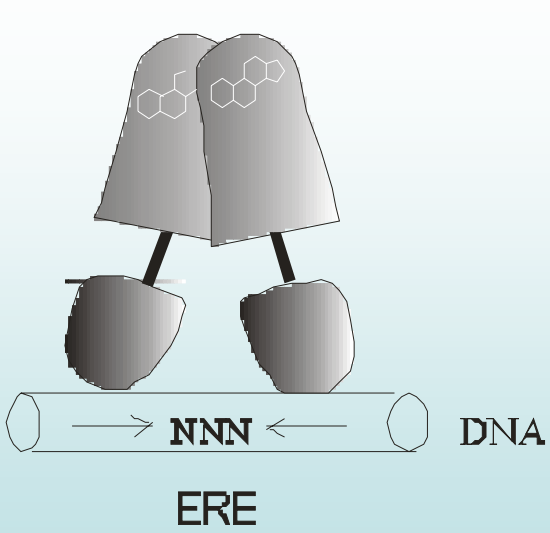
estriol

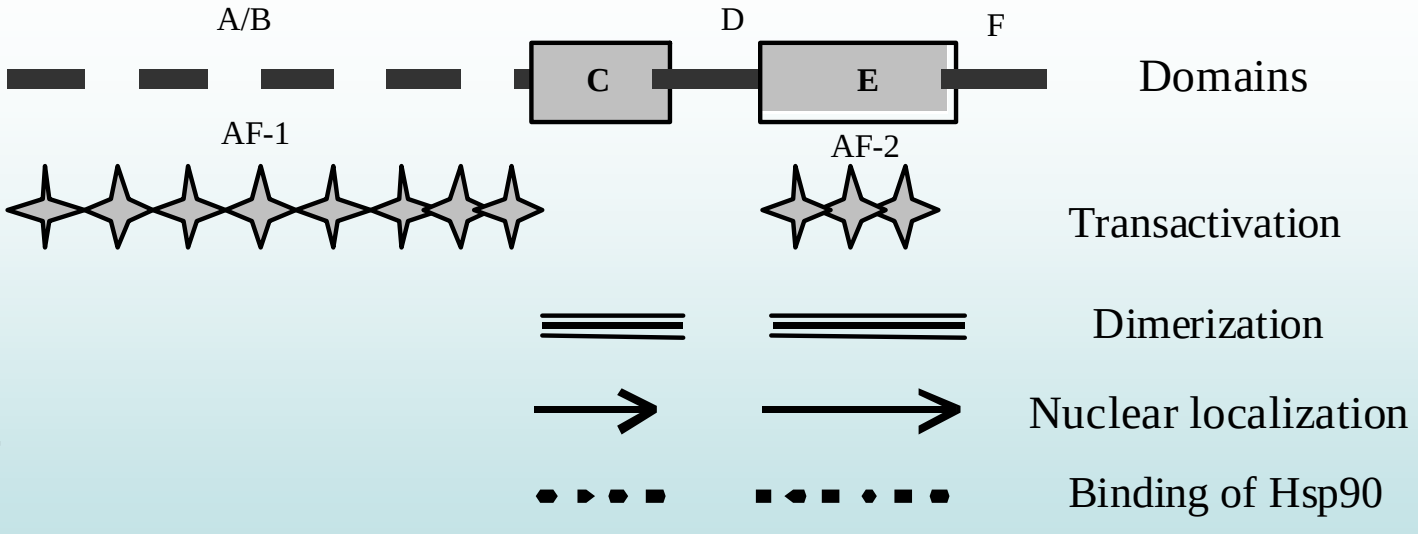
- play a key role in female hormone regulation and signalling
- are responsible for metabolic, behavioural and morphologic changes occurring during stages of reproduction
- are involved in the growth, development and homeostasis of a number of tissues
- control the bone formation, regulation of homeostasis, cardiovascular system and behaviour
- regulate production, transport and concentration of testicular liquid and anabolic activity of androgens in males

Estrogen receptor:

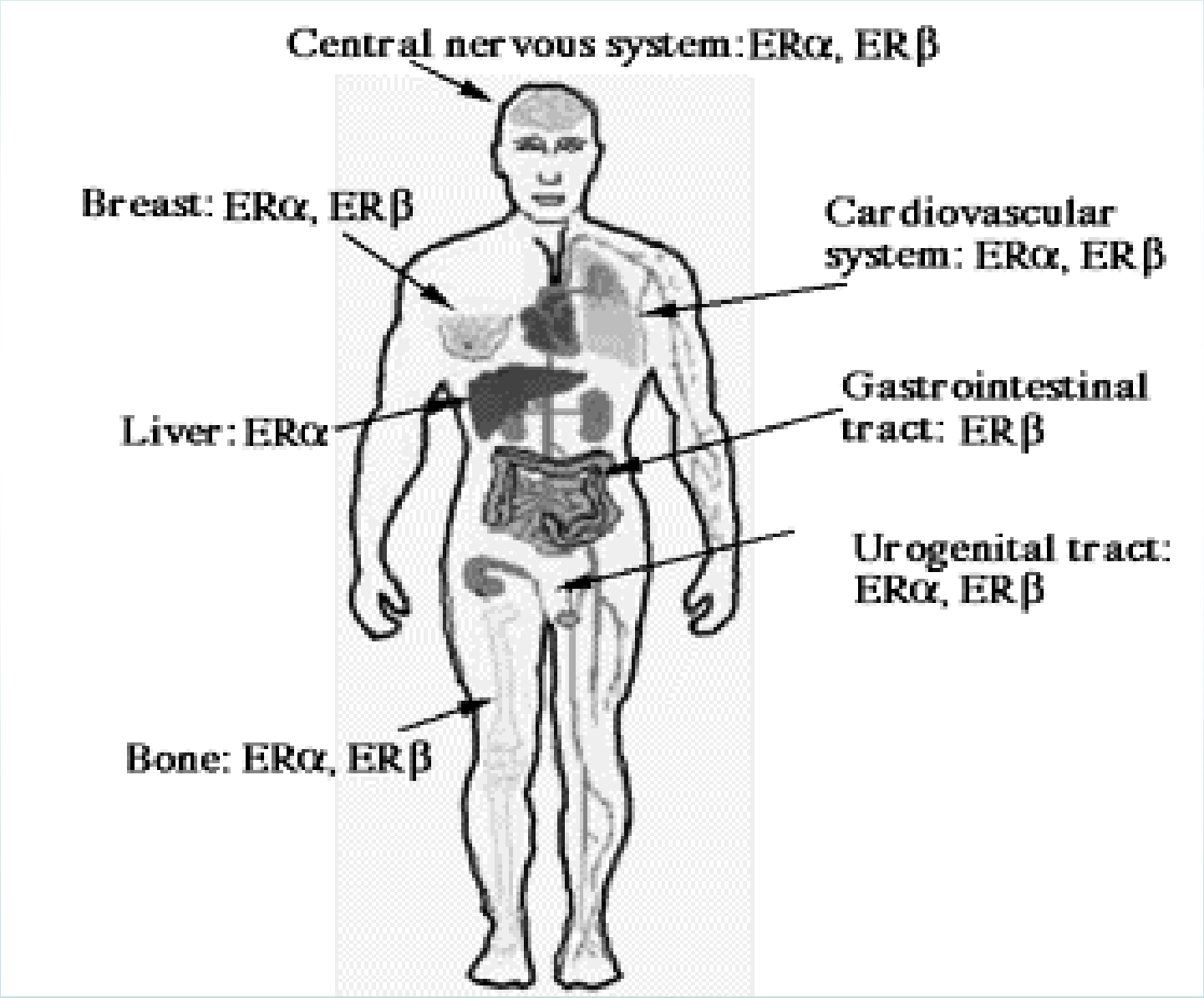
- a member of the nuclear hormone receptor superfamily
- a ligand – inducible transcription factor

- subtype: ER-α (in breast, ovary, brain, liver, bone and cardiovascular system, adrenals, testis and urogenital tract)
  ER-β (in kidneys, prostate and gastrointestinal tract)
  ER-γ (in fish)

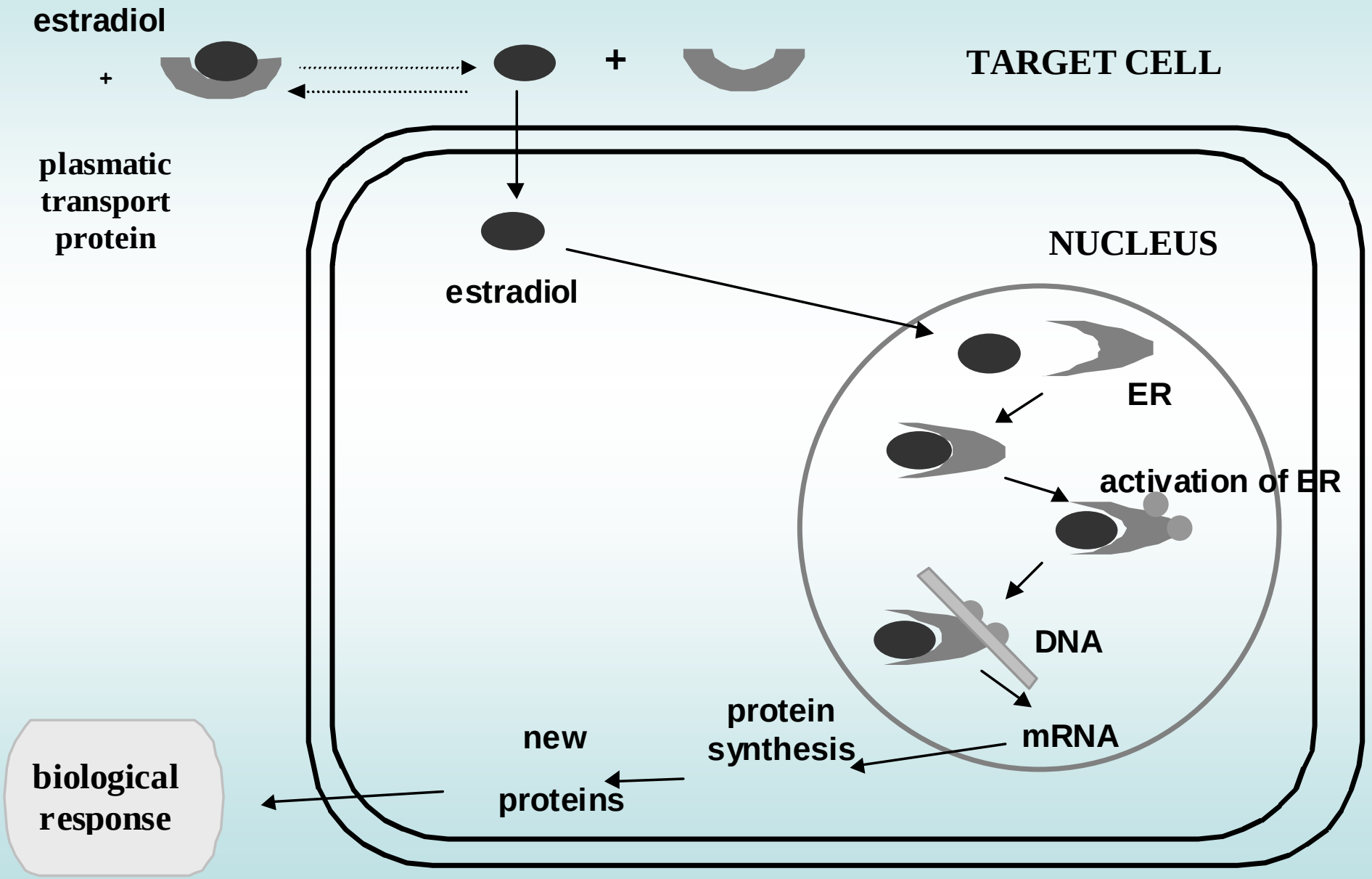




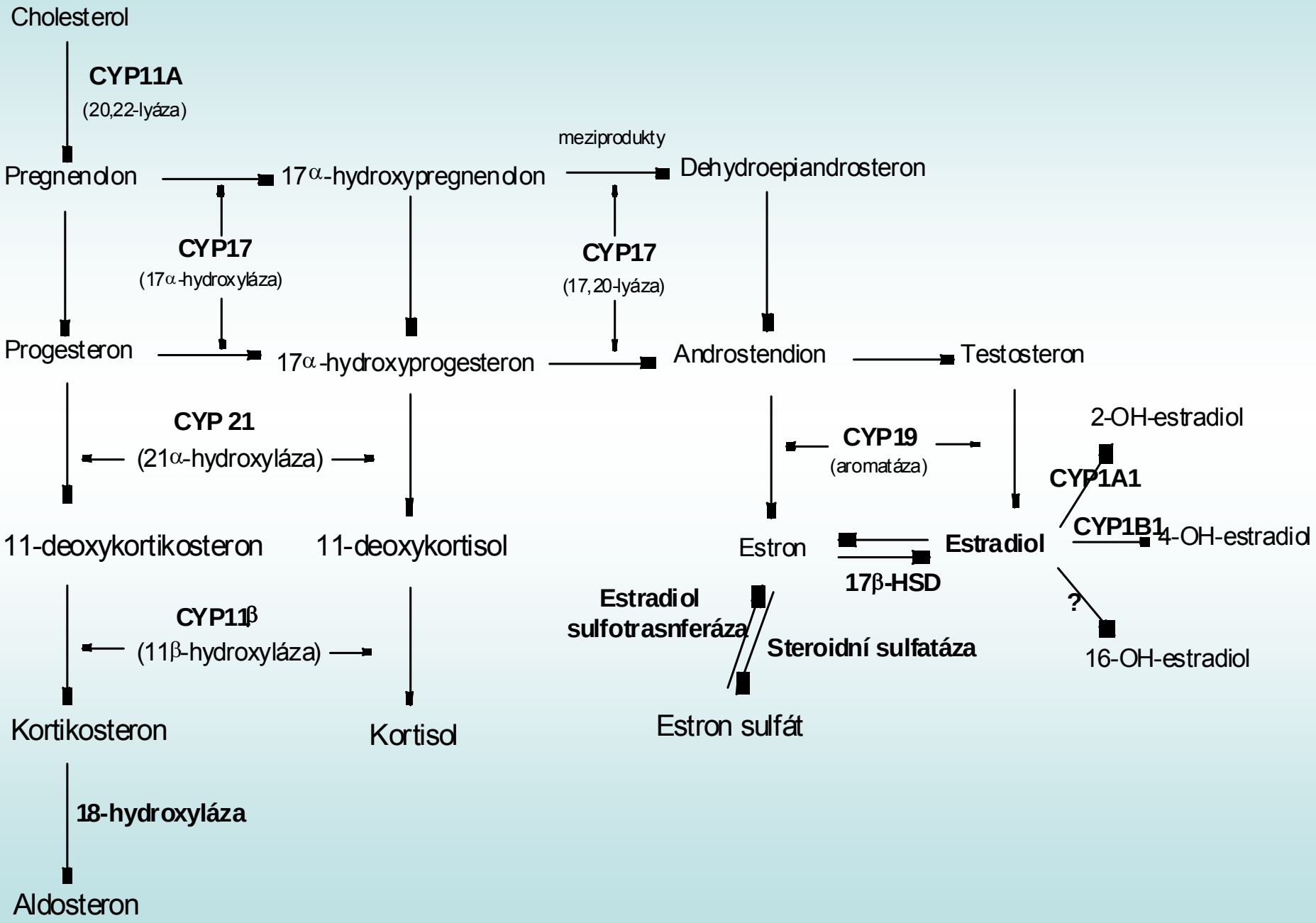
ESTROGEN RECEPTORS - ER-α & ER-β:



Mechanism of action of the estrogen hormones



Synthesis and metabolism of estrogens



Environmental estrogens (xenoestrogens, exoestrogens)

are a diverse group of substances that do not necessarily share any structural resemblance to the prototypical estrogen (17β-estradiol) but evoke effects resembling those of estrogen

- **estrogenic substances (estrogen agonist)**
- **ANTI-estrogenic substances**

Exoestrogens - examples

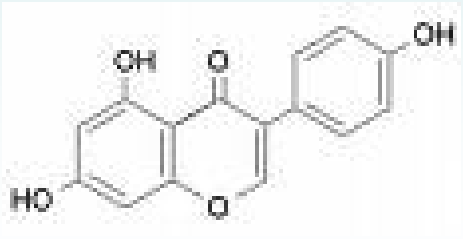
Natural products

genistein

naringenin

coumestrol

zearalenone



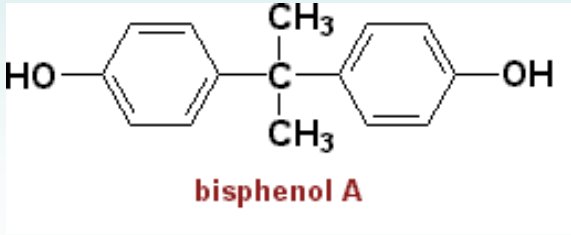
Industrial chemicals

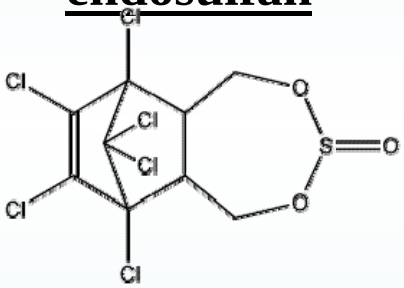
Bisphenol A

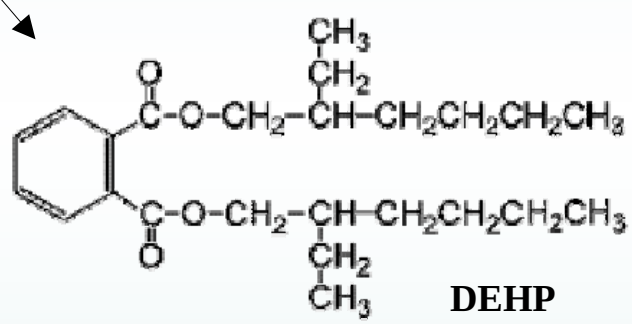
Nonionic surfactants

Pthalate esters

endosulfan







DEHP

Environmental pollutant

DDT

kepone

PCBs/OH-PCBs

PAHs and dioxins

Pharmaceuticals

Ethinyl estradiol

Diethylstilbestrol

gestodene

norgestrel

Exoestrogens - Relative Potencies to bind to ERα (REPs)

Chemical group	Substance	REP
Endogenous hormones	Estradiol	1
	Estriol	$6{,}3.10^{-3}$
	Testosteron	$9{,}6.10^{-6}$
Phytoestrogens	Cuomestrol	$6{,}8.10^{-3}$
	Genistein	$4{,}9.10^{-4}$
Pesticides	o,p´-DDT	$1{,}1.10^{-6}$
PCBs	2,4,6-trichlorbiphenyl-4´-ol	1.10^{-2}
	2,5-dichlorobiphenyl-4´-ol	$6{,}2.10^{-3}$
	3,3´,5,5´tetrachlorobiphenyl-4,4´-diol	$1{,}6.10^{-4}$
alkylphenoles	4-tert-oktylphenol	$3{,}6.10^{-6}$
phthalates	butylbenzylphthalate	4.10^{-6}

REP (RElative Potencies) of selected compounds related to 17-β-estradiol derived from reporter yeast assay

Toxicity assessment - in vivo and in vitro methods

Assay (ref.)	Exposure type	Detects ER-dependent agents?	Detects non-ER-dependent agents?	Distinguishes agonist versus antagonist?	Pharmacokinetic and metabolism included?
Receptor-based assays					
Receptor binding assay (27)	Cell lysate	Yes	No	No	No
Receptor activation assay (32–34)	Cells in vitro	Yes	No	Yes[a]	No
In vitro estrogen-regulated response assays					
MCF-7 cell proliferation assay (41)	Cells in vitro	Yes	Limited	Yes[a]	No
Induction assays (46,48)	Cells in vitro	Yes	Limited	Yes[a]	No
DNA synthesis assays (47)	Cells in vitro	Yes	Limited	Yes[a]	No
In vivo estrogen-regulated response assays					
Uterotrophic response assay (49)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal cornification assay (50)	Whole animal	Yes	Limited	Yes[a]	Yes
Vaginal opening (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine fluid imbibition (11)	Whole animal	Yes	Limited	Yes[a]	Yes
Uterine epithelial hypertrophy (51)	Whole animal	Yes	Limited	Yes[a]	Yes
Inhibition of steroid synthesis assays					
In vitro ovarian steroid assay (55)	Minced tissue	No	Yes	Yes	No
Ex vivo ovarian steroid assay (56)	Whole animal	No	Yes	Yes	Yes

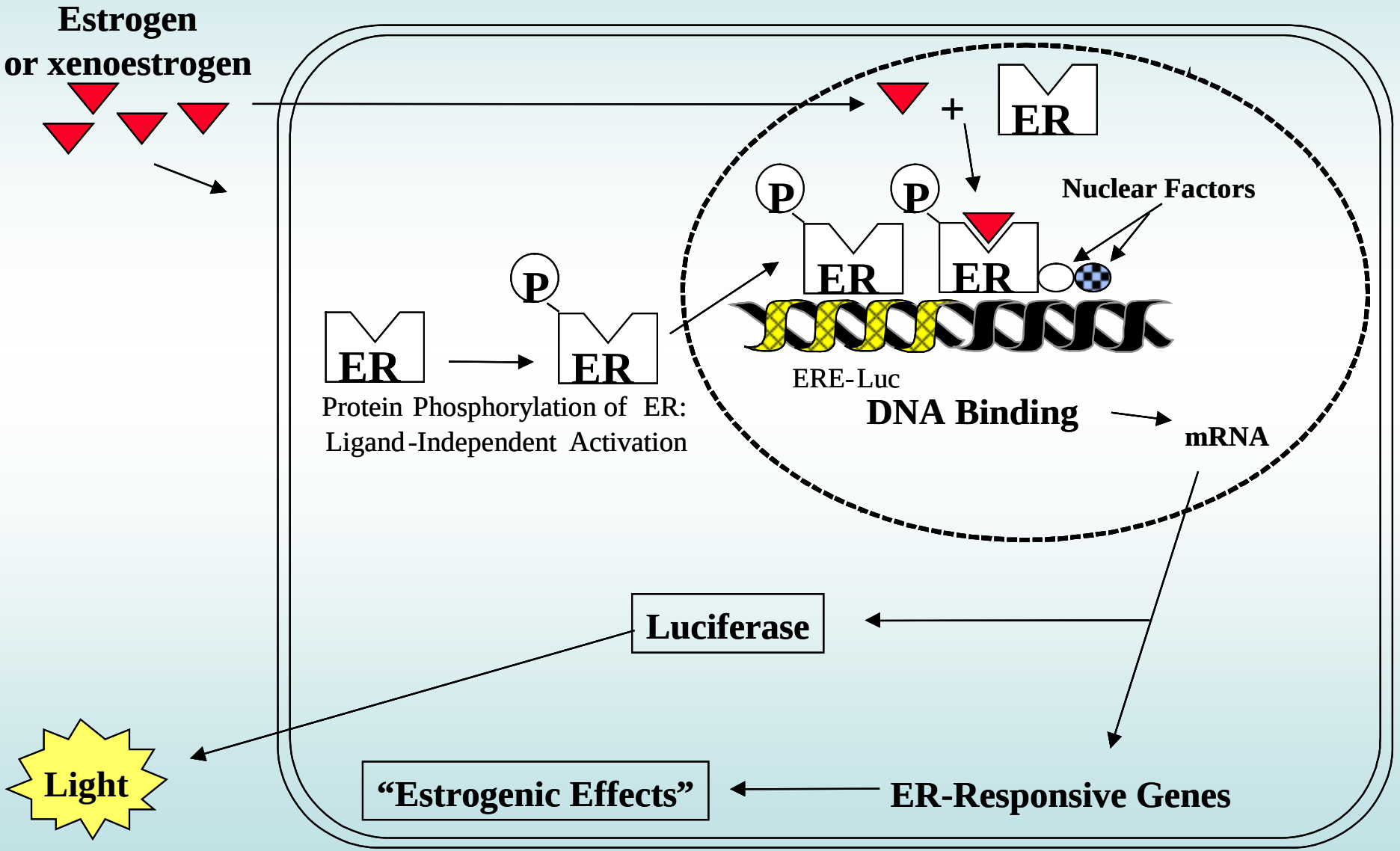
[a]Detection of antagonists requires use of additional groups with test material + estradiol.

In vitro assay

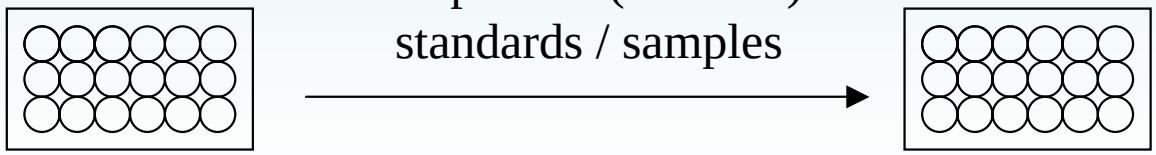
- competitive ligand binding assay
- cell proliferation assay
- endogenous protein expression (or enzyme activity) assay
- reporter gene assay

In vitro ER- mediated effects

luciferase reporter assay



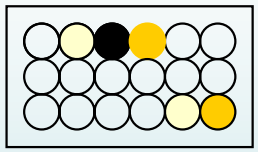
ER- mediated effects
luciferase reporter assay

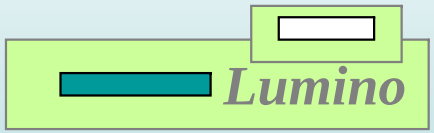


96 microwell plate
cultivation of transgenic cell lines

ER: breast carcinoma MVLN cells

Cell lysis
-> extraction of induced luciferase





Luminescence determination
(microplate luminescence reader)

SIMILAR DESIGN FOR
OTHER RECEPTORS:

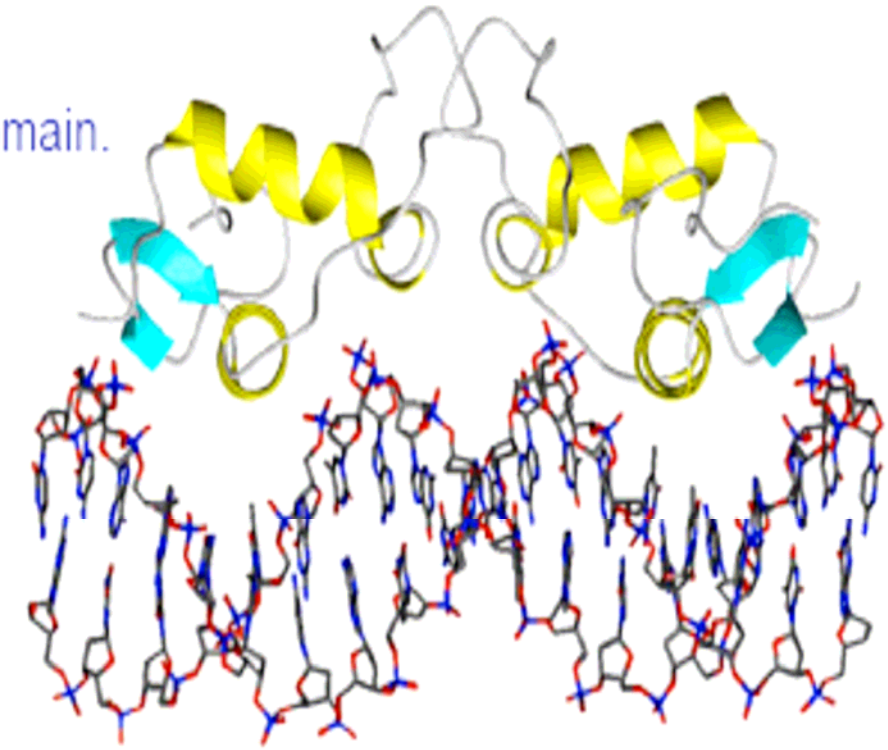
AhR (H4IIE.luc cells)
AR (MDA cells)
RAR/RXR (P19 cells)

In vivo assay

- uterotropic assay
- vaginal cornification assay
- standard test procedures for reproductive and developmental toxicity (e.g. FETAX)
- production of estrogen-inducible proteins (e.g. vittelogenin and zona radiata protein)

ANDROGEN RECEPTOR (AR)

Androgen receptor DNA binding domain.

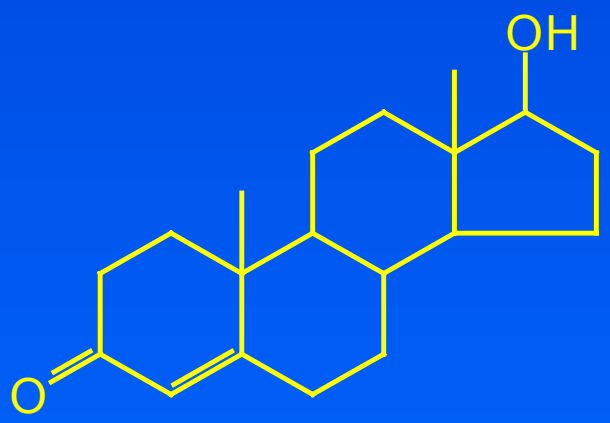


Androgens

- **Role in males similar to the of estrogens in females**
  - development of male sexual characteristics
  - stimulating protein synthesis, growth of bones
  - cell differenciation, spermatogenesis
  - male type of behaviour

Androgens

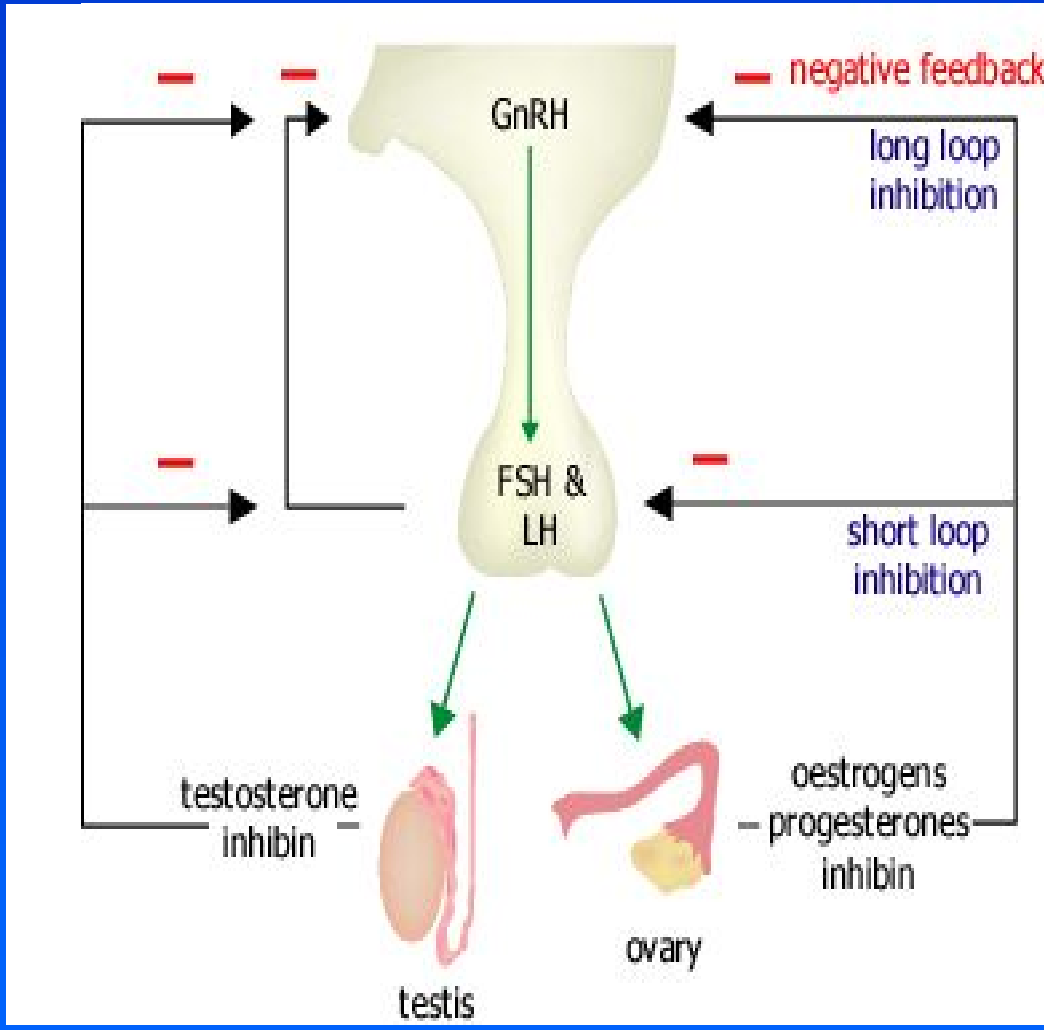
- Endogenous ligands – androgen hormones
- testosterone
- dihydrotestosterone (DHT)
- androstanediol
- dehydroepiandrosterone
- androstenedione



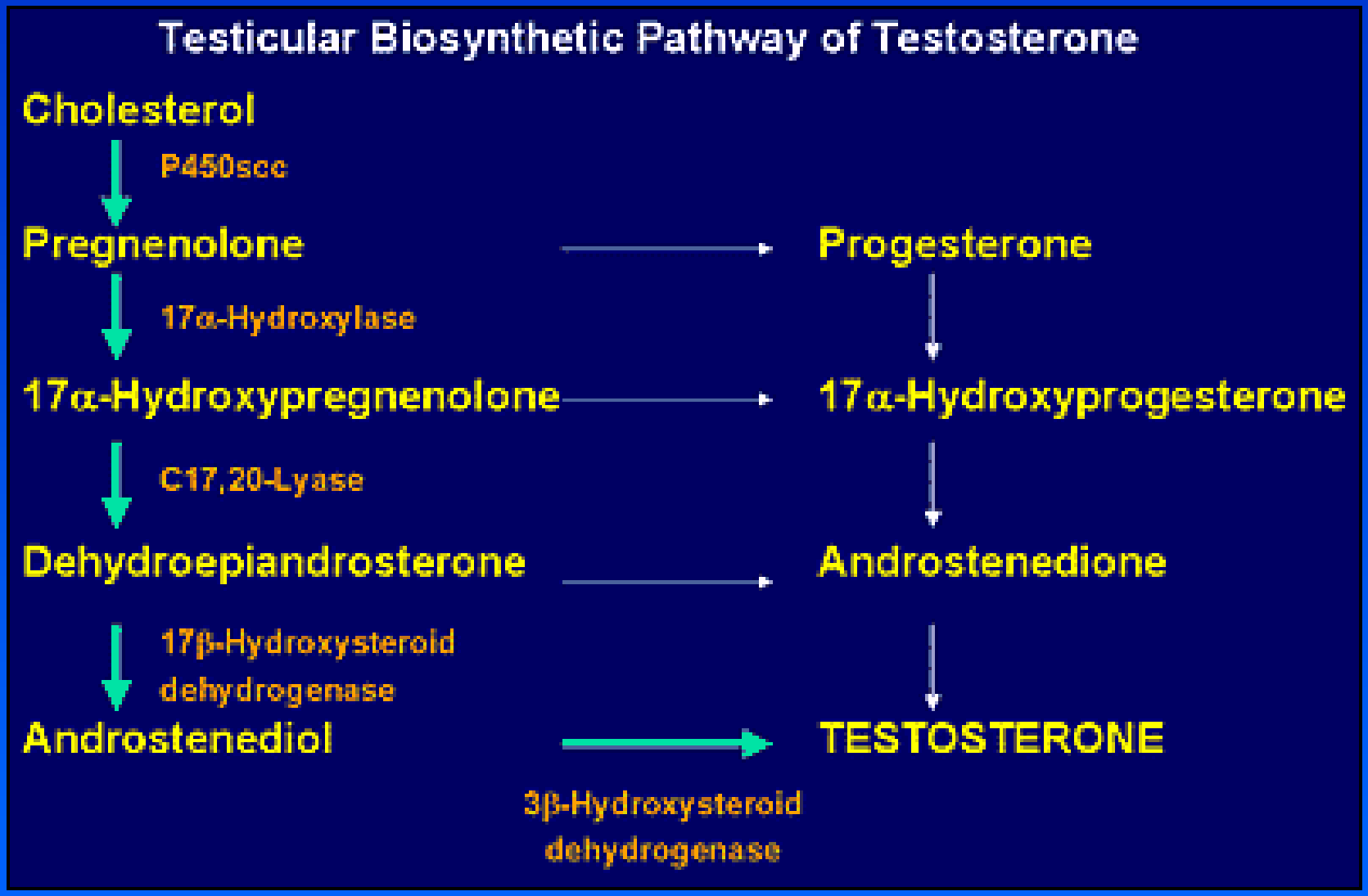
Testosterone

Hypothalamo-pituitary axis

- Folicle stimulating hormone
  - Stimulates synthesis of androgen binding proteins and spermatogenesis in Sertoli cells (testis)
- Luteineizing hormone
  - Stimulates testosterone production in Leydig cells



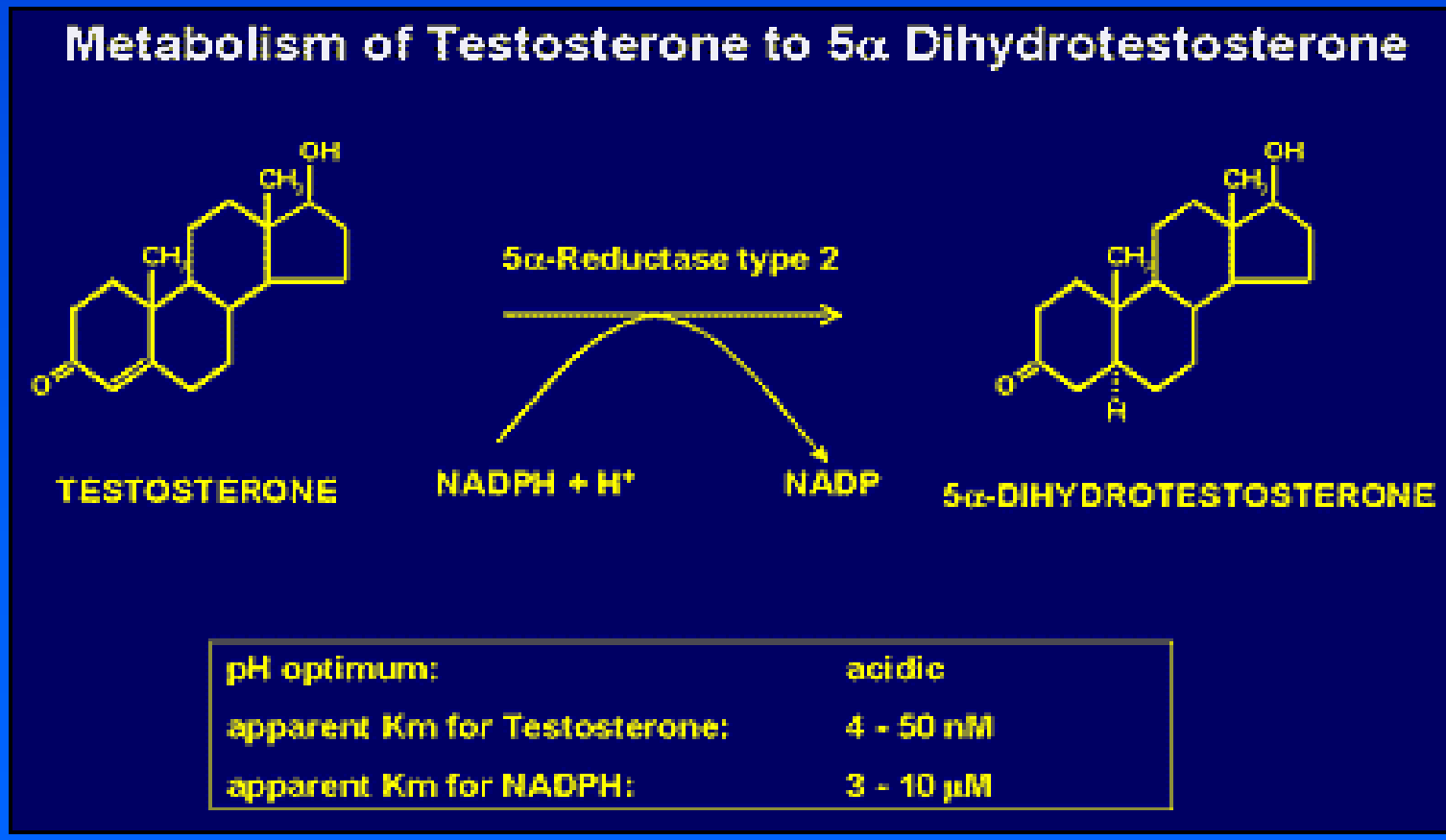
Testosterone



- synthetized in testis (Leydig cells)
- in lesser extent in adrenals

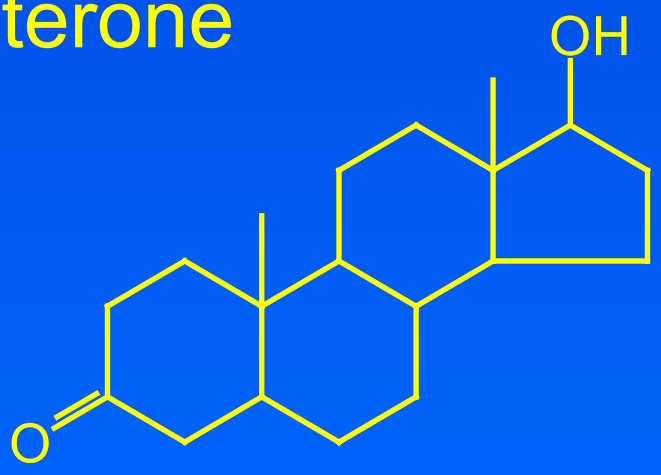
Dihydrotestosterone

- The most important derivative of testosterone
- Formed **<u>extratesticulary</u>** from testosterone
- 5α-reductase



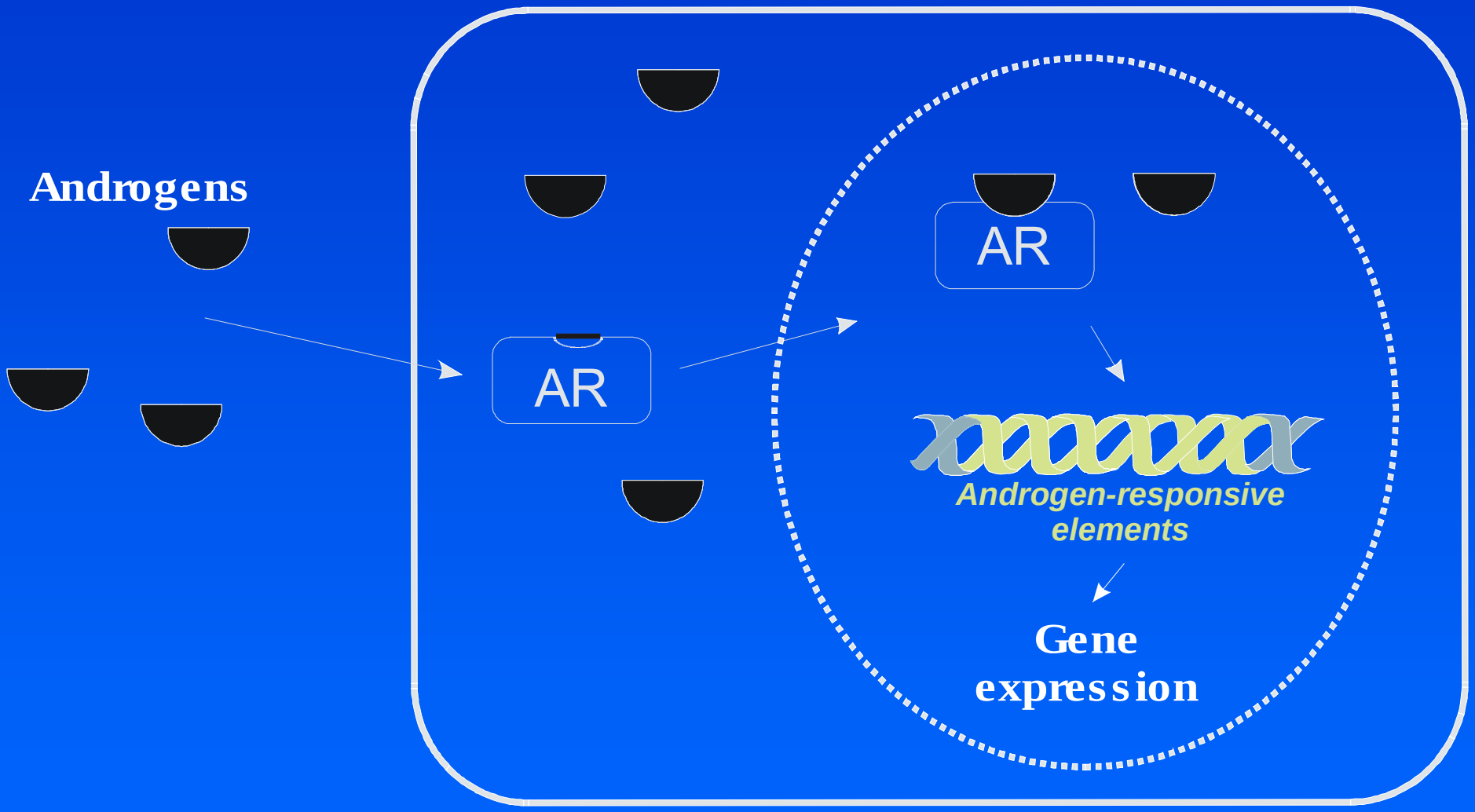
Dihydrotestosterone

- In several tissues (seminal vesicles, prostate, skin) higher affinity to androgen receptor than testosterone
- Daily production 5-10% of testosterone



Dihydrotestosterone

Mechanism of action



Mechanisms of androgen signalling disruption

Binding to AR

- Mostly competitive inhibition – xenobiotics do mostly NOT activate AR-dependent transcription

- Few compounds are able to activate AR in absence of androgen hormones x in presence of T/DHT antiandrogenic (**metabolites of fungicide vinclozoline**, some PAHs)

FSH/LH (gonadotropins) signalling disruption

- FSH/LH expression - regulation via negative feedback by testosterone

- Suppressing leads to alterations of spermatogenesis

Mechanisms of androgen signalling disruption

Alterations of testosterone synthesis

- Inhibition of P450scc needed for side chain cleavage of cholesterol (fungicide **ketoconazol**)

- Inhibition of 17- α-hydroxylase and other CYPs - – enzymes needed for testosterone synthesis (**ketoconazol**)

Testosterone metabolic clearance

- Induction of UDP-glucuronosyltransferase or monooxygenases CYP1A, 1B involved in androgen catabolism

- Pesticides **endosulfan, mirex, o-p´-DDT**

Effects of male exposure to antiandrogens

Exposure **during prenatal** development:

- malformations of the reproductive tract
  - reduced anogenital distance
  - hypospadias *(abnormal position of the urethral opening on the penis)*
  - vagina development
  - undescendent ectopic testes
  - atrophy of seminal vesicles and prostate gland

Effects of male exposure to antiandrogens

Exposure in prepubertal age:

- delayed puberty
- reduced seminal vesicles
- reduced prostate

Exposure in adult age:

- oligospermia
- azoospermia
- libido diminution

AR-binding - potencies

(Ref: DHT EC50 ~ 0.1 uM)

Compound	IC_{50} (µM)
Benz[a]anthracene	3.2
Benzo[a]pyrene	3.9
Dimethylbenz[a]anthracene	10.4
Chrysene	10.3
Dibenzo[a,h]anthracene	activation in range 0.1-10µM
Bisphenol A	5
vinclozolin metabolites	9.7
hydroxyflutamide	5
Aroclor typical values	0.25-1.11
Individual PCBs typical values	64 - 87
tris-(4-chlorophenyl)-methanol	0.2

Antiandrogenic compounds

tris-(4-chlorophenyl)-methanol

- Ubiquitous contaminant of uncertain origin
- Probable metabolite of DDT-mixture contaminant
- Levels in human blood serum cca. 50nM
- EC50 – cca. 200nM

In vivo antiandrogenicity assessment

Hershberger assay

- castrated rats treated with examined substance

- Endpoint – after 4-7 days – seminal vesicles and ventral prostate weight

Measurement of testosterone concentration in serum

In vitro
antiandrogenicity assessment

Most often employed – prostatic cell lines

Cell proliferation assays – cell lines with androgen-dependent growth;

- Treatment with tested chemical only (androgenicity) or cotreatment with DHT (antiandrogenicity)

  - mammary carcinoma cell lines

  - prostatic carcinoma cell lines

In vitro antiandrogenicity assessment

Receptor-reporter assays

- Gene for luciferase or GFP synthesis under transcriptional control of AR
- Luciferase:
- AR-CALUX (human breast carcinoma T47D)
- PALM (human prostatic carcinoma PC-3)
- CHO515 (Chinese hamster ovary CHO)

In vitro antiandrogenicity assessment

GFP

- Possibility of nondestructive measurement (fluorescence of intact cells)

X

Less sensitive – lack of enzymatic amplification

- Human prostatic cell lines

Yeast assays

- Mostly **β-galactosidase** as reporter enzyme
- Easy cultivation and experimental design

X

- Cell wall may obstruct transport of chemical into cell=>

=> false negatives

Thyroid hormones

Thyroid hormones

Thyroxine
Triiodothyronine
Calcitonin

Play crucial roles in stimulating metabolism and influencing development and maturation

<u>Regulation of metabolism</u>

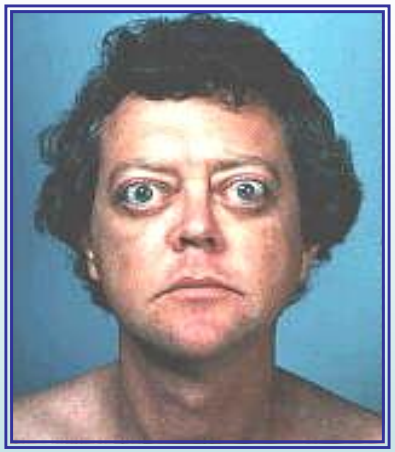
- increasing oxygen consumption
- modulating levels of other hormones (insulin, glucagon, somatotropin, adrenalin)
- important in cell differenciation
- crucial role in development of CNS, gonads and bones

The Thyroid Gland

Thyroid hormones bind to nuclear receptors

- regulate carbohydrate & lipid metabolism
  - adults with **hypothyroidism** have low production of thyroxine
  - reduced metabolism and overweight

  - adults with **hyperthyroidism** have high production (excessive secretion) of thyroid hormones (thyroxine)
  - high metabolism and weight loss
  - trigger metamorphosis in amphibians



Effects of thyroid disruption

Thyroid hormones

- if absent during fetal development or for first year:

  - nervous system fails to develop normally

    - mental retardation results

- In prenatal development - severe damage of CNS (cretenism, delayed eye opening, cognition)
- Megalotestis
- Histological changes in thyroid gland (goitre)

if T4 concentrations decline before puberty:

- normal skeletal development will not continue

Thyroid hormones

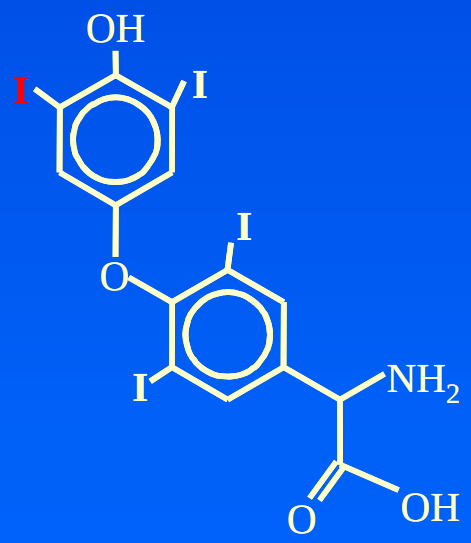
Thyroxine (T4)

Also called tetraiodothyronine
Contains 4 iodide ions

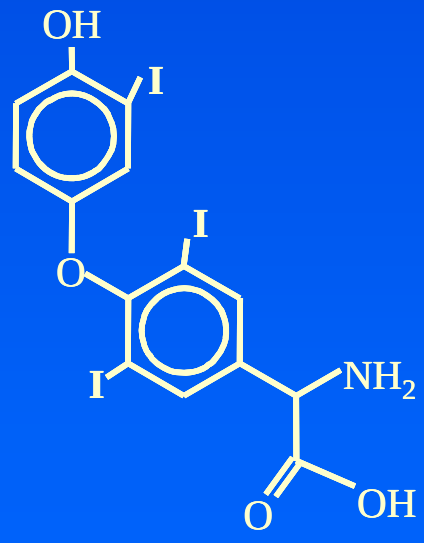
Triiodothyronine (T3)

Contains 3 iodide ions

- T4 – prohormone
- 5´-deiodination leads to active form, T3



Thyroxine (T_4)

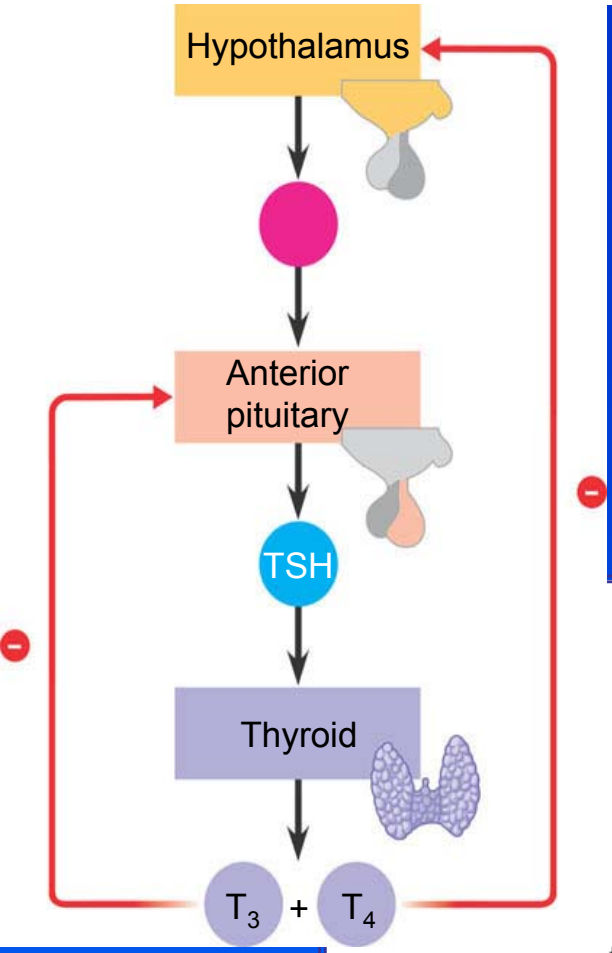


3,5,3'-Triiodothyronine (T_3)

Thyroid hormones

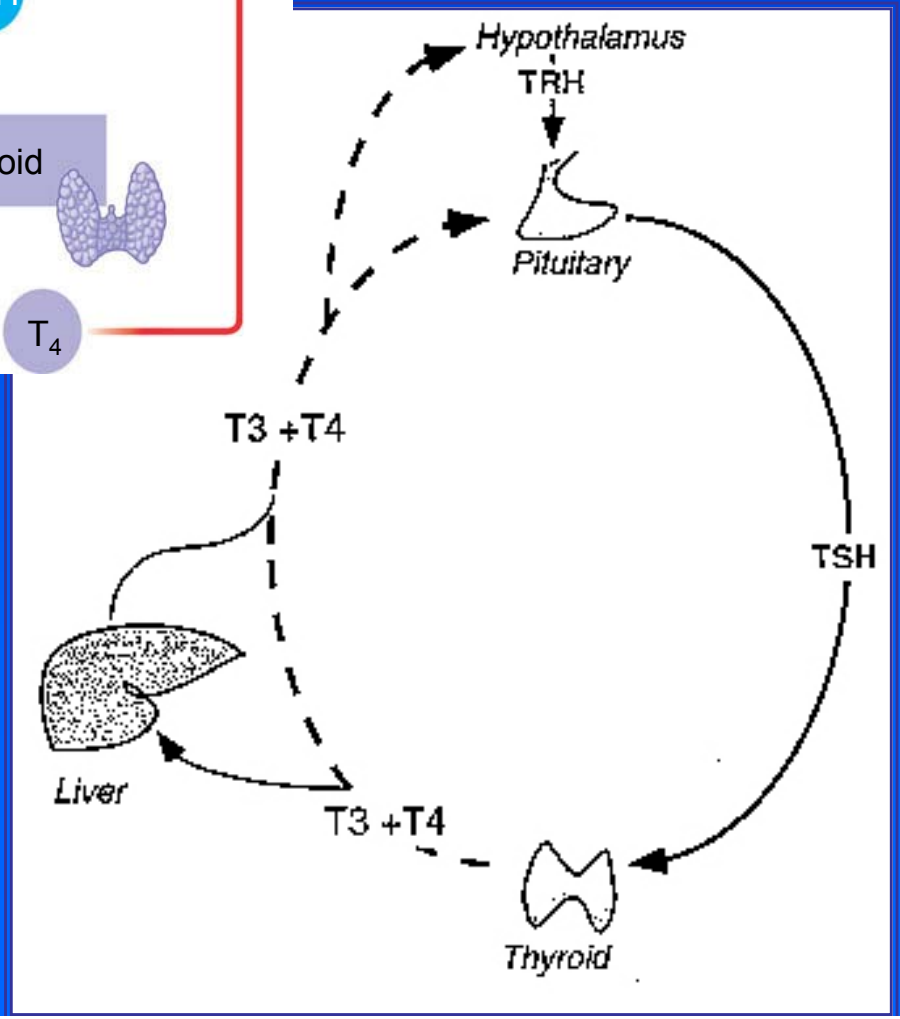
- Enter target cells by transport system
- Affect most cells in body
- T4 and small amount of T3 produced in thyroid gland

- **Most T3 produced by deiodination in target tissues (deiodinases)**
  - T4 synthesis - iodination of tyrosin residues on tyreoglobulin
  - coupling of two iodotyrosines conducted by thyroid peroxidase

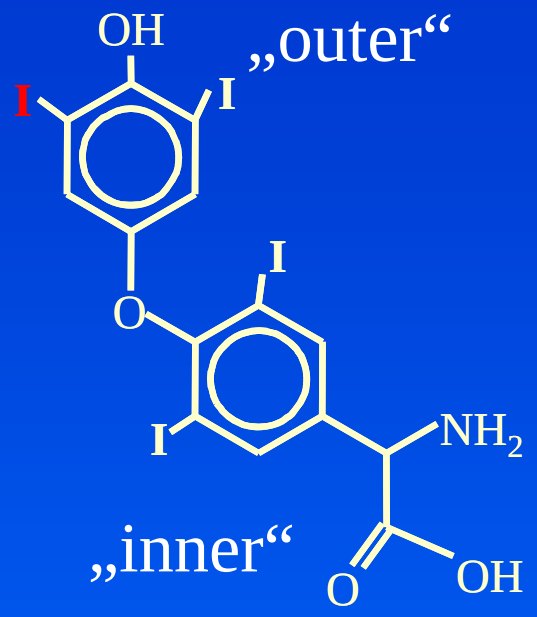


Pituitary-thyroid axis

- Regulation of thyroid synthesis
- Control the secretion of thyroid hormones through two negative feedback loops
- Pituitary TSH (thyroid stimulating hormone) stimulates both I^- uptake and iodination of tyrosine resides on Tg



Enzymes involved in thyroid metabolism



- Thyroid peroxidases
  - iodination of tyrosyl residues
  - coupling of iodinated tyrosyl residues
- Thyroid deiodinases
  - D1, D2 - activation of T4 into T3 via deiodination on „outer“ ring (formation of T3)
  - D3 - deactivation into rT3 via deiodination on „inner“ ring

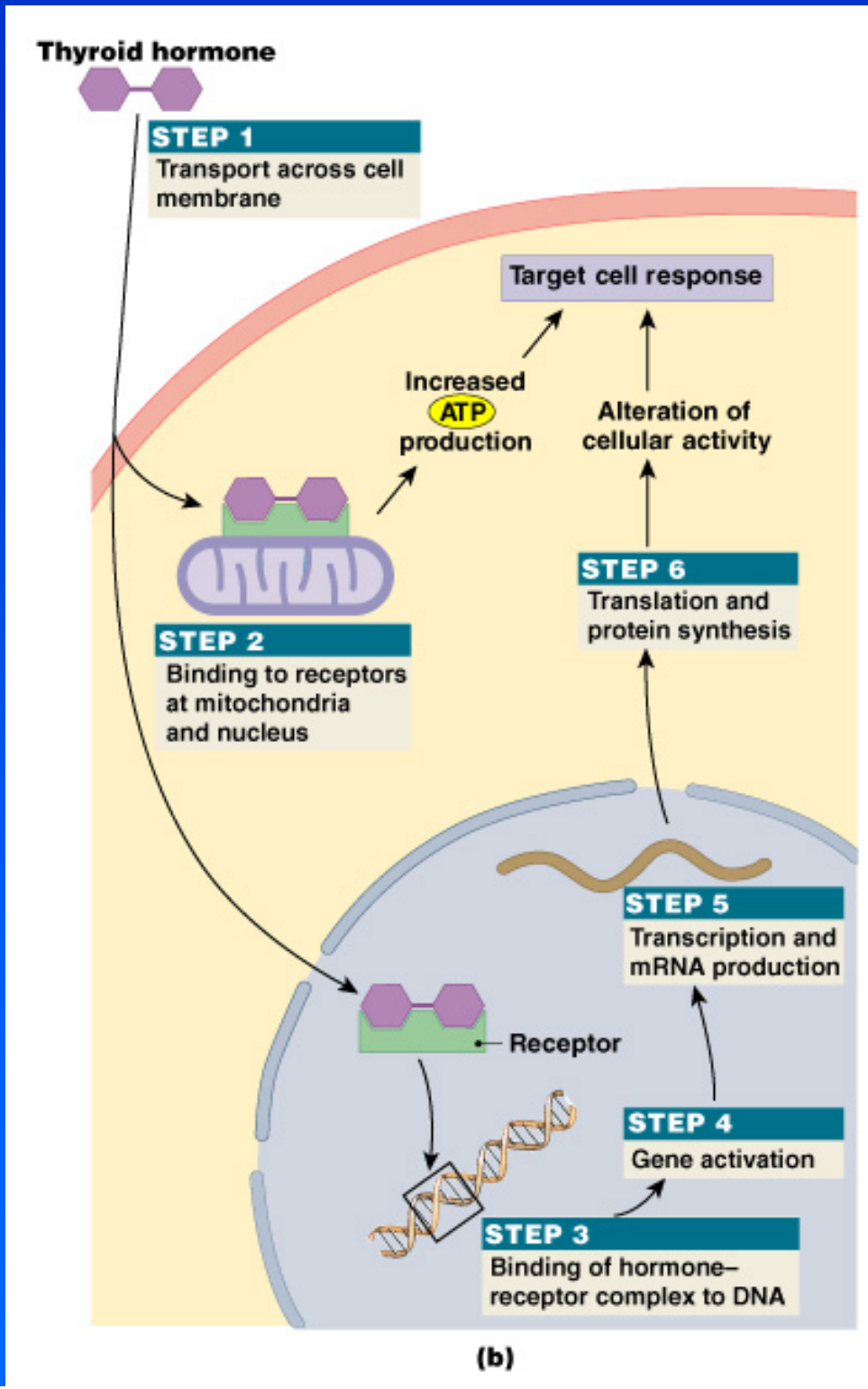
Thyroid receptors - Mechanism of action

Thyroid hormones bind to receptors in:

- cytoplasm
- surfaces of mitochondria
- nucleus

Alike other nuclear receptors

- 5 isoforms of TR
- After activation formation of homo- and heterodimers
- Binding to thyroid responsive elements (TRE)
- Gene expression



Thyroid binding proteins

- Regulating free T4 and T3 levels in blood
- 3 types :
  - -Thyroid-binding prealbunin (transthyretin) (20-25%)
  - -Albumin (5-10%)
  - -Thyroid binding globulin (75%)

Competitive binding to thyroid binding proteins

- OH-PCBs, brominated and chlorinated flame retardants, DDT, dieldrin
- OH-PCBs – equal affinity to TBP as T4 and T3
- More of free T4 in blood => negative feedback to TSH release **=>** increased depletion => increased weight, histological changes in thyroid gland (after exposure to POPs in mammals, birds, fish)

Competitive binding to TR

- **Probably less important than binding to TBP**
- Chemicals that affect thyroid signalling in vivo mostly don´t bind to TR (DDT, PCBs) or bind with much lesser affinity than T3 (OH-PCBs – 10000x)

Accelerated depletion of TH

- UDP-glucuronosyltransferase – detoxication enzyme (II.biotransformation phase)
  - Induced by PCBs, dioxins
  - Key enzyme in thyroid catabolism
- Increased by disruption of TBP binding

In vivo assessment

- **TH serum levels** – simple, nondestructive x variation within time of day, age, sensitive to other than biochemical stresses
- Thyroid gland weight and folicular cells number
- Developmental toxicity assays - delayed eye opening, abnormalities in brain development and cognition, increased testis weight and sperm counts
- Perchlorate discharge test (TH synthesis)
- Hepatic UDP-glucuronosyltransferase activity (marker of enhanced TH clearance from serum)

In vitro assessment

- Enzyme inhibition assays *(thyroid peroxidase, deiodinases)* – assessment of thyroid metabolism

- Competitive binding assays with TBP

- TH- dependent proliferation assay (pituitary tumor GH3, thyroid tumors like FRTL-5 cell line) or TSH-dependent proliferation assay (thyroid tumors)

- Receptor-reporter gene assays with luciferase (monkey kidney CV-1, chinese hamster ovary CHO or insect Sf9 cell lines)

Retinoids

Vitamin A and its derivatives

Retinoids

Regulation of development and homeostasis in tissues of vertebrates and invertebrates

Development of embryonic, epithelial cells (gastrointestinal tract, skin, bones)

Necessary for vision

Suppressive effects in cancer development

Important for cell growth, apoptosis and differenciation

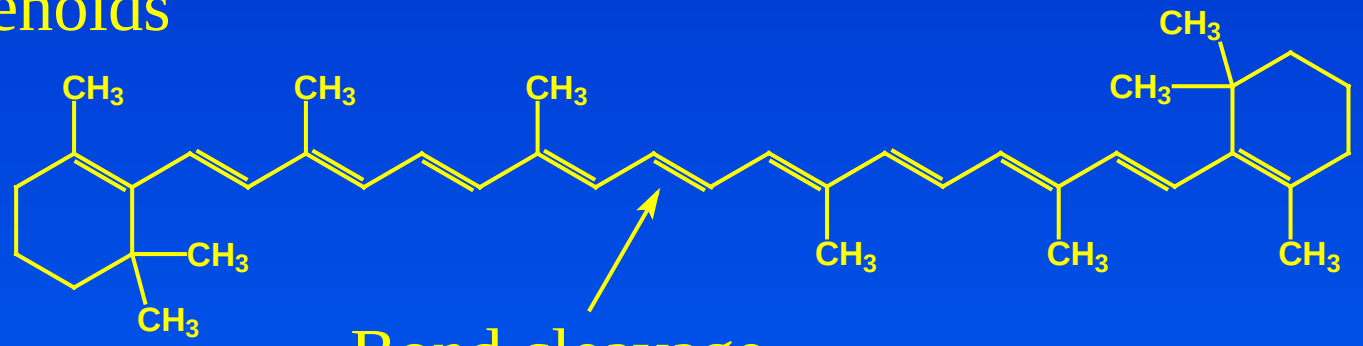
Antioxidative agent

Affect nervous and immune function

Retinoids

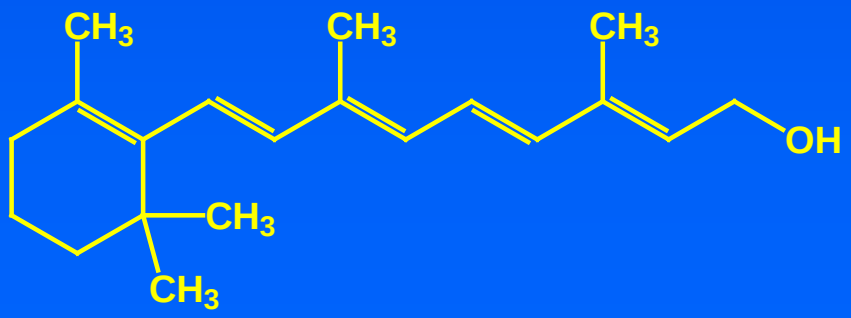
Sources: from diet (dietary hormones)
Retinyl esters – animal sources
Plant carotenoids

β-karoten

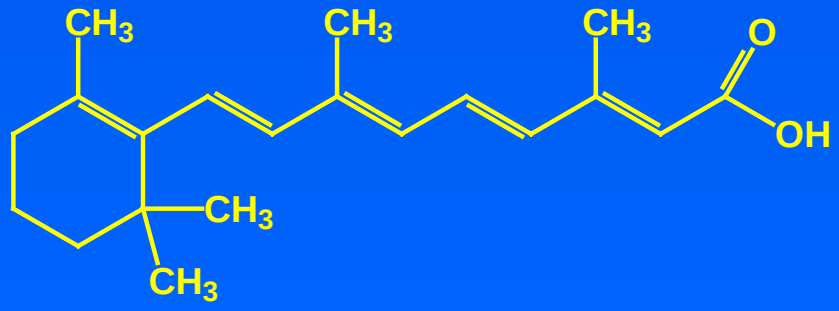


Bond cleavage

Retinol (vitamin A)



Retinoic Acid

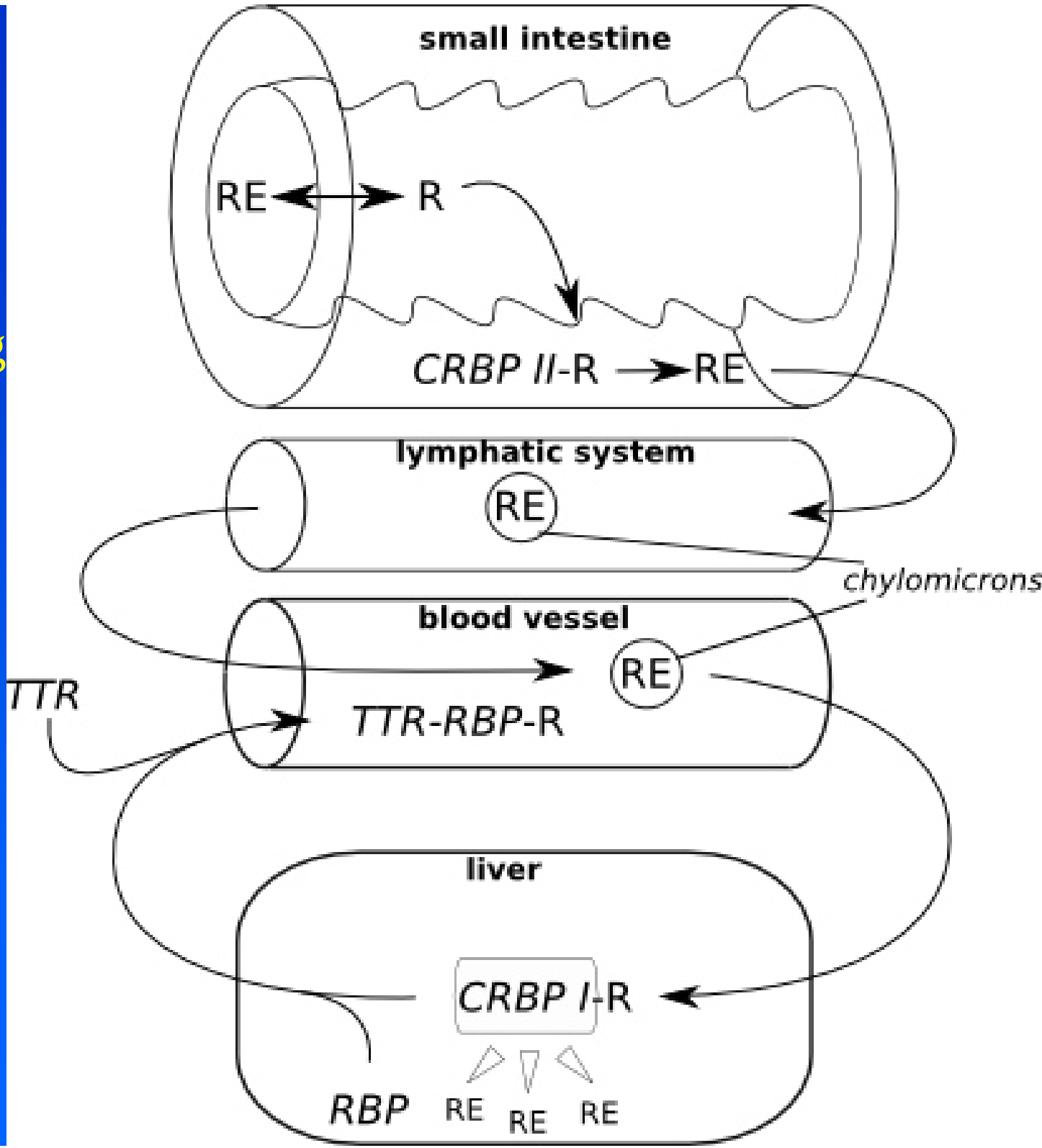


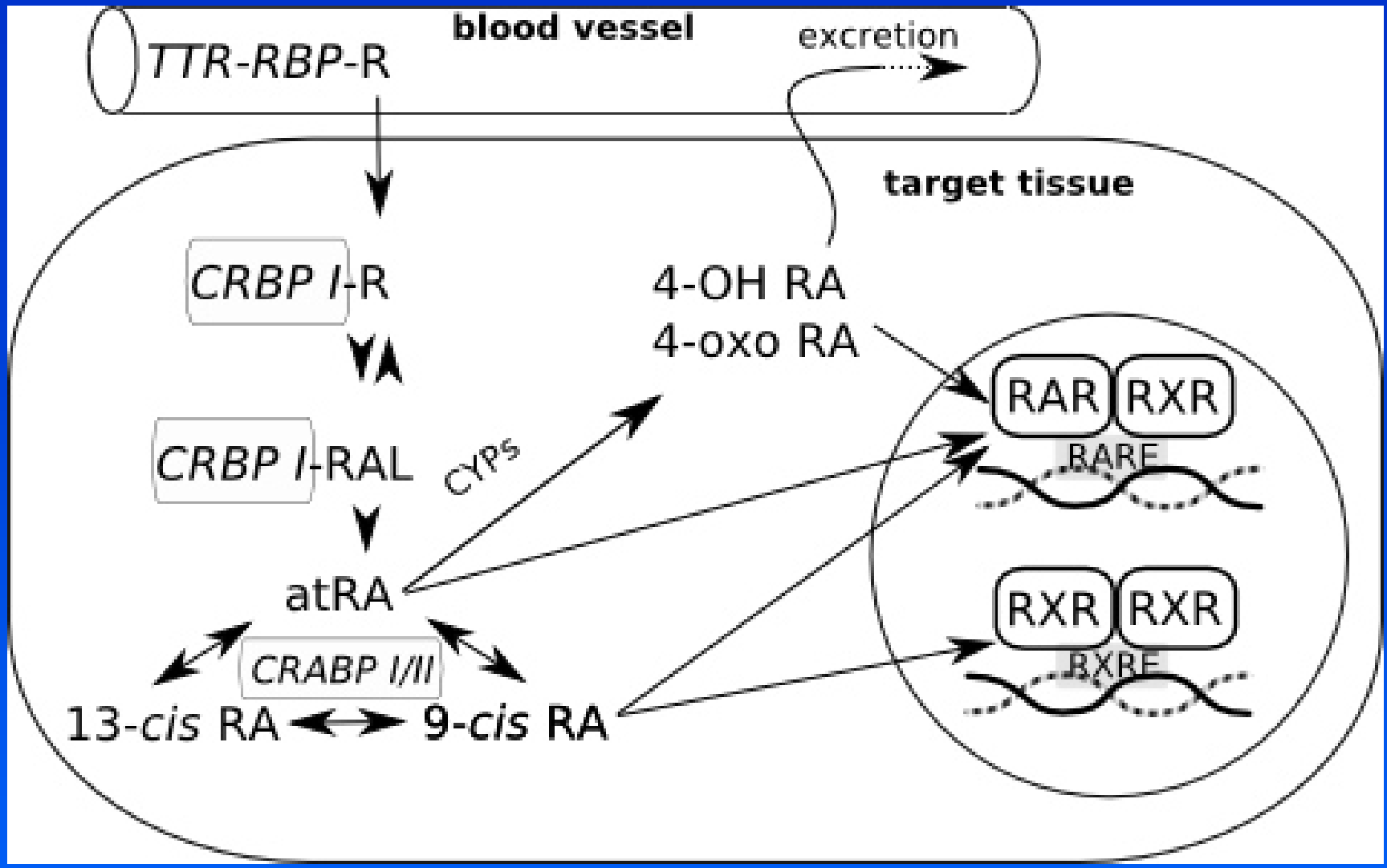
RE: Retinol-Ester

R: Retinol

RBP: Retinol Binding Protein (*LMW*)

TTR: Transthyrethin (*HMW*)





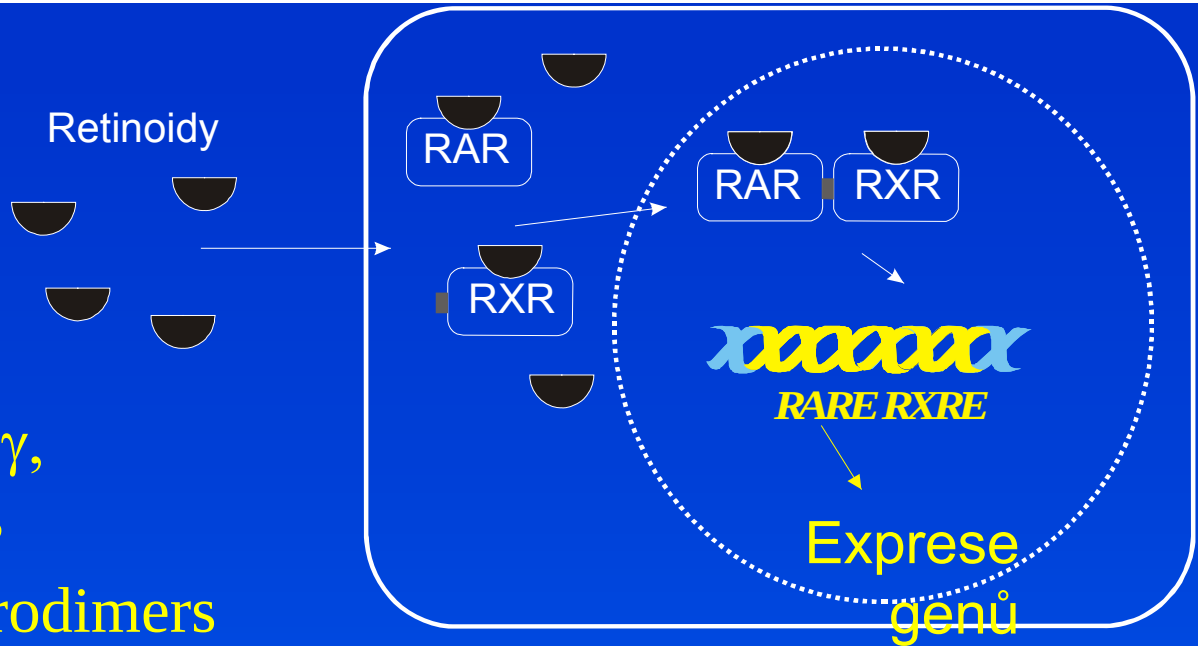
Retinoid binding proteins

CRBP – cellular retinol binding protein
- binding of retinol, immediate decrease of retinol concentration

CRBAP – cellular retinoic acid binding protein
- Controlling ratio free retinol/free retinoic acid

RAL - Retinal

Mode of action



- Isoforms of RAR a RXR
- Both have isoforms α, β and γ, each of them several subtypes
- Formation of homo- and heterodimers
- 48 possible RAR-RXR heterodimers

=>sensitive regulation of gene expression

- RXR – heterodimers even with other receptors like VDR, TR, PPAR

Retinoic acid

- 3 basic subtypes
- all-trans-, 9-cis- and 13-cis-retinoic acid
- All-trans RA binds selectively to RAR
- Cis RA bind to both receptor types

Disruption of retinoid signalling by xenobiotics

- Relatively little is known
- Possible modes of action:
  - Metabolization of retinoids by detoxication enzymes
  - Disruption of binding retinoids to retinoid binding proteins
  - Retinoids as antioxidants may be consumed cause of oxidative stress caused by xenobiotics
  - Interference of chemicals (binding to RAR/RXR)

Consequences of retinoid signalling disruption

Decreased retinoid levels in organisms

- Downregulation of growth factors
- Xerophtalmia, night blindness
- Embryotoxicity, developmental abnormalities

X

Increased ATRA concentration – teratogenic effect

Change may cause severe developmental anomalies (both excess and deficiency)

Disruption of retinoid signalling by xenobiotics

Polluted areas – mostly decrease of retinoid levels in aquatic birds, mammals and fish

Disruption of retinoid transport: PCBs

Effects on retinoid receptors:

- RAR, RXR binding and/or transactivation – pesticides (chlordane, dieldrin, methoprene, tributyltin…)
- Effect on ATRA mediated response – TCDD, PAHs

Disruption of retinoid metabolism:

– PCDD/Fs, PAHs, PCBs, pesticides

- changes of serum concentrations of retinol and RA
- mobilization of hepatic storage forms
- in kidney, concentration of all forms elevated

Tests to assess retinoid signalling disruption

In vivo

- Mostly derived from classical toxicity tests, particularly of developmental toxicity
- Direct measurements of various retinoid forms in living organisms (laboratory and wildlife)

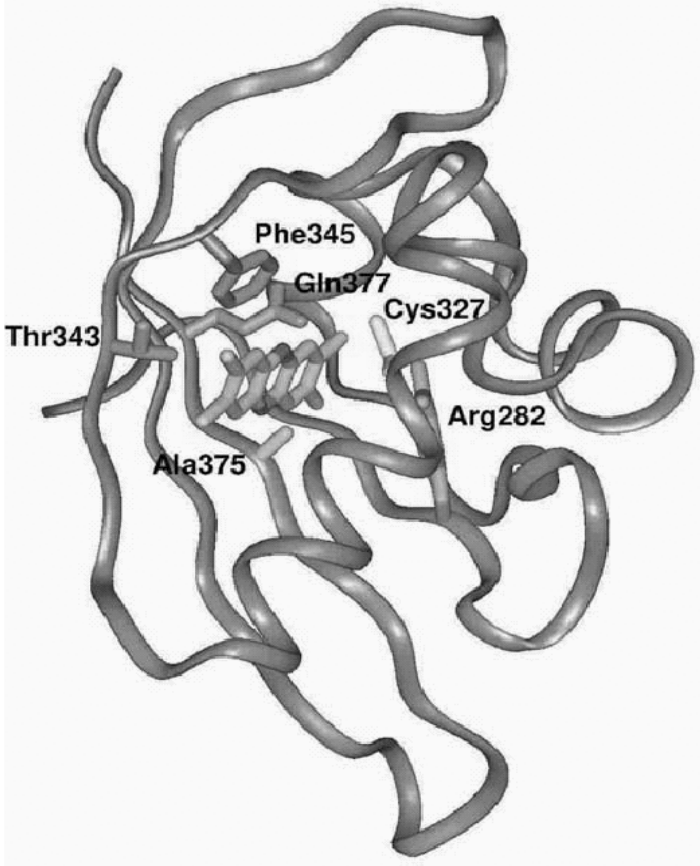
In vitro

- Mostly epithelial cell lines (keratinocytes)
- Mouse embryonic cell lines P19
  pluripotent cells
  differentiation dependent on circumstances, triggered by ATRA
  - reporter gene assay P19/A15
  - Other cell lines – rainbow trout gonads, human salivary gland, breast or prostatic carcinomas etc.

AhR (Arylhydrocarbon receptor)

AhR structure

Denison et al., Chem. Biol. Interact. 141: 3



?? Physiological role for AhR

⟶ Effects in AhR-deficient mice:

- significant growth retardation;
- defective development of liver and immune system;
- retinoid accumulation in liver;
- abnormal kidney and hepatic vascular structures.

- resistant to BaP-induced carcinogenesis and TCDD-induced teratogenesis;
- no inducible expression of CYP 1A1 and 2.

AhR

- ligand-activated transcription factor
- activation of different responsive elements (genes)
- important mediator of toxicity of POPs – primary target of coplanar aromatic substances
- regulator of xenobiotic metabolism and activation of promutagens
- crossactivation/crosstalk with other receptors
- strongest known ligand TCDD

Biological responses to TCDD

Schmidt & Bradfield, Annu. Rev. Cell Dev. Biol. 12:55

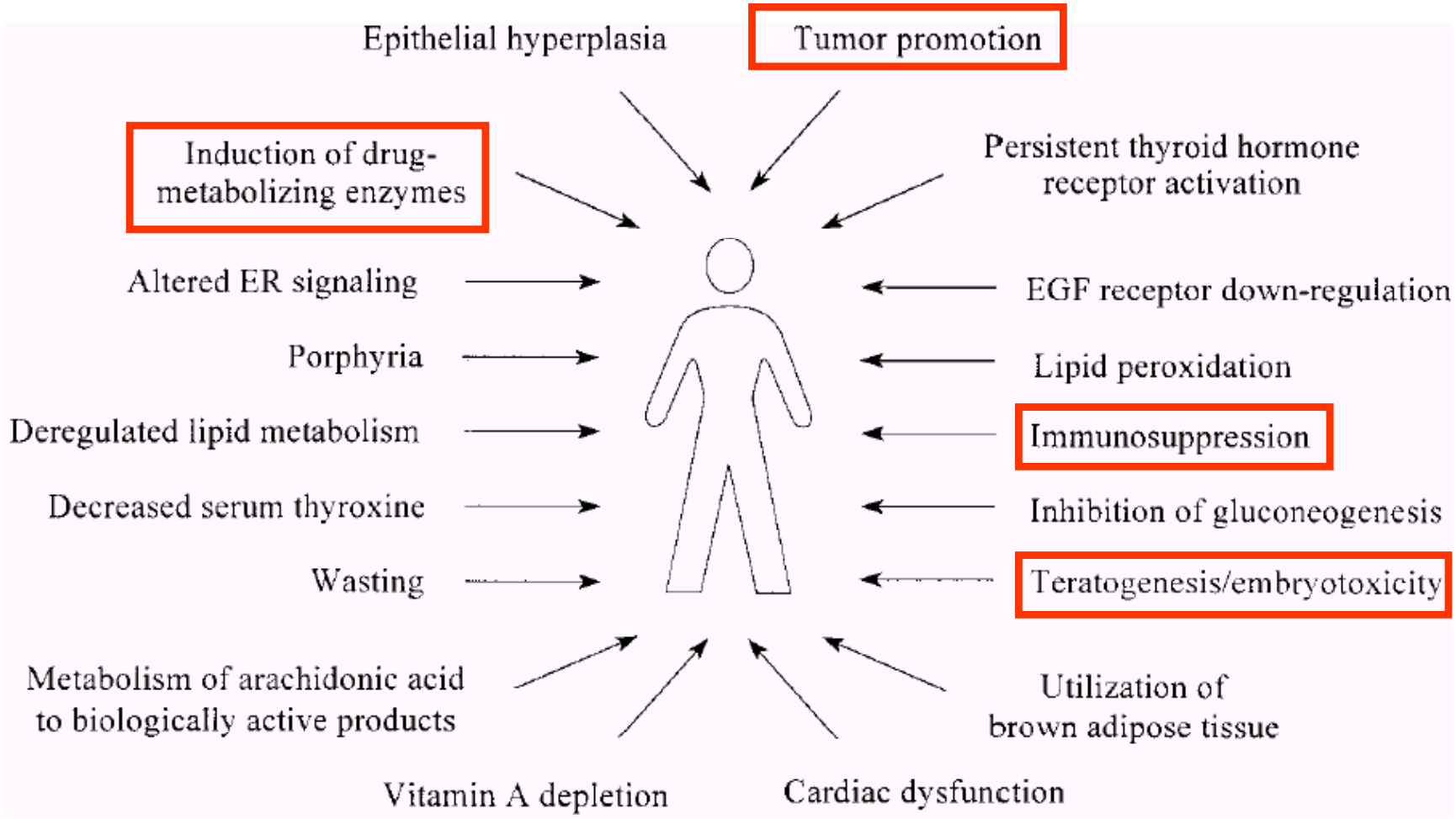


Figure 1 Biological responses to TCDD. A wide variety of cellular processes have been shown to be affected by TCDD.

AhR = cytosolic helix-loop-helix/PAS protein

PAS proteins:

R.J. Kewley et al. / The International Journal of Biochemistry & Cell Biology 36 (2004) 189–204

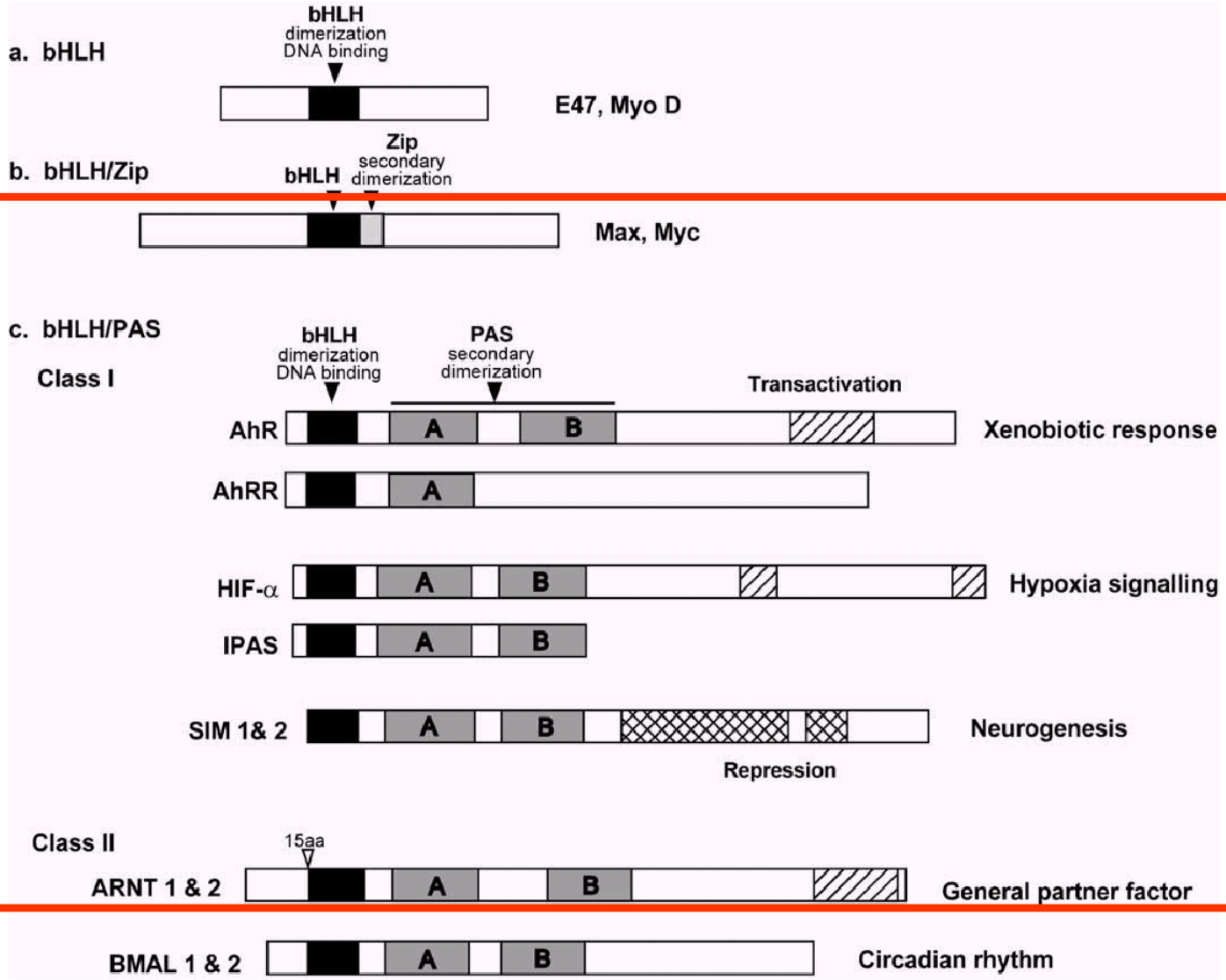


Fig. 1. Schematic representation of the domain structure of some bHLH transcription factor family members.

AhR domain structure:

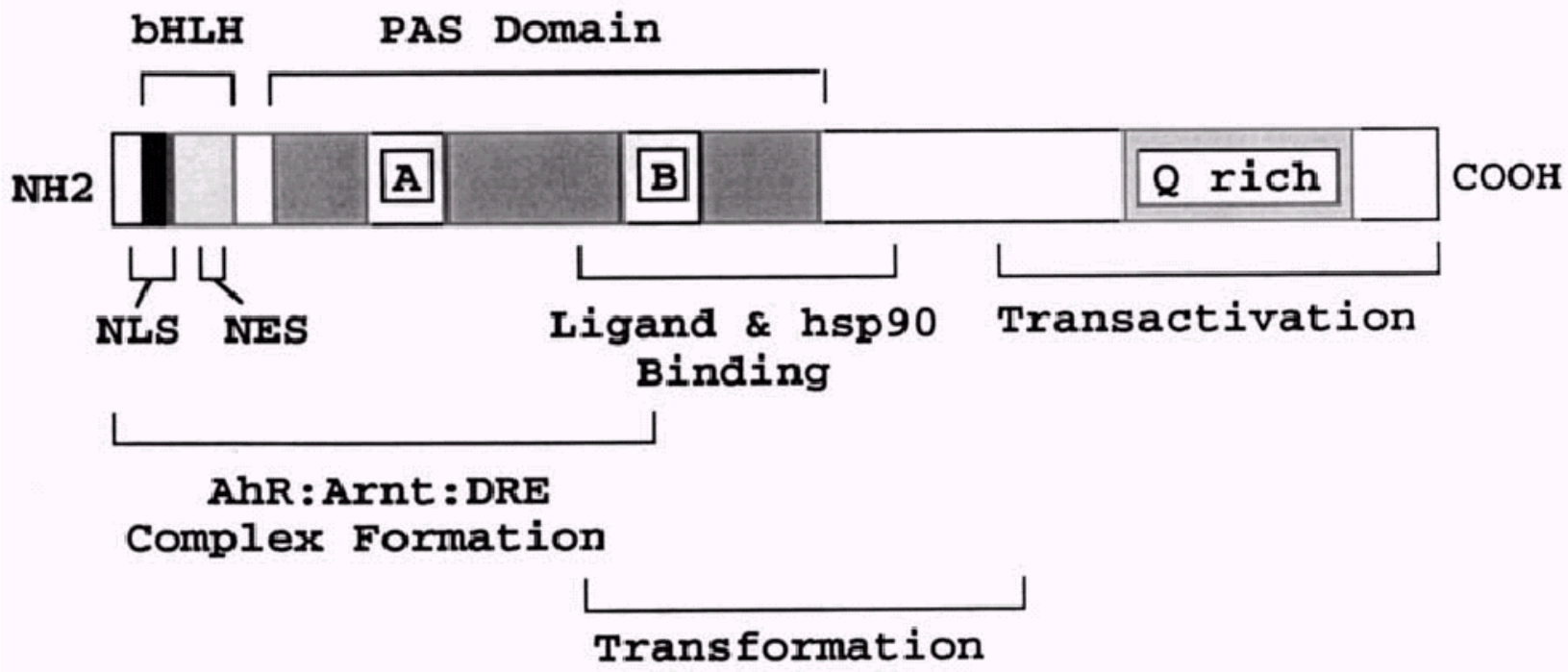
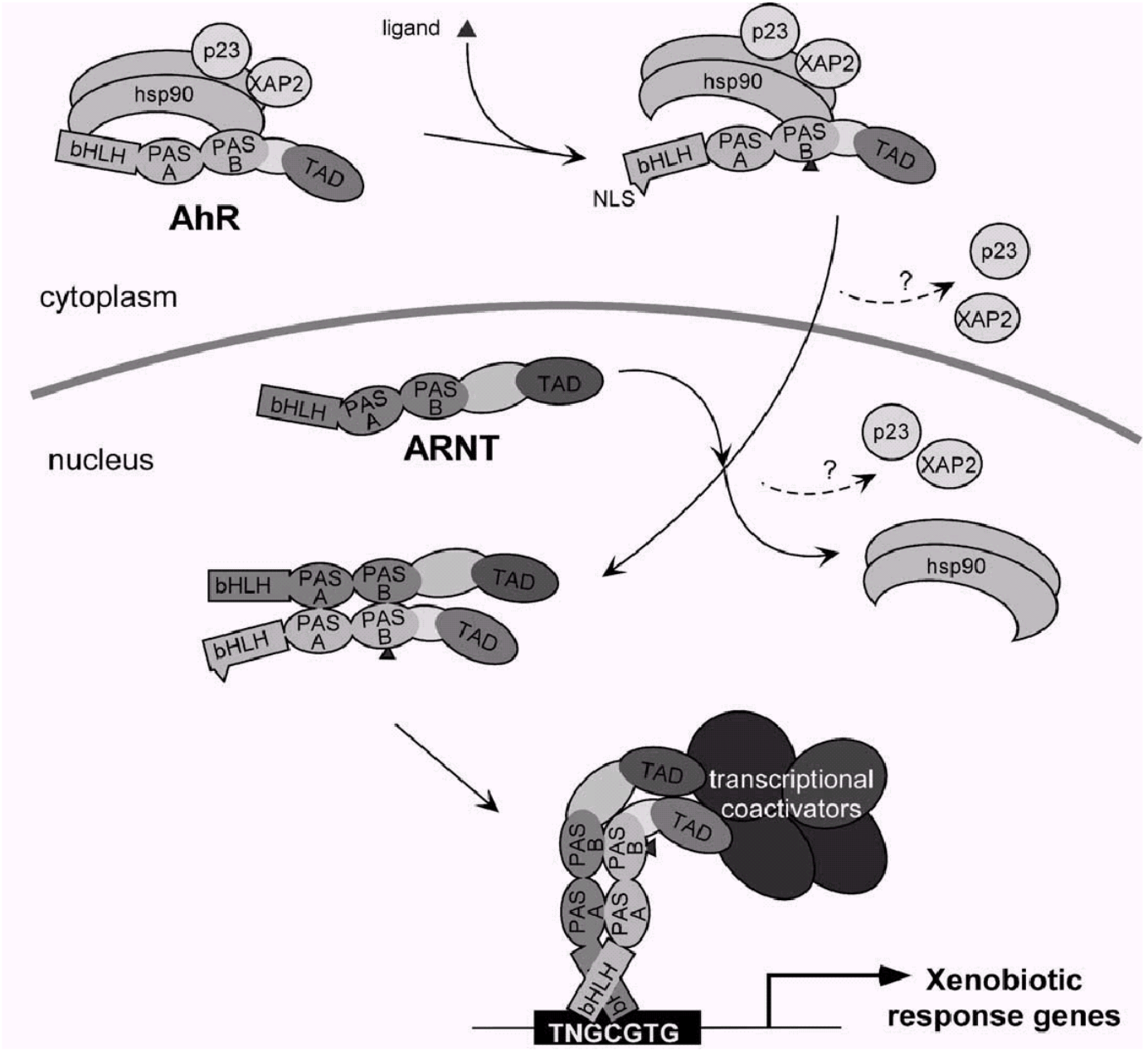


Fig. 2. Domain structure of the AhR.

Denison et al., Chem. Biol. Interact. 141: 3

AhR activation:

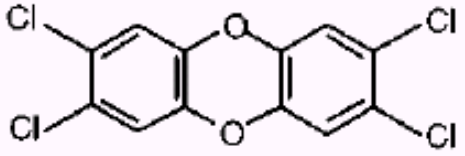


AhR regulated genes:

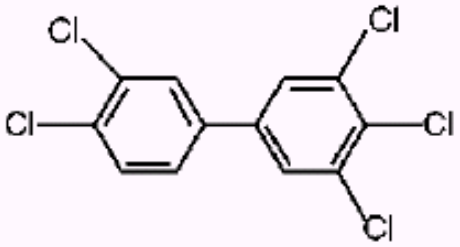
contain xenobiotic response elements (XRE) or dioxin responsive elements (DRE) in their promoter region:

- **phase I enzymes - *CYP 1A1, CYP 1A2, CYP 1B1;***
- **phase II enzymes - *UDP-glucuronosyltransferase, GST-Ya, NADP(H):oxidoreductase;***
- **other genes - *Bax, $p27^{Kip1}$, Jun B, TGF-β* - regulation of cell cycle and apoptosis;**

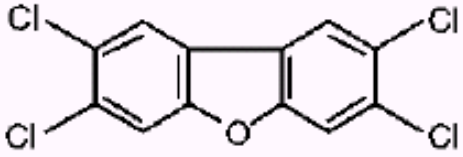
"Classical" AhR Ligands and CYP1A1 Inducers



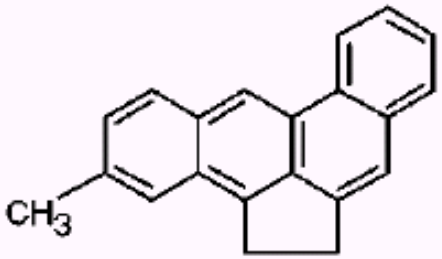
2,3,7,8-Tetrachlorodibenzo-p-dioxin



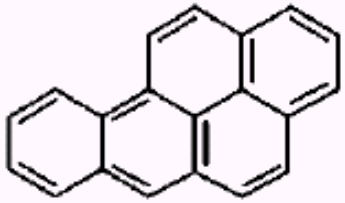
3,4,3',4,'5-Pentachlorobiphenyl



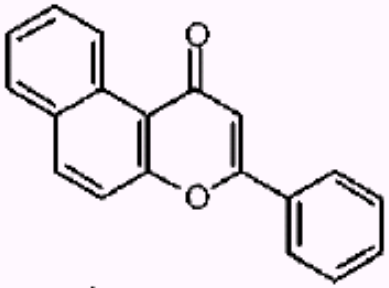
2,3,7,8-Tetrachlorodibenzofuran



3-Methylcholanthrene



Benzo(a)pyrene

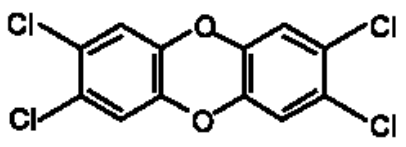


β-Naphthoflavone

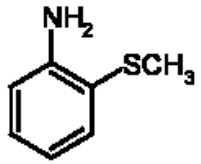
Denison & Nagy, Annu. Rev. Pharmacol. Toxicol. 43:309

„Non-classical“ AhR ligands

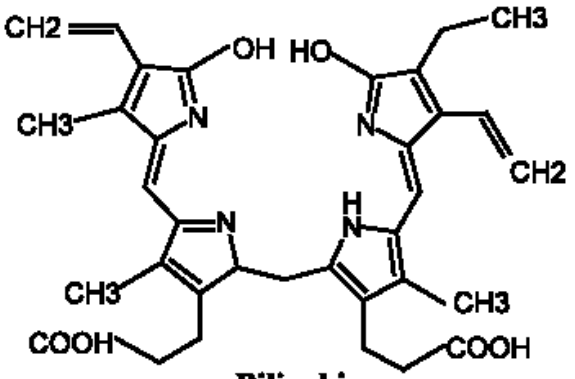
M.S. Denison et al. / Chemico-Biological Interactions 141 (2002) 3–24



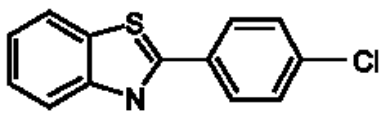
2,3,7,8-Tetrachlorodibenzo-p-dioxin



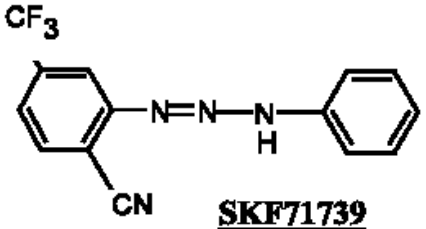
2-(Methylmercapto)aniline



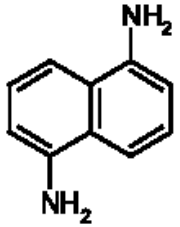
Bilirubin



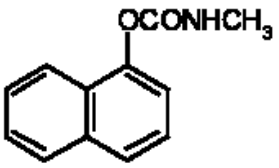
2-(4'-Chlorophenyl)benzothiazole



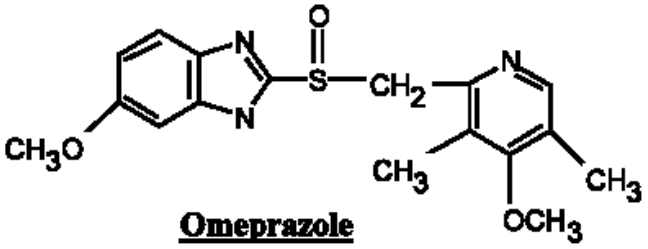
SKF71739



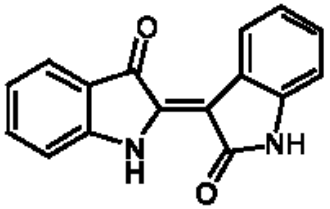
1,5-Diaminonaphthalene



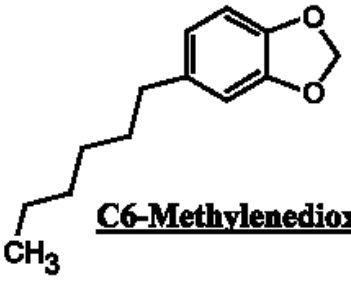
Carbaryl



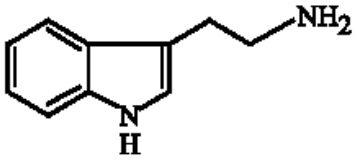
Omeprazole



Indirubin



C6-Methylenedioxybenzene



Tryptamine

Biomarkers/bioanalytical methods:

• ***in vivo*: liver enlargement, reduction of thymus weight, wasting syndrome, reproductive and developmental disorders**

• ***in vivo* biomarkers: EROD activity, CYP 1A1 and 1B1 expression;**

• ***in vitro:***

➔ **EROD in H4IIE rat hepatoma cells;**

➔ **CALUX/CAFLUX assays;**

➔ **GRAB assay (AhR-DNA binding)**

➔ **yeast bioassay;**

➔ **immunoassays;**

➔ **detection of CYP1A mRNA or protein**

Detection of EROD activity:

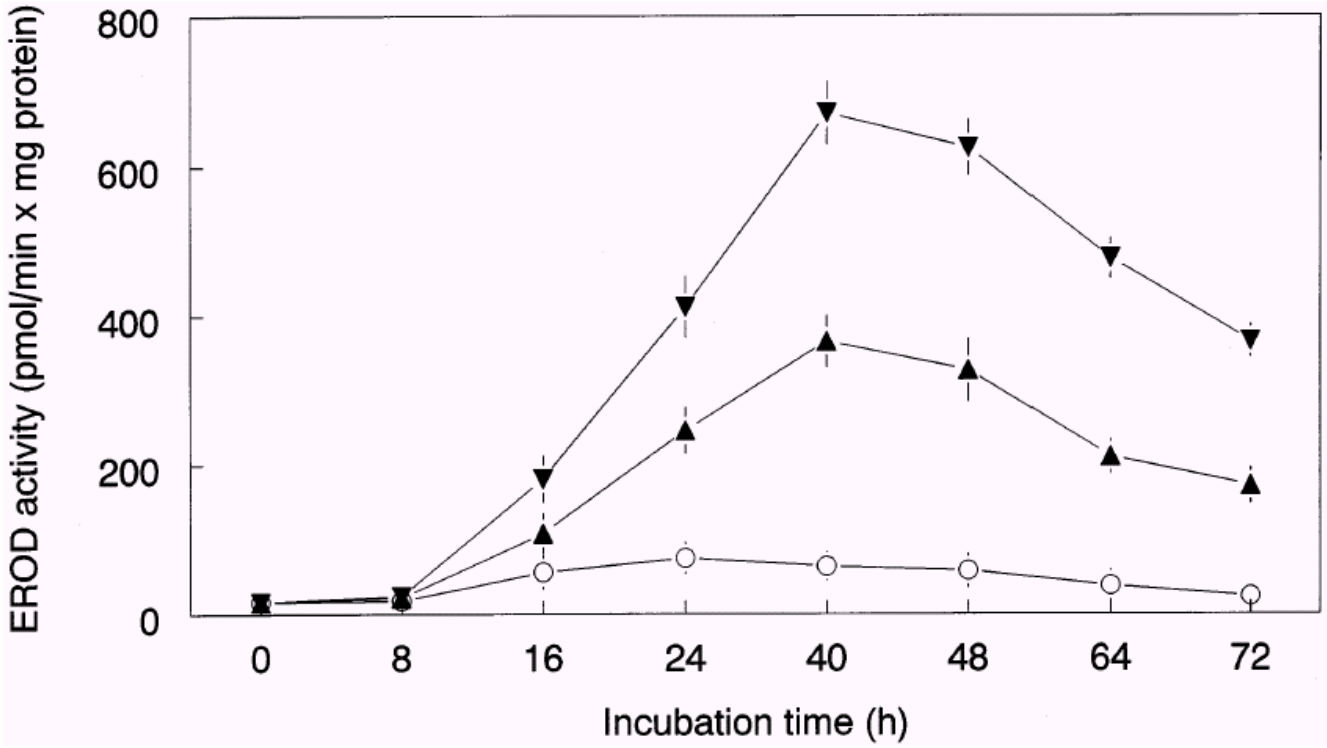
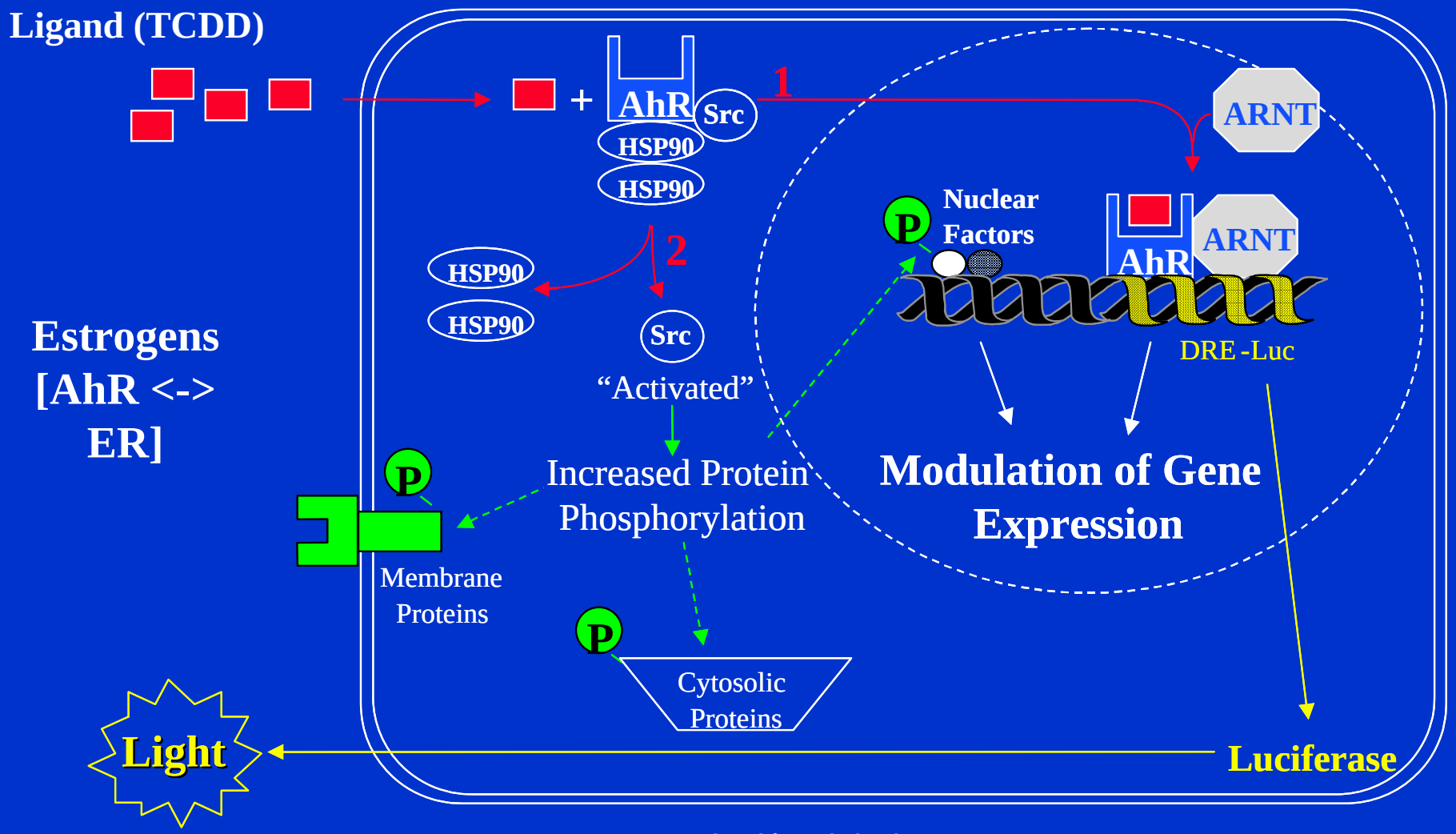


Fig. 2. Time course of induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity in primary cultures of rat hepatocytes, after addition of 1.7×10^{-5} M benzo[*a*]pyrene (-▼-), 1.9×10^{-6} M benzo[*k*]fluoranthene (-▲-) or 9.4×10^{-5} M acenaphthylene (-○-). EROD activity was determined in cell homogenates. The data represent means ± S.D. from four independent experiments.

In vitro assays for nongenotoxic (epigenetic) effects

AhR-mediated effects

luciferase reporter assay - H4IIE.luc cells

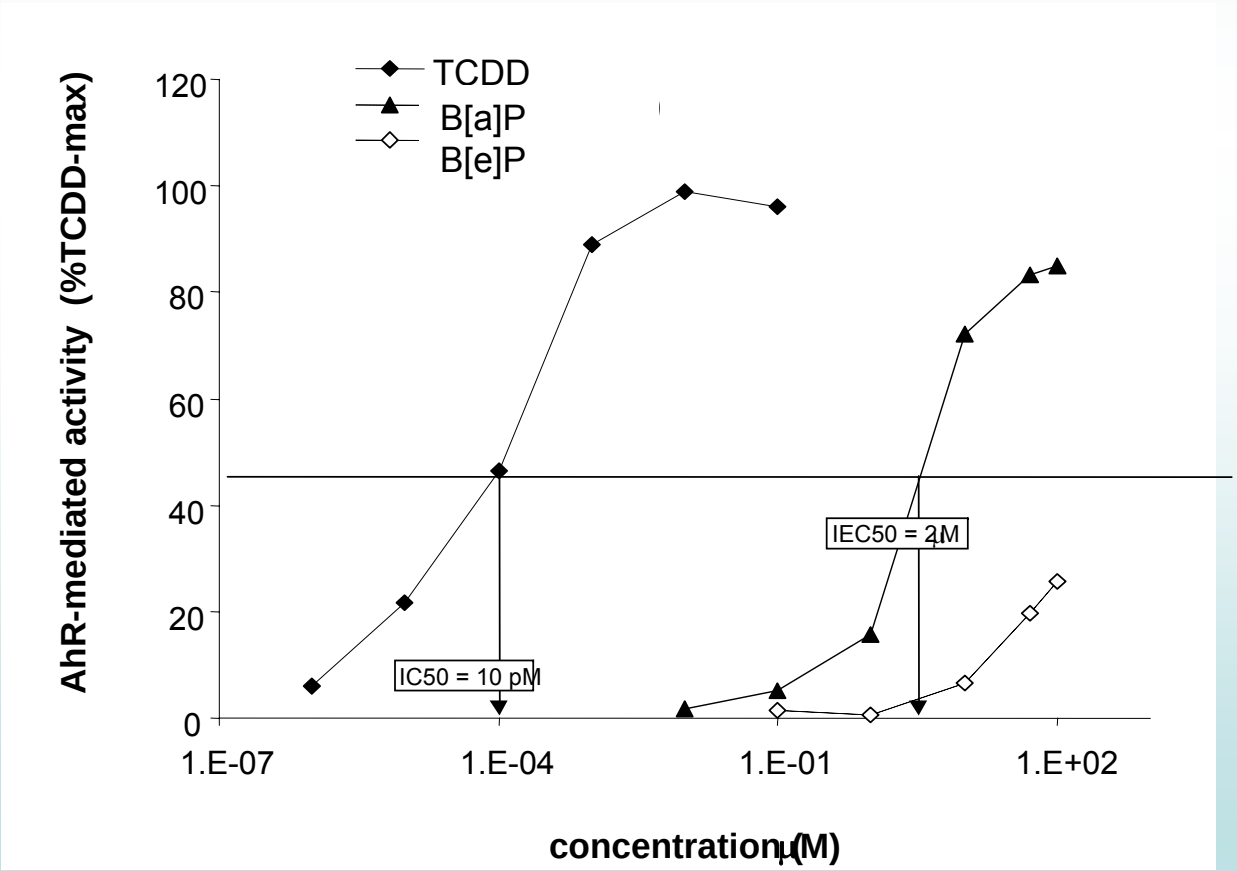


Adapted from Blankenship (1994)

CALUX/CAFLUX assays

Comparing compounds
-> Application in Risk Assessment

- Quantification of effects (EC_{50}) - relative potencies
- Comparison with the effect of reference toxicant (2,3,7,8-TCDD)
  - Expression as Equivalency Factors (~ TEFs)



TCDD: IC_{50}

PAH: IEC_{50}

Induction Equivalency Factor

$IEF = IC_{50} / IEC_{50}$

How many times is the compound "weaker" inducer than TCDD ?

Toxic equivalency factors (TEF)/TEQ concept:

TEFs provide a simple, single number that is indicative of overall toxicity of a sample containing a mixture of dioxins and dioxin-like compounds. TEFs are consensus values based on REPs across multiple species and/or endpoints. TEFs are based upon a number of endpoints, from chronic in vivo toxicity to in vitro toxicity with the former having the greatest importance in determining overall TEF.

The total potency of a mixture can be expressed in TCDD TEQ concentration:

$$\mathrm{TEQ} = \Sigma\{\mathrm{compound_1} \times \mathrm{TEF_1} + \ldots$$

$$+ \mathrm{compound_n} \times \mathrm{TEF_n}\}$$

Toxic equivalency factors for PCDDs, PCDFs and PCBs:

Table 4. Toxic Equivalent Factors established by the WHO (WHO-TEFs) for dioxins and dioxin-like PCBs [4]

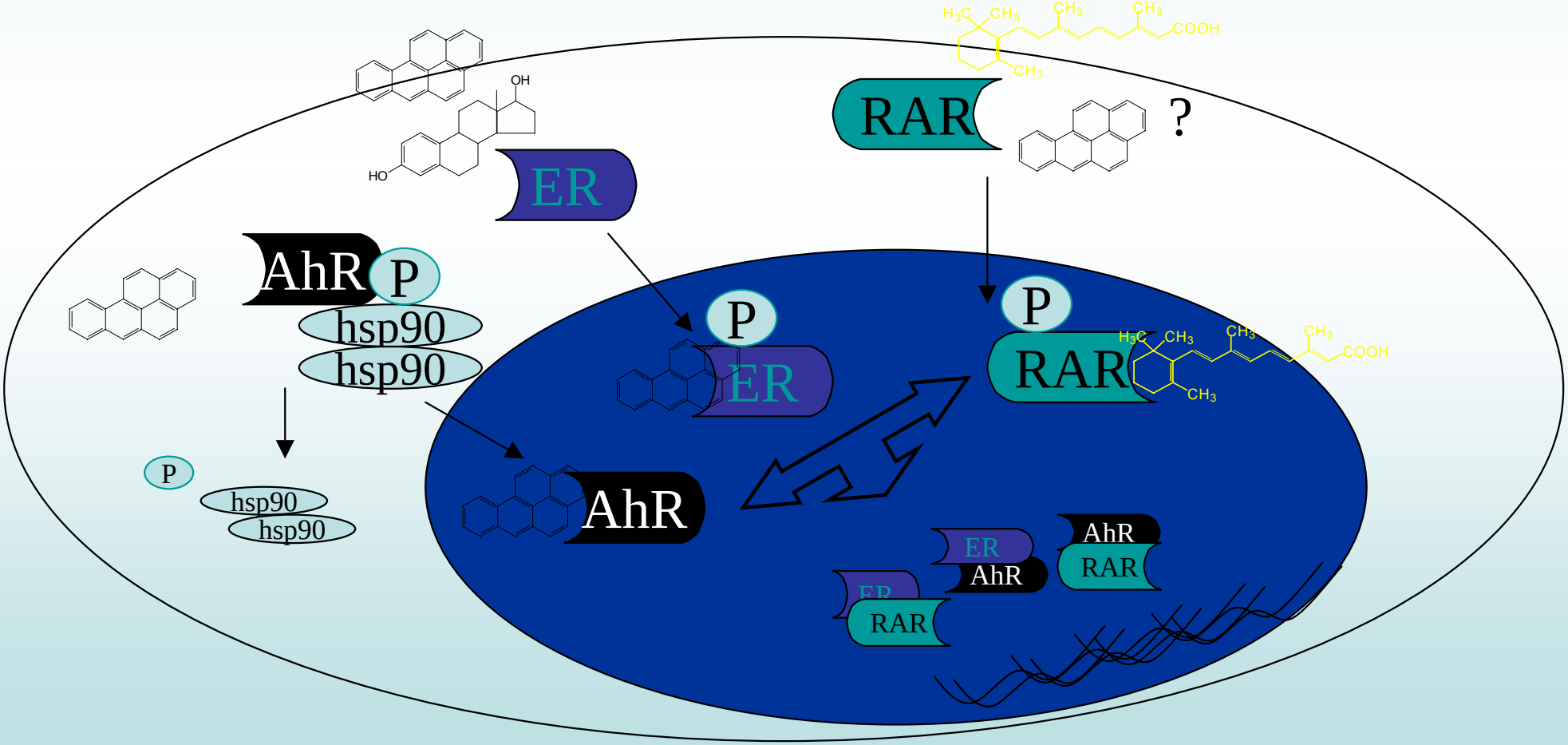
PCDD Congener	WHO-TEF	PCDF Congener	WHO-TEF	PCB Congener	WHO-TEF
2,3,7,8-TCDD	1	2,3,7,8-TCDF	0.1	*Non-ortho*	
12,3,7,8-PeCDD	1	12,3,7,8-PeCDF	0.05	PCB#81	0.0005
123478-HxCDD	0.1	23478-PeCDF	0.5	PCB#77	0.0005
123678-HxCDD	0.1	123478-HxCDF	0.01	PCB#126	0.1
12,3,7,89-HxCDD	0.1	123678-HxCDF	0.1	PCB#169	0.01
1234678-HpCDD	0.01	234678-HxCDF	0.1	*Mono-ortho*	
OCDD	0.0001	12,3,7,89-HxCDF	0.1	PCB#105	0.0001
		1234678-HpCDF	0.01	PCB#114	0.0005
		1234789-HpCDF	0.01	PCB#118	0.0001
		OCDF	0.0001	PCB#123	0.0001
				PCB#156	0.0005
				PCB#157	0.0005
				PCB#167	0.00001
				PCB#189	0.0001

Eljarrat & Barceló, Trends Anal. Chem.22: 655

Nuclear Receptors & Signalling Crosstalk

poorly characterized (toxicity) mechanisms

Nuclear receptors (AhR, ER, RAR/RXR ...) = Transcription factors with numerous cofactors and interactions (crosstalk)



Cross-talk between estrogen signalling pathways and other receptors

- estrogen signalling pathways and
  other members of nuclear receptor superfamily
- estrogen signalling pathways and AhR
- estrogen signalling pathways and receptors for EGF and insuline

Modulation of RAR/RXR : retinoic acid signalling

ATRA – important regulatory molecule

: cellular differentiation (embryotoxicity, teratogenicity), other biological events

Concentrations of retinoids are known to be modulated by PCBs (*? mechanism*)

In vitro assay for modulation of ATRA - RAR/RXR effects

Luciferase reporter gene assay (embryonic P19/A15)

RAR- dependent gene transcription