CELL SIGNALING

Professor Maria Luisa Genova – Medicine and Surgery Unibo 2020-2021

This document is a collection of the material that I used to prepare the cell signaling exam; it contains material provided by professor Genova, some of the sbobine of UniBo's Medicine and Surgery program (2019; 2020, 2021), and some personal notes. These notes do not follow the exact same order of Professor Genova's lectures, but I re-ordered them according to a personal preference.
I hope this can be useful for the preparation of the cell signaling exam! – Sofia Mincolelli

Note: info written in *italic* were not directly mentioned in class by the professor, but were present in her slides or in past-years' sbobine.

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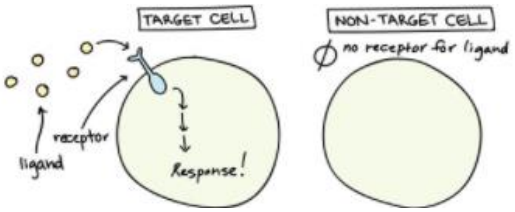
INTRODUCTION TO CELL SIGNALING

1 Lecture (15/03)

General features of cell signal transduction

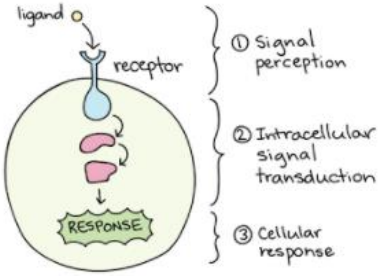
Cells need to communicate with each other: at any very moment, living cells are sending and receiving millions of messages in the form of physical signals or chemical signaling molecules.

Every **target cell** needs to be *responsive* to a signal, and to have a specific **receptor** for that signal, which is also called **ligand** (because it binds to the receptor). The binding of ligand to the receptor results in an altered shape of the receptor (because of its proteic nature). This conformational change will eventually lead to the desired **response** inside the target cell.



Remember that not all cells can sense a particular message: in the same environment, the same signal is recognized by a target cell that has a specific receptor, while it is not recognized by a cell that does not have the receptor for that particular ligand *(some proteins are synthesized only in some cells, depending on chromatin conformation)*.

The signals are often relayed through a chain of chemical messenger inside the cell.
Basically, extracellular signals are converted in intracellular signals that are often related through a chain of chemical reactions: this pathway of different chemical steps leads to a final response (*alterations in gene expression*, metabolic pathways etc.).



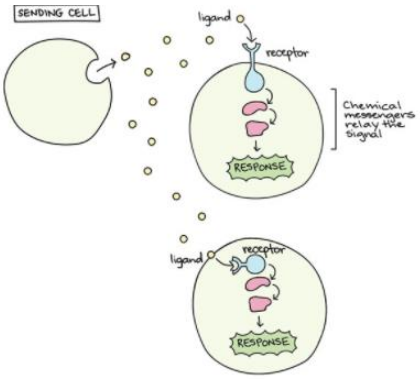
All kinds of signals (even physical signals) are converted to chemical signals by the receptor; e. g. photoreceptors and pressure-sensing channels.

Ligands come in many different varieties and interact with the receptor either from the surface of the cell, or from the inside**.**

Receptor location

Receptor location is important: a receptor can either be placed in the cytoplasm or on the cell membrane.

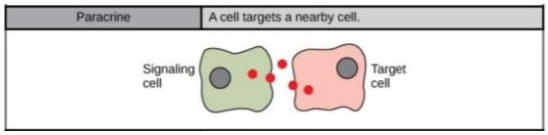
- Receptor on the cell membrane: the receptor has a domain that faces the extracellular environment and interacts with the ligand; and an intracellular domain that is able to activate the cascade of signals.
- Receptors in the cytoplasm: they can respond only to ligands that can cross the cell membrane *(mostly hydrophobic compounds)*.



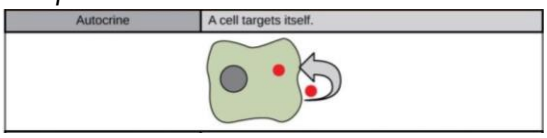
Signaling Classification

There are four basic categories of chemical signaling found in multicellular organisms: paracrine signaling, autocrine signaling, endocrine signaling, and signaling by direct contact. The main difference between the different categories of signaling is the distance that the signal travels through the organism to reach the target cell. This different is not important at a molecular level but is more significant at a physiological one.

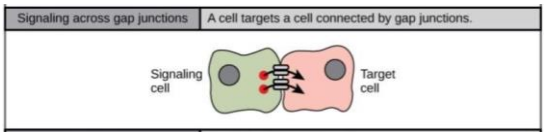
1) **Paracrine signal**: *often, cells that are near one another communicate through the release of chemical messengers (ligands that can diffuse through the space between the cells).* Cells communicate over relatively short distance (immediate surrounding area) *to locally coordinate activities with nearby target cells. These signals are especially important during development, when they allow one group of cells to tell a neighboring group of cells what cellular identity to take on*.



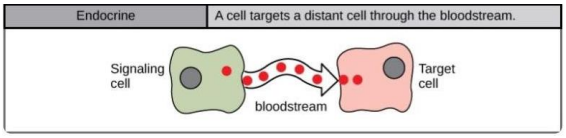
2) **Autocrine signal***: a cell signals to itself, releasing a ligand that binds to receptors on its own surface (or, depending on the type of signal, to receptors inside of the cell). For instance, autocrine signaling is important during development, helping cells take on and reinforce their correct identities. From a medical standpoint, autocrine signaling is important in cancer and is thought to play a key role in metastasis (the spread of cancer from its original site to other parts of the body).*
Usually it is used for negative feedback.



3) **Cell-cell contact**: *small signaling molecules called intracellular mediators diffuse through neighboring cells that are connected through gap junctions, that are tiny water-filled channels that directly connect the two neighboring cells (small molecules, such as calcium ions, are able to move between cells, but large molecules like proteins and DNA cannot fit through the channels without special assistance).*
This allows a group of cells to coordinate their response to a signal that only one of them received.



4) **Endocrine signal**: the molecules travel a long distance to reach the target. When cells need to transmit signals over long distances, they often use the circulatory system as a distribution network for the messages they send. In long-distance endocrine signaling, signals are produced by specialized cells and released into the bloodstream, which carries them to target cells in distant parts of the body. Signals that are produced in one part of the body and travel through the circulation to reach far-away targets are known as hormones.



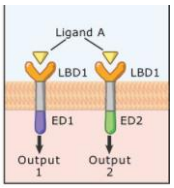
PDF: Extracellular movement of signaling molecules - Patrick Müller, Alexander F. Schier.

PRINCIPLES OF LIGAND-RECEPTOR INTERACTIONS

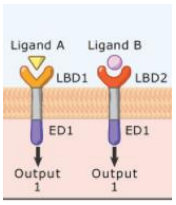
The **receptor** consists of two different **domains** *that allow the cell to regulate the binding of the ligand and the effect of the ligand* <u>*independently*</u>:

- Ligand-binding domain (LBD): involved in signal interaction
- Effector domain (ED): involved in second messenger interaction (response triggered inside the cell)

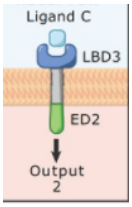
Expression of a receptor that is normally not expressed in a cell is often sufficient to confer responsiveness to that receptor's ligand: this responsiveness occurs because the cell already produces the other components necessary to propagate the signal.



- The two receptors have the same Ligand Binding Domain (LBD) and therefore bind the same ligand. However, they have different Effector Domains (ED), which means that they will lead to two different outputs, even if the ligand is the same. This is the case of **isoforms**, e. g. acetylcholine receptors.



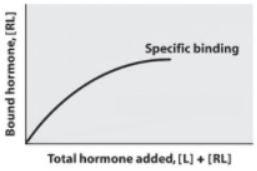
- The two receptors have different LBD, and therefore bind different ligands. But because they have the same ED, they will initiate the same pathway with the same output.
  Two different ligand-binding domains (combined with two specific hormones) might have the same effect; e. g. cAMP receptors.



- Chimeric receptors can be synthesized in the lab to obtain specific responses to a given hormone or to other specific ligands (such as drugs, even in particular pathological conditions.) *[Chimera = (in Greek mythology) a fire-breathing female monster with a lion's head, a goat's body, and a serpent's tail. In this case we could say "hybrid receptors", made with different components of receptors existing in nature.]*

Receptor can act to accelerate intracellular functions and are analogous to enzyme or other catalysts, but not all receptors catalyze reactions. The interaction between receptor and ligand is a stereospecific recognition, similar to the one between substrate and enzymes, thus, receptors and enzymes share all the basic **properties**:

- ✓ **Specificity**: ligand and receptor are complementary to each other, and are bound by weak, reversible bonds. *In multicellular organisms, there is also cell type tissue specificity (a specific receptor is not always the same in all the cell types of the organism).*
- ✓ **Affinity**: usually receptors have a high ka (association, affinity), and low Kd (dissociation): Kd=[r][l]/[rl] <10^(-7). This means that once the ligand is bound to its receptor it is very difficult for it to dissociate. The receptor detects micromolar to nanomolar concentration of the ligand (e.g. very low amount of hormone can be detected).
- ✓ **Saturation**: *occurs when the amount of ligands is greater than the amount of receptors.* The saturation curve is very similar to the ones of enzymes. After saturation, [L] does not affect [RL]: *there is no need of very high amounts of hormone to trigger a signaling pathway.*



- ✓ **Cooperativity**: small changes in ligand concentration can create large changes in receptor activation. *The binding of two (or more) similar molecules increases/decreases the receptor affinity for that molecule (/those molecules).* All the protein system of a receptor can adapt to the first ligand and become more prone to activate other protein receptors so that the signal will be higher thanks to a positive modulation (further enhancement).

Receptor states

In absence of the ligand:

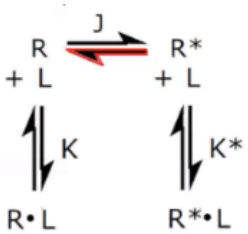
Receptors can exist in inactive (**R**) or active (**R***) molecular conformations, these two forms are found in equilibrium. The equilibrium constant is indicated as **J**:

- **J** is very low: **R**, the inactive form (with no response in the cell), is the most probable and frequent. The reaction is shifted towards the inactive form.
- **J** must be greater than one: there are few receptors (less than 1%) in the active form (**R***) without the ligand binding. This due to the fact thar receptors are proteins, and they can change their conformation due to different conditions, such as changes in temperature or PH, even in absence of a ligand.

  Note: Overexpressed receptors frequently display their intrinsic low activity. When there are too many receptors, the total amount of active receptors would be high (like the amount of activated receptors with ligand in a normal condition), although the ratio would stay low.

  E.g. this problem can be found in cancer cells that have multiple copies of particular receptors that overstimulate specific signals even without the hormone in the bloodstream.

In presence of the ligand:

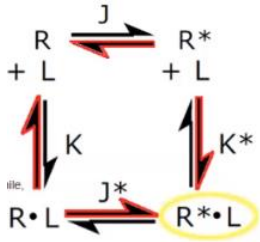


Ligand binding changes the receptor conformation and its relative affinities for the receptor's **R*** and **R** conformations is described by association constant K for the RL states and K* for the R*L state. Chemical manipulation of a ligand's structure varies its selectivity between R and R* and can often alter its affinity for the two conformations

- Ligands that bind equally well to both the R and R* states, reaching the equilibrium between RL and R*L, are called <u>competitive inhibitors (antagonists)</u> because they do not cause activation but may still occupy the binding site and thereby competitively inhibit binding of an activating ligand. *They are frequently used as drugs to block unwanted activation of a receptor in various disease states. Some drugs can stay bound to receptors and leave them in the R state (i.e. inactive state) for long periods: this is a way to intentionally block the response in the cell. The same process can happen in diseases.*
- Ligands that bind with higher affinity for the **R*** conformation *(J*/J>>1* and K*/K>>1*)* are called **ligand activators (agonists)**. The ligand stays bound to the R* state instead of being separated, the favored shift is towards the active form, R*L.
- Ligands that bind with higher affinity for the **R** conformation (J/J*>>1 and K/K*>>1), are called **inverse agonist**. They will shift the equilibrium to the inactive state causing the net inhibition.

  *They can separate from the inactive receptor or force changing shape and reach the **R*** conformation:* in this case the RL pair remains inactive for a short while, and then becomes active (shifting to the **R*L** state): J*/J>>*1*.

  → *coupling of ligand binding and effector domains isomerization.*



Following these two different pathways, the sum of the energetic steps to reach the R*•L state is the same (thermodynamically speaking): ***path independence in a system of coupled equilibria:***

J x K = K x J* and J*/J = K*/K:* these are two pathways for getting to R*•L.

<u>PDF: 100 years of modelling ligand–receptor binding and response: A focus on GPCRs - David B. Finlay, Stephen B. Duffull, Michelle Glass</u>

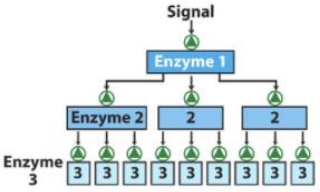
2 Lecture (16/03)

Multistep signalling pathways

Multistep signaling pathways allow cells to:

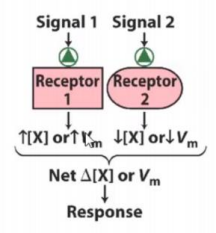
- Amplify signals
- Integrate multiple signals
- Insert control points (the more steps, the more control points there will be)
- Route signals to distinct effectors (cell can deviate the signals from one pathway to another)

Amplification:



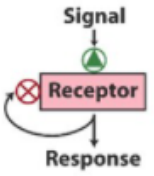
In all signaling reactions, receptors use their catalytic activities to function as molecular amplifiers.
Molecular amplification is a hallmark of receptors: they generate a chemical signal and typically initiate a sequence of regulatory events that is huge with respect to the number of molecules recruited by a single receptor.
One ligand can interact with many partners (forming RL complexes) in a branched pathway, and this results in multiple copies of a second enzyme and so on (enzyme cascade). *The number of affected molecules increases geometrically within milliseconds.* A signal can have several levels of amplifications by different enzymes, thus, increase the concentration within the cells. *In this way cells can react "strongly" even if the concentration of hormones is very low in the blood.*

Integration:

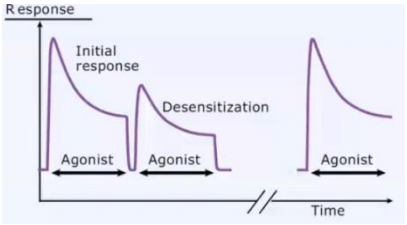


Multiple signals (with opposite/similar effects) combine to produce a unified response.
There can be different types of signals reacting with different types of receptors but at the end within the cell they result to be integrated to produce a unified response.
The reactions function as mathematical logic to integrate information.
The final output corresponds to the summation of the different phenomena.

Desensitization/Adaptation:



The cell can decide what is the destiny of hormonal signals that come from the outside. If the cell is not able to produce the molecular intermediates itself, then any stimulus coming from the outside will stop. This is a very important point, because it means that the cell has an active role in establishing the responsiveness to the outside signal.
Once the cell has received and processed the signal arrived from outside, it can give a **feedback** to that signal in order to abandon it for a certain period of time. That is called desensitization or adaptation or circuit feedback.



When a signal is present continuously, the receptor no longer responds until the stimulus falls below a certain threshold, then sensitivity slowly returns to normal. After the process of desensitization, the cellular response has a lower plateau than the initial peak response, but after a specific period of time the receptors will turn to their original state and the cell will give a more efficient response. A second similar stimulus will elicit a smaller response unless adequate time is allowed for recovery.

More sources for this course:
Cell signaling | Biology library | Science | Khan Academy
Cellular Landscapes: Receptor Tyrosine Kinase (cellsignal.com)

CLASSIFICATION OF RECEPTORS

There are two major categories of cellular receptors:

Intracellular receptors (localized inside the cell):

- Steroid receptors: intracellular receptors (they are not located in the plasma membrane, but in the nuclear environment of the cell or in the cytoplasm). The ligand is usually a steroid (hydrophobic and lipophilic) able to cross the membrane and reach the receptor: the interaction activates a cascade that usually has the genome as main target.

Membrane receptors (localized in the plasma membrane):

- **Metabotropic receptors**:

  • Serpentine receptors: the external ligand, when bound to the receptor, activates a G protein, that stands for "GTP-binding protein", which is already inside the cell. The activated G-protein regulates an enzyme, whose purpose is to build a certain product, the second messenger, that has its own target too. This interaction results in alteration of the pre-existing enzyme and of its final product, affecting metabolism.

- **Catalytic receptors:**

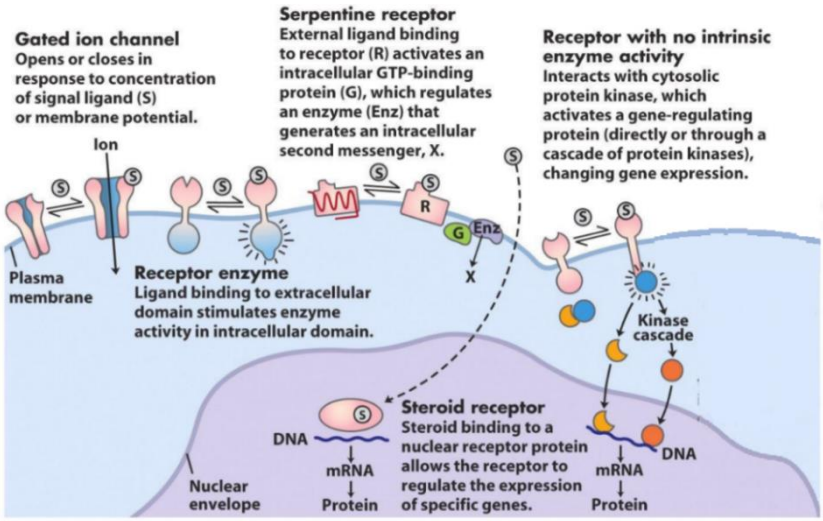
  • Receptor enzymes: transmembrane receptors that can bind the ligand on the outside of the cell; after the interaction, the inner domain of the receptor becomes an active enzyme with a binding site for a substate that is converted into a specific product.

  • Receptors with no intrinsic enzyme activity: as a consequence of the ligand binding, the inner portion of the receptor can bind to an "additional subunit", that has enzymatic activity. In some cases, the signal created can also reach DNA in the nucleus and change gene expression.

- **Ionotropic receptors:** ion channels that open or close in presence of a ligand:

  •Acetylcholine receptors
  • GABA receptors
  • Glutamate receptors
  • Serotonin receptors
  • Glycine receptors

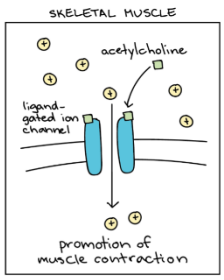
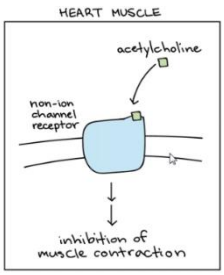
All the ligands for the receptors mentioned here are neurotransmitters, *but some of them are excitatory (acetylcholine for the nicotinic receptors) while others are inhibitory (GABA for GABA receptors)*



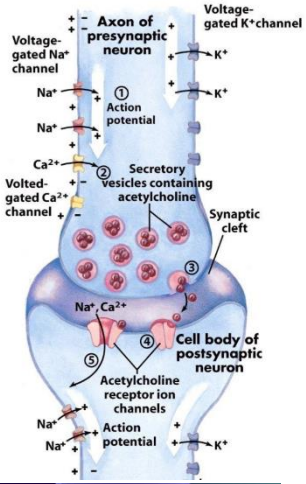
IONOTROPIC RECEPTORS

Acetylcholine receptors

Acetylcholine is a key excitatory neurotransmitter in neuromuscular junctions and in some synapses. There are two main types of receptors involved in the interaction with acetylcholine.

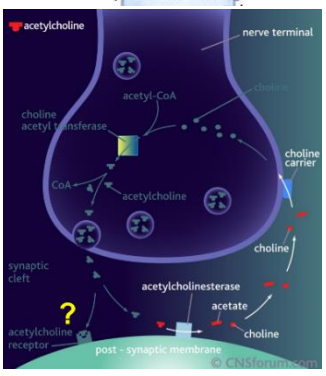
Ionotropic receptor: Nicotinic receptor/ Ligand gated ion channel/ Cationic gated channels Able to shift from a close to an open conformation in presence of the ligand	
Metabotropic receptor: Muscarinic receptor/ Non-ion channel receptors (Do not create a channel, but a different type of response). *Inhibit muscle contraction.*	

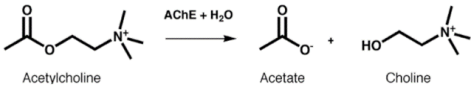
Nicotinic receptors are post synaptic ligand-gated cation channel that convert chemical signal into electric modifications. *This type of receptor is found in neuromuscular junction, sympathetic and parasympathetic ganglia, adrenal medulla, and central nervous system.*



Choline acetyl transferase is an enzyme that adds choline to acetyl-coA to form acetylcholine. The neurotransmitter is then released from the presynaptic neuron into the synaptic cleft. A-Ch can have two destines:

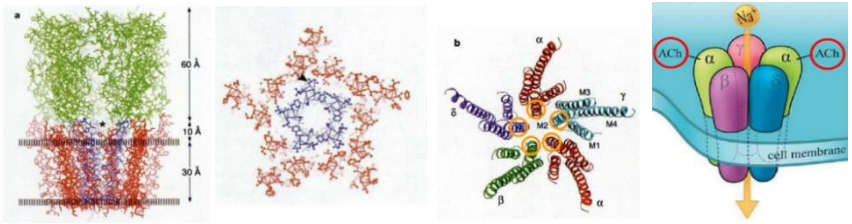
- Acetylcholine reaches the receptors located on the membrane of the postsynaptic neuron (*or myocyte*): it stays on the outside of the cell, and its interaction with the **acetylcholine receptor ion channels** causes the channel to <u>open</u>, so that Na^+ and Ca^{2+} enter the postsynaptic neuron (*the cations enter the cell thanks to the concentration gradient, not to the electrical gradient*). The equilibrium of charges changes, resulting in <u>depolarization</u> of the cell and formation of an action potential.
- The molecules of acetylcholine that do not bind to the receptor, interact with acetylcholinesterase, an enzyme located on the extracellular surface of the postsynaptic neuron: this enzyme converts acetylcholine into:
    - **Choline**: this molecule re-enters the presynaptic neuron thanks to choline carriers and is recycled
    - **Acetate**: is transported and metabolized by <u>astrocytes</u>, where it can be converted by Acetyl-CoA synthetase enzyme into Acetyl-CoA, now being able to enter mitochondria being a substrate in the <u>Three Carboxylic Acid cycle</u> (TCA cycle <u>or Krebs cycle</u>).
      It can be also converted into GABA.





NOTE: Acetylcholine never enters the postsynaptic neuron: all the reactions occur in the extracellular space. It does not *pass through the nicotinic channel, it only binds to the channel's ligand-binding site in the synaptic cleft, opening it. It has more than one receptor in different tissues (is an exception to the <u>1 hormone → 1 receptor</u> rule).*

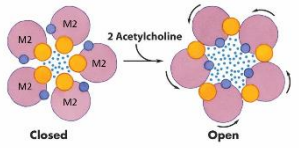
A nicotinic receptor has a pentameric structure, meaning that it is composed of 5 subunits (2 α subunits, β, γ, δ) which shape the core of the channel). Each subunit is a transmembrane protein that has 4 α-helix structures called M1, **M2**, M3, and M4 (the M2 ones delimit the inner surface of the channel) that can create a lipophilic domain on the surface of the protein, interacting with the membrane. *The ligand-binding domain, in the peripheral portion of the receptor, comprises β-sheet structures.* Only the 2 α-subunits are able to bind ACh (since there are 2 α-subunits, to open the channel, 2 molecules of ACh are needed).



PDF: Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore

The ligand interacts only with the surface of the receptor, so how can it affect the conformational changes on the inside of the receptor? The binding of the ligand on one subunit, affects other subunits through the transmission of a conformational change (allosteric interaction).
Nicotinic receptors have an open and a closed conformation. The closed one is determined by the presence of 5 α-leucine 257 sidechains (hydrophobic aa because it has an apolar sidechain) on the M2 helices in the center of the channel, which blocks the passage of cations.

An allosteric interaction between 2 molecules of ACh and a nicotinic receptor (through its α subunit) induces a rotation in the M2 helices, which now will expose in the center of the channel a different residue's side chain. The new residue will be hydrophilic, having a polar sidechain.
In this way the diameter of the pore is bigger and the flow of cations through it is allowed.
Changing the diameter of the pore also changes its functional state:
small diameter → closed conformation / big diameter → open conformation.



Although the concentration of ACh is high, the channels can still switch to their closed conformation due to the desensitization process. In this case ACh is defined as **transient**, because its effect is limited to a specific time period; after some time, it can't open the channel's pores anymore (similar to GABA receptor, in the image):

- No ligand: small diameter and close conformation
- With ligand: larger diameter and open conformation
- Intermediate state: the aa in specific regions move again to close a specific section of the receptor: ions can enter only the first part of the receptor but are not able to cross the membrane (even in presence of ACh). When this happens ACh accumulates in the synaptic cleft where it interacts with acetylcholinesterase.

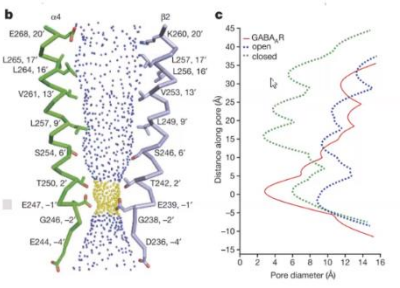


Figure 3 | Ion permeation pathway.
b, M2 α-helices from opposing α and β subunits with side chains shown for pore-lining residues. Blue spheres indicate pore diameters >5.6 Å, yellow are >2.8 Å and <5.6 Å.
c, Pore diameter for representative receptors in distinct functional states: desensitized/closed (red, $GABA_AR$), activated/open (blue) and resting/closed (green). Structures were aligned using the M2 helix 9′ leucine, which occurs at *y*=15 Å. The zero value along the *y* axis in the plot is aligned with the α-carbon of the M2 helix −1′ glutamate residue.

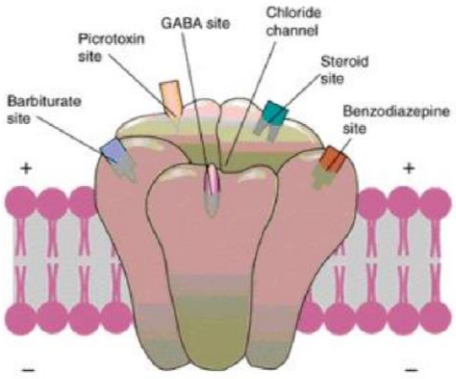
PDF: Unwin. Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy
PDF: Unwin; Fuji Yoshi. Gating Movement of Acetylcholine Receptor Caught by Plunge-Freezing
PDF: Morales; Perez. X-ray structure of the human α4β2 nicotinic receptor

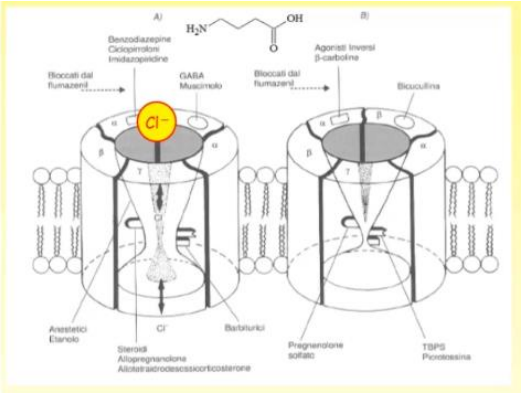
GABA receptors

GABA (γ-aminobutyric acid) is a type of neurotransmitter, and it is the ligand for GABA receptors.
GABA receptors have a pentameric structure and are ligand-gated ion channels which allow the passage of $\mathbf{Cl^-}$ only when the ligand is bound. This is an important difference with the Acetylcholine receptors where only positive charges can pass: since the selectivity for the ions is different, there will be a different effect on the distribution of charges and on the membrane potential. When chlorine passes it emphasizes the basal polarity of the membrane (hyperpolarization), which already has negative charges in the intracellular part. Therefore it increases the transmembrane potential of that neuron. *Remember that the higher the transmembrane potential is, the less active that neuron is*. GABA as a ligand when bound to its receptors allows the passage of chlorine making the neuronal transmission less effective: GABA is the main inhibitory neurotransmitter in CNS.

GABA channels, as many others, have many binding sites other than the GABA site; on other subunits of the receptor there are in fact allosteric binding sites for modulators.
In the case of GABA receptors, different drugs, such as benzodiazepines, steroids, alcohol, barbiturates and picrotoxins can affect the possibility of GABA to open the channel, increasing or decreasing GABA effectiveness.

- Those that increase the effectiveness of GABA are called **anxiolytic** (more chloride enters in the cell)
- Those that reduce the effectiveness of GABA are called **anxiogenic** (they do not allow chloride ions to enter in the cells)**.**





Glutamate, serotonin and glycine are other neurotransmitters that have ionotropic receptor.

CLASSIFICATION OF LIGANDS

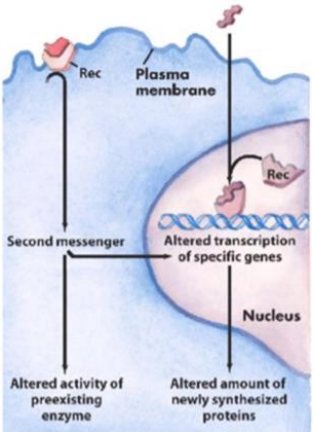
3 Lecture (18/03)

There are two schools of thought about the classification of chemical signals:

Type: the classification by type is more helpful from the physiological point of view. It considers the function, but the molecular structure is not defined. ➢ Neurotransmitters ➢ Hormones ➢ "Local hormones" (autacoids) e.g. cytokines ➢ Growth factors	**Structure**: this classification instead is much clearer as it is based on the biochemical properties of the signals. • Amino acid derivatives • Polypeptides • Eicosanoids • Steroids

Hormones

Hormones are small molecules or proteins produced at very low dosage by an endocrine tissue and carried to target tissues through the bloodstream. The amount of hormones usually available in the bloodstream is quite low: the physiological concentration is around **10^{-9}-10^{-15} M**.
Why? Target cells usually remove extra hormones to prevent the amplification of their signal: this is why only few extra molecules (in respect to those that are actually needed by the target cell) are synthetized. In this way, the cell does not have to remove many extra molecules and can save some energy.



Hormones are typically divided into two categories based on their solubility.

Hydrophilic hormones *(left side of the image)* Hydrophilic hormones are polar and water soluble, thus they are not able to cross the plasma membrane: they bind to receptors on the exoplasmic face of the membrane and **do not enter the cell**. When they bind, they trigger conformational modifications of their receptors and cause a change in the intracellular concentration (increase/decrease) of low molecular weight metabolites, called second messengers. The function of second messengers is to: • **Alter the activity of pre-existing enzymes**, already available in the cytoplasm. *The alteration could be positive, resulting in an activation of the enzyme, or negative, resulting in an inhibition.* • Enter the nucleus and affect the transcription of genes (not the major function). Metabolic response: usually **rapid** because they interact with pre-existing enzymes. They are usually: Amino acid derivatives; Peptides (insulin, glucagon, epinephrine).	**Lipophilic hormones** *(right side of the image)* Lipophilic hormones can cross the cell membrane and enter the cell. Their receptors are found in the cytoplasm or in the nucleus, according to the specific type of hormone. They form a complex with their receptor and **interact with DNA.** Their action alters the expression of specific genes: the different transcription, results in translation of a **different enzyme complement** of the cell. Metabolic response: the effects are observed after hours or even days because transcription and translation need time to change the amount of proteins in the cells. The signal itself is fast, but all the cascade processes involved **requires time.** They are usually: Steroids; Retinoids; Thyroid hormones

It is possible to distinguish hormones from a biochemical point of view:

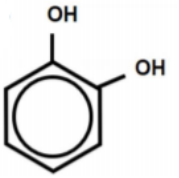
1. Peptides:

Peptides that act as hormones are usually very small; and can be formed by a variable amount of amino acid residues, from 3 to more than 200.

They are usually synthetized and stored into high-density secretory vesicles as precursor proteins (pro-hormones), that are inactive. When the hormone has to be released, the pro-hormone is proteolytically cleaved in the gland to form the active and mature peptide. *The cleavage usually cuts the carboxyl- and amino-terminal residues, in order to remove charges.* This is a case of *regulated* exocytosis because the hormone can be released only after a specific signal.

E.g. Pancreatic hormones (insulin, glucagon, somatostatin); Parathyroid hormones (calcitonin); Hypothalamus and pituitary gland hormones

2. Catecholamines:



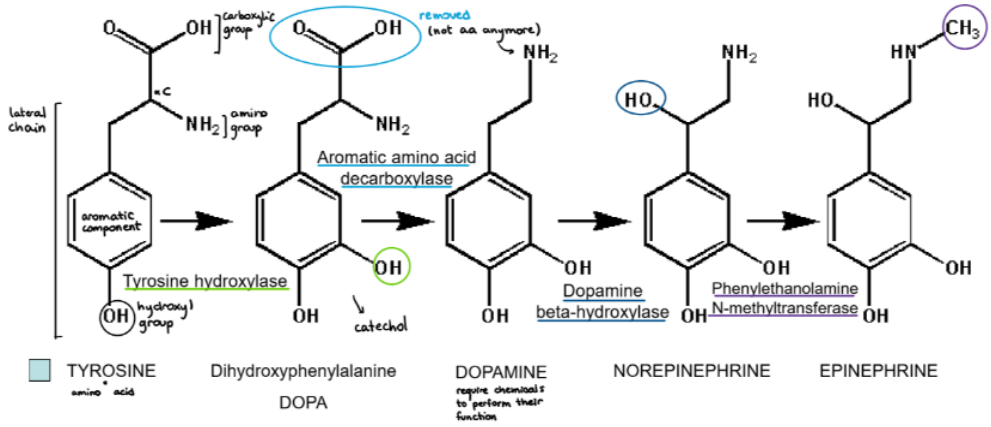
Catecholamines are named after the structurally related compound catechol (1,2-dihydroxybenzene), a structure which has six carbons and two hydroxyl groups. *They are not built up around the catechol, which thus is NOT the precursor of catecholamines, but it is a recurrent structure in these particular molecules.*

They are mainly produced in the brain and other neural tissues, and they serve as **neurotransmitters**. *There is a controversial debate, regarding whether catecholamine should be seen as neurotransmitters or hormones. As we will see, they can be classified under both categories.*

They can be seen as hormones when they are produced by the **adrenal glands**, in which exocytosis takes place. After they are released, they flow in the bloodstream looking for metabotropic receptors.

E.g. epinephrine (adrenaline), norepinephrine (noradrenaline)

Biosynthesis: the amino acid tyrosine is the substrate required for the biosynthesis of both nor-and epinephrine. All the intermediate products seen in the biosynthetic process are classified as **catecholamines**. *The most important step is the cleavage of the carboxylic group from the tyrosine, which is still present in DOPA, but it is absent in the following steps. Also, tyrosine misses one hydroxyl group (characteristic of catechol molecule) fundamental for the molecule to be considered a catecholamine.* It is important to consider that adding a molecule and forming a new covalent bond is a process that requires energy; therefore, all the steps of the process need to be catalyzed.



Also Dopa and Dopamine are elements in the category of hormones.

3. Eicosanoid

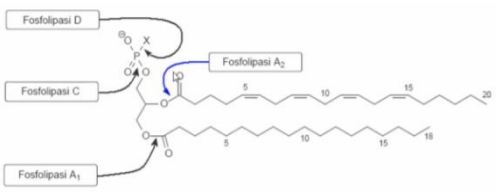
Eicosanoids are all derived **from arachidonate/arachidonic acid**, that is a polyunsaturated fatty acid (20:4), which has 4 double bonds and is composed of 20 carbon atoms. Fatty acids are the basic components of lipid, thus eicosanoids (hormones that derive from them) are lipophilic. Because of this characteristic they can easily cross membranes and cannot be stored in vesicles *or endocrine tissue.* Most of them are **paracrine hormones**: they are secreted into the interstitial fluid of cells and act nearby. They are highly produced in mammalian tissues.

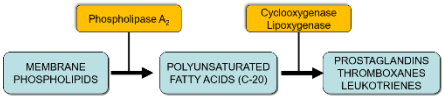
E.g. prostaglandins, thromboxane, leukotrienes.

Biosynthesis: the biosynthesis of eicosanoids depends on the availability of hydrolases (**phospholipases** A2 and others), able to release arachidonate from the middle carbon of glycerol**.**

Usually the concentration of **free** arachidonic acid is very low. It is mainly stored as a phospholipid and inserted into the membrane. For this reason, the main source of arachidonic acid is the hydrolysis of a glycerol phospholipid which has arachidonic acid as fatty acid chain.

Phospholipases A, C and D can cleave phospholipids in different positions: especially phospholipase A can cleave the ester bond between the carbonyl of the fatty acid chain (arachidonic acid in this case) from the oxygen of the glycerol backbone. Note that A1 phospholipase releases the acyl acid linked to the Carbon 1; while phospholipase A2 releases the acyl acid attached to Carbon 2.

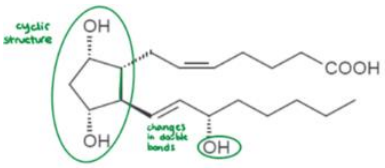




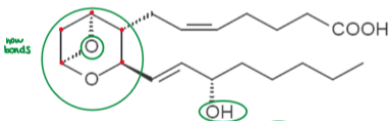
The polyunsaturated fatty acids (C20) obtained from the previous cleavage have to be processed by **lipoxygenases** and **cyclooxygenases**, in order to produce the 3 different types of eicosanoids. Both the enzymes involve the addition of oxygen atoms to the arachidonic acid: the final molecules are a little more hydrophilic than the arachidonate, thanks to the presence of -OH groups. These additions are also necessary for the recognition between their receptors and these hormones.

The three types of eicosanoids are:

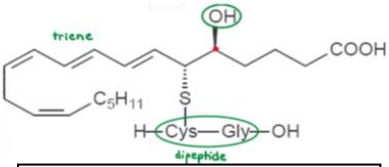
- Prostaglandins [PG]: the complete name of the prostaglandin in the image is **PGF2** α; it is a vasoconstrictor produced by the uterus when stimulated by oxytocin: it acts on the uterine and bronchial smooth muscles; used to induce labor and terminate pregnancy (pharmaceutically termed Dinoprost).



- Thromboxane's [TX]**: TXA2** in this case, are also vasoconstrictors produced by endothelial cells, macrophages, and platelets. *They amplify platelet activation and recruit additional platelets to the site of injury :* "blood cell signaling".

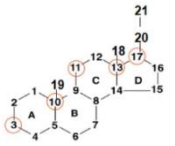


- Leukotrienes [LT]: they are inflammatory mediators. *The arachidonic acid is oxidized by 5-lipoxyngenases on the nuclear envelope to form leukotrienes.* They have a role in airway inflammation, producing mucus hypersecretion, *increasing vascular permeability and mucosal edema, inducing bronchoconstriction.* Their name suggests their origin and structure: in fact, leukocytes produce them, and they present at least 3 conjugated double bonds, alternated with single bonds.



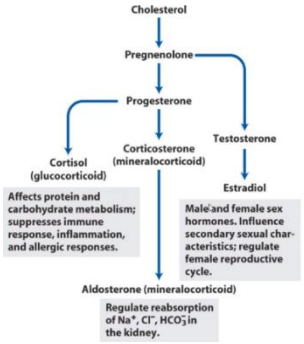
F, A, D = kinds of substitutions in the ring
2, 4 = n° of unsaturation

4. Steroids



Steroids are lipophilic hormones derived from the cyclopentane perhydro phenanthrene (<u>sterane</u>), which is a component of cholesterol (cholesterol is an amphipathic molecule that has a hydrophilic OH group, and a big hydrophobic component). Sterane, the basic structure of the steroid molecules, is composed by 21 carbon atoms and 4 rings. Steroid hormones need to have the 4 rings, and at least 17 carbon atoms; they are hydrophobic and are not storable in cells but travel in the blood for long distances thanks to carrier proteins that allow them to circulate in an aqueous solution (blood and lymphatic system). *Some carrier proteins are albumin, which binds with low affinity to the hormone, and α-globulins. The role of steroids carriers is to carry them to the target cells/tissue, and to protect them from metabolites that could inactivate them: if this happens, the hormone would not be able to recognize its specific receptor, blocking the entire metabolic pathway.*

Hormones derived by cholesterol (will be explained in future lectures):

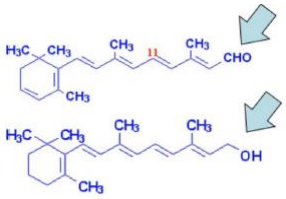


- ***Pregnenolone****: is not released in the blood, it is an intermediate molecule that will produce two different proper hormones (progesterone and testosterone);*
- ***Glucocorticoid*** *(e.g. cortisol) is a category that derives form progesterone: they affect carbohydrate and protein metabolism; are involved in the immune, inflammation and allergic responses;*
- ***Mineralocorticoid*** *is another category deriving from progesterone: from corticosterone we can obtain aldosterone, which is involved in the reabsorption process of ions in the kidneys;*
- ***Sex hormones*** *(e.g. estradiol) derive from testosterone: they influence the secondary sexual characteristics and regulate the female reproductive cycle.*

5. Retinoid

Retinoid hormones derive from vitamin A. They are lipophilic hormones, so they act at a nuclear level.

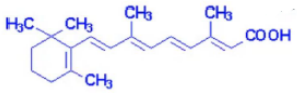
<u>Biosynthesis of retinol</u> (Intestine)



The human body does not produce endogenous β-carotene, so an exogenous uptake is necessary, in order to produce Vitamin A.

β carotene is cut by β carotene dioxygenase to obtain two molecules of **retinal**, which is an aldehyde molecule that is converted into **retinol** (**vitamin A**) by *retinaldehyde* reductase (NADPH requiring enzymes), replacing the aldehydic group with the alcoholic group. In mammalian cells, vitamin A (acquired by the nutrients) can be esterified and delivered to the liver, then to the blood via chylomicrons.

<u>In the liver</u>: the uptake of chylomicron remnants results in delivery of retinol to this organ for storage as a lipid ester within lipocytes. Tansport of retinol from the liver occurs by binding of hydrolyzed retinol to aporetinol binding protein (RBP). The retinol-RBP complex is then transported to the cell surface within the Golgi and secreted. Plasma transport of retinoic acid is accomplished by binding to albumin.

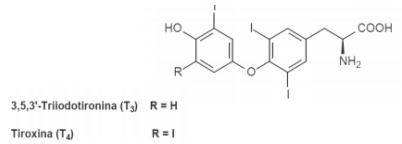


<u>Extrahepatic tissues:</u> retinol is bound to cellular retinol binding protein (CRBP). It is converted to all-trans-retinoic acid (the alcoholic group is transformed into a carboxylic group), that can reach the nucleus and be recognized by nuclear receptors, altering gene expression. In this capacity retinol and retinoic acid are considered lipophilic hormones.

6. Thyroid

Thyroid hormones are lipophilic molecules (have nuclear receptors), but they derive from peptides. **Thyroglobulin**, a protein precursor with numerous enzymatically condensated residues of iodinated-Tyr, accumulates in thyroid follicles (colloid), found in thyroid glands.

The precursor is modified before being released in the blood by a proteolytic process stimulated by factor TSH: the modification leads to two different hormones that are **triiodothyronine** (T3) and **thyroxine** (T4).

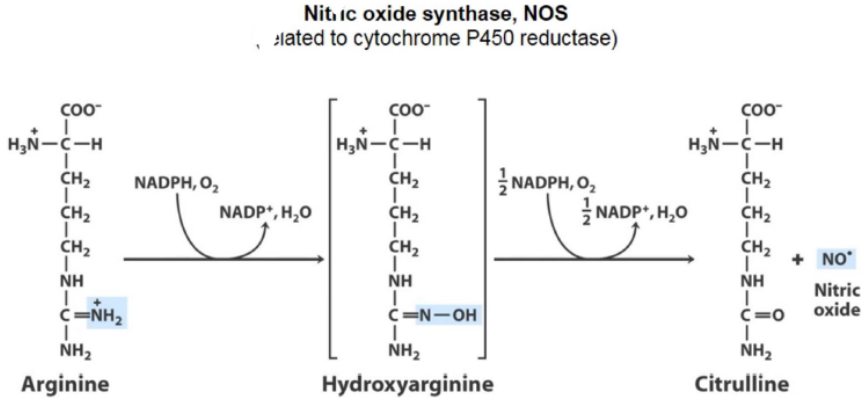


Biosynthesis: *starting from thyroglobulin, the hydroxyl groups of the original tyrosine can be replaced by iodine groups: in order to obtain the hormone, it is necessary to separate these residues from the whole protein.*

7. Nitric oxide

Nitric oxide is a gas composed by nitrogen and oxygen (NO), a free radical looking for other molecules that can bind. Given that it is very reactive, it is not carried for long distances in the blood and for this reason, it is not considered as a proper hormone. However, it has its proper receptor, thus it can generate bio-signalling processes in target cells. It acts near its synthesis/release site, and it is freely diffusible through membrane because it is a small molecule. Nitric oxide is also referred to as the endothelium-derived relaxing factor (EDRF).

Biosynthesis: it is obtained from arginine in a cascade of reactions that remove the nitrous functional group and add molecular oxygen, obtaining nitric oxide. The enzyme that catalyze the reaction is called **nitric oxide synthase** (**NOS**). In different cells, there are several families of NOS: the common feature of all the members is that the prosthetic group is related to cytochrome P450 reductase.



The 3 different genes that encode for different NO synthases are:

- ***nNOS*** *(NOS1): found in neurons;*
- ***iNOS*** *(NOS2): found in macrophages (the "I" stands for "inducible" because its synthesis can be induced by environmental factors).*
- ***eNOS*** *(NOS3): found in endothelial cells that line the lumen of blood vessels.*
  In these 3 types of cells, nitric oxide is synthesized as a hormone.

INSULIN

4 Lecture (07/04)

Frederick Sanger (awarded with the Nobel prize in 1958) is considered nowadays the father of the chemistry of insulin. Its 12-year work on the structure of insulin, allowed him to identify the primary structure of the protein, and to observe that:

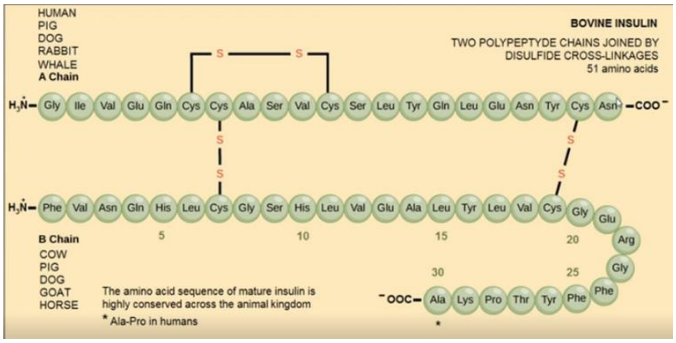
- "Two of the commonly occurring amino acids, tryptophan and methionine, were absent". This conclusion resulted from his accurate analysis of the aa sequence.
- The molecule presents a "high content of free α amino groups": he used the number of N-terminal residues to determine the number of chains (*2*).

PDF: "The chemistry of insulin" -Frederick Sanger (Nobel Lecture, December 11, 1958)

Primary structure of insulin:

Insulin is a very small protein (51 amino acids), composed of two polypeptide chains:

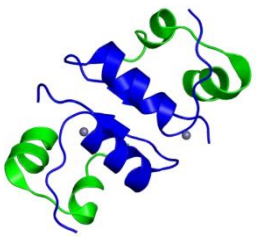
A chain (acid): composed of 21 amino acids.
B chain (basic): composed of 30 amino acids.
The two chains are connected by 2 disulfide cross-linkages (that link cysteine residues of the two peptides); a third disulfide linkage connects two amino acids belonging to the same chain.
Since the polypeptide chains are two, there are two amino terminal groups ($-NH_3^+$) and two carboxyl terminal groups ($-COO^-$).



The amino acid sequence of mature insulin is highly conserved across the animal kingdom. Many animal species share exactly the same amino acid sequence. For example:

- The A chain is typical of humans, pigs, dogs, rabbits, and whales.
- The B chain is typical of cows, pigs, dogs, goats, and horses. Humans possess almost the same sequence, but the last amino acid (the one signaled with a star) is a threonine instead of an alanine.

Secondary, tertiary and quaternary structure of insulin:

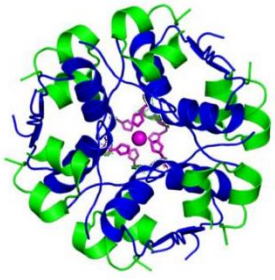


Secondary structure reveals that:

- The longer chain, the B chain, forms an α-helix and a β-strand.
- The shorter chain, the A chain, consists of two α-helices.

This is the mature form of insulin molecule, as secreted by the pancreas.

The two disulfide-linked polypeptides associate with another insulin molecule to form a *homo*dimer (*through electrostatic interactions*).



The dimer then aggregates with two additional dimers to form a hexamer.
The hexamer is assembled around two zinc ions at its center which are coordinated by a histidine residue from each monomer (*6 His residues in total*). Insulin can bind more zinc (four zinc hexamer). *The bond between zinc and the pentameric structure is not covalent, but it stabilizes the hexameric geometrical conformation.*

Within the pancreatic β cells, proinsulin is stored in this hexameric, inactive and very stable form. The hexameric form cannot be found in blood because the mature form secreted by pancreas is the monomeric form.

Biosynthesis

1. **Translation and translocation:**

   The gene encoding for insulin is transcribed into a mRNA, which is then translated into pre-proinsulin by ribosomes bound to the membrane of the endoplasmic reticulum. The protein is directly delivered into the lumen of this organelle.

   Pre-proinsulin is a single, non-mature peptide chain (110 amino acids) composed of:

   - A-chain (21 aa)
   - C-peptide (31+4 aa) that will be removed in mature insulin
   - B-chain (30 aa)
   - Signal sequence (23+1 aa): it is a transmembrane domain found at the N terminus, that anchors the immature protein to the ER membrane and directs the passage into secretory vesicles; it is removed in mature insulin

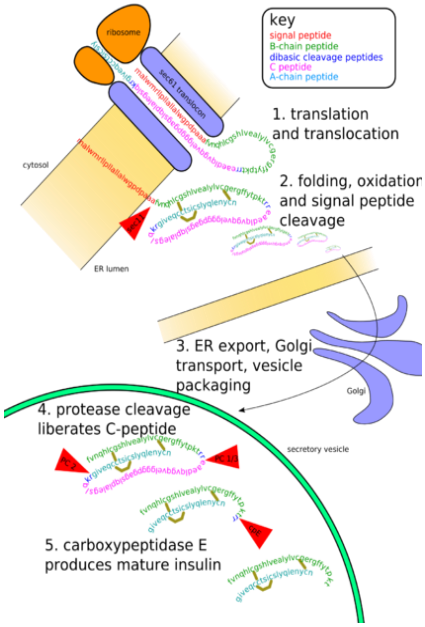
   Note: A and B chain are encoded by the same chain and separated only after the maturation process

2. **Folding, oxidation, and signal peptide cleavage**

   After the proteolytic removal (sec11) of the sequence signal, the peptide is no longer anchored to the ER membrane; *also the oxidation of cysteine residues results in the* creation of the 3 disufide bonds: proinsulin is formed.

3. **ER export, Golgi transport, vesicle packaging**

   Vesicles containing proinsulin bud from the ER and move into the Golgi apparatus, where proinsulin is stored, in hexamer form, in the secretory granules.



Only when blood glucose is sufficiently elevated proinsulin is converted to mature (active) insulin:

4. **Protease cleavage liberates C peptide**.

   Proteases, also known as prohormone convertases (PC-1, PC-2), cleave the protein at specific positions (*dibasic cleavage peptides*) by disrupting the peptide bond between two specific amino acids: they remove the C-peptide (31 amino acids), detaching the A-peptide from the B-peptide.

5. **Carboxypeptidase E (CPE) produces mature insulin**.

   CPE removes aa at some terminus, resulting in the creation of mature insulin, made by 51 amino acids. Active insulin is then released into the blood by exocytosis, together with the C-peptide

Since only monomeric insulin is able to bind to its receptor in the liver and adipose tissue, insulin hexamers must dissociate to be biologically active. This process happens by the removal of the zinc in the bloodstream once insulin is released from the β-cells. ***The zinc dissociation occurs spontaneously****: as soon as the Zinc meets with the high water-content in the blood, it is diluted (surrounded by water molecules). The coordinative bond is broken, and active insulin is released. Different pH values between the blood and the storage vesicles cause a difference in dissociation efficiency because the molecules are kept together by electrostatic interactions and different pH means different proton levels.*

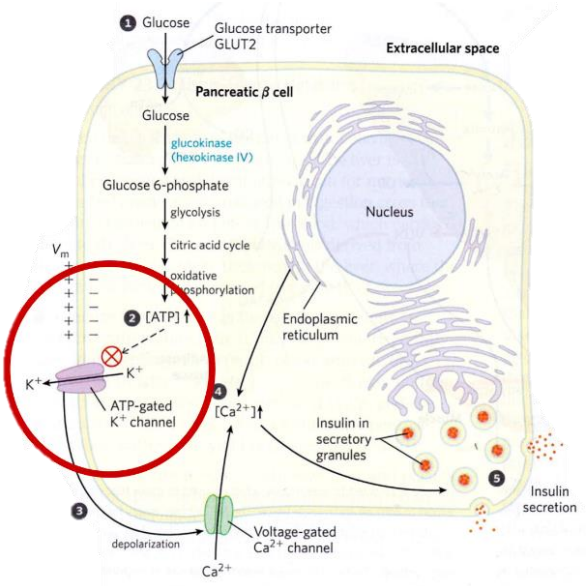
Q: What type of insulin should be injected in the derma (skin) as a drug? How will it reach the active state? (PDF)

Secretion

Pancreatic beta-cells secrete insulin in response to changes in blood glucose:

1. An increased glucose concentration in the bloodstream lets glucose diffuse into the pancreatic β-cells through GLUT2 (uniporter) transporters. The facilitated diffusion of glucose is a type of passive transport: glucose moves from a region at higher concentration (outside, bloodstream) to a region at lower concentration (inside of the cell). Once in the cell, glucose is converted into Glucose 6-phosphate by hexokinase IV (glucokinase). G6P is then processed by several metabolic pathways: glycolysis; Citric acid cycle; Oxidative phosphorylation (that synthesizes ATP).

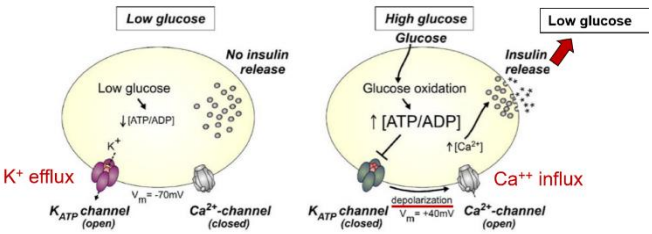
2. After oxidative phosphorylation, ATP concentration inside the cell increases. ATP acts as an inhibitory ligand for the Ligand-Gated Channel called ATP gated potassium channels *(KATP):* ATP closes the channel that is usually open. By closing the channel, K+ can no longer leave the cell. Intracellular accumulation of K+ disrupts the membrane potential of -70mV, causing a depolarization.
[Cells are usually (–) negatively charged on the inside of the membrane and (+) positively charged outside.]



3. Depolarization of the cell, activates another type of channels: Voltage Gated Channels for Ca++. Usually, Ca++ channels are closed, and the ion is much more concentrated outside the cell membrane, *because Ca is seen as a disturbing element for cell homeostasis*.
Voltage Gated Channels can sense different distribution of charges on the membrane: the decrease in membrane potential causes these channels to open and bring to an influx of cations (note that calcium enters the cell because of the chemical gradient, even if the electrical gradient is not favorable).

4. Ca2+ concentration inside the cell increases, activating the secretory pathway of insulin, causing the cell to perform exocytosis with the granules and free the insulin molecules in the blood.
The signal stops due to the fact that calcium is a stress factor for the cell; hence Ca is pumped out either to the mitochondria or to the outside of the cell. Those pumps use ATP, thus reducing Ca concentration inside the cell and reopening the K+ channels.

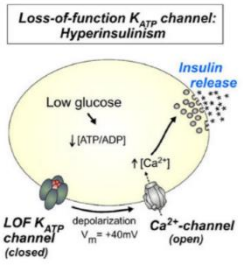
ATP sensitive potassium channels (KATP)
At physiological conditions (as previously said):



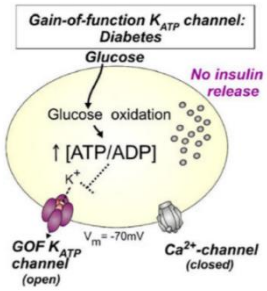
- Low glucose: the channel is open: K+ efflux maintain the membrane potential constant (-70 mV), so that Calcium does not enter the cell and insulin is not secreted.
- High glucose: the channel closes: K does not exit the cell, resulting in depolarization of the cell membrane and Ca++ influx. Insulin is secreted

Pathological conditions: some rare genetic mutations can affect the physiological functioning of KATP

- **Hyperinsulinism**: loss of function for KATP.
  The mutation causes the channel proteins to be regularly closed, independently of the [ATP]. Therefore, the cell is constantly depolarized, and calcium freely enters the cell.
  As a result, insulin is continuously released in the blood, causing hypoglycemia. *This mutation can cause irreversible brain damage in new-born and be fatal* if not treated within hours by surgically removing a portion of the pancreas tissue.



- **Neonatal diabetes *mellitus***: gain of function of KATP.
  The channels are no longer ligand gated and always open, causing the cell to never depolarize, thus insulin is never released in the blood, and there is an excess of glucose in the blood.
  Treatments include insulin therapy or sulfonylurea drug (pharmaceutical therapy), an allosteric modulator of the channel: it binds to the KATP and forces the channels to open, activating the pathway.



5 Lecture (09/04)

About the K_{ATP} channel, it is appropriate to point out that:

- The **K_{ATP} channel** is an octamer made by four **Kir6.2** subunits and four **SUR1** subunits.

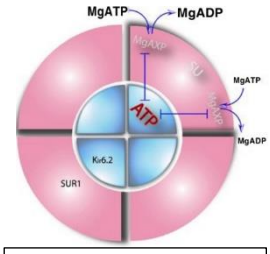


Fig.1, The K_{ATP} channel octamer: SUR1 (pink) can hydrolyze MgATP, Kir6.2 (blue) is able to bind ATP

- *Subunit (KCNJ11) confers the characteristic ATP-inhibition of the channel.* **Kir6.2** subunits are responsible for binding ATP causing the closure of the channels; a single molecule of ATP binding to just one subunit is enough to make this happen.

  The value indicating the concentration of ATP needed for the K_{ATP} channels to close is clearly lower compared to ATP concentration in normal condition inside the cell (without high glucose concentration)[2]: paradoxically, greater than 99% of KATP in the pancreatic β-cell are predicted to be closed at any one time *since (KATP,1/2) in isolated membrane patches is ~10 μM, yet, cellular ATP concentrations in the energized cell resides in the low mM range.*
  However, measured KATP activity "on-cell" is significantly higher: **the [ATP] / [ADP] ratio** needs to be considered to understand if a channel will be open or closed.

- **SUR1** subunits *confer sensitivity of the β-cell KATP to stimulatory Mg2+nucleotides (MgADP and MgATP).* They interact with MgATP hydrolyzing it to MgADP which itself can stabilize the opening state of the channel *(it reduces ATP-inhibition in the cellular milieu.)*
  The conformational changes associated with MgATP binding/ hydrolysis at the NBFs are presumably transduced to the activation gates of Kir6.2, stabilizing the opening state.

  Cell in normal condition: the amount of ATP is sufficient to cause the closure of the K_{ATP} channels → ADP can balance ATP → the channels stay open.

  High glucose concentration: ATP concentration increases → ADP cannot balance ATP anymore, thus [ATP]/[ADP] increases → the channels close.

Final step of Insulin secretion (and Ca^{2+} role)

Schekman, Südhof and Rothman won the Nobel prize in Physiology in 2013 for discovering the mechanism of vesicles docking, fundamental in the **secretion of insulin**.

This phenomenon occurs in less than 1 msec.

- When glucose enters the cell, Ca2+ concentration inside the cell increases
- **Synaptotagmin** *is a synaptic* Ca^{2+} *sensor essential for* Ca^{2+}*-triggered vesicle fusion:* it can interact with **Ca^{2+}** stimulating the SNARE proteins to initiate the docking process
- **v-SNAREs** (in the vesicle) and **t-SNAREs** (in the target membrane) can interact and bind to each other when they are close enough forcing the secretory vesicle containing insulin to come in contact with the membrane and fuse together, releasing the content.

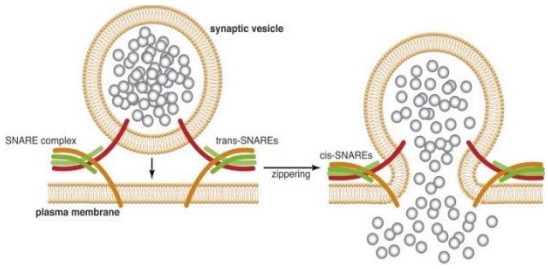
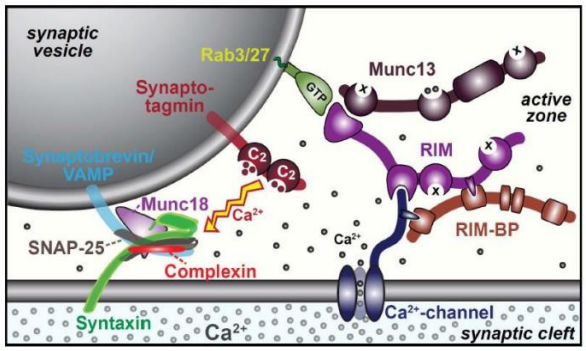


Fig.2 v-SNAREs and t-SNAREs interact causing the vesicle fusion into the membrane. Synaptotagmin is not present in the illustration

SNARE: SNAP (Soluble N-ethylmaleimide-sensitive factor Attachment Protein) Receptor



Insulin is not the only type of peptide hormone; the most common are:

- pancreatic hormones (insulin, glucagon, somatostatin);
- parathyroid hormon (calcitonin);
- hypotalamus and pituitary hormones.

Glucagon

Glucagon is a single polypeptide chain of 29 amino acids organized in a single α-helix, it is initially **synthesized** as a **prohormone**[3] called proglucagon, a longer chain that can give rise to different hormones. Proglucagon needs to be processed by specific enzymes called prohormone convertases (e.g. furin) to become mature glucagon. From proglucagon conversion we also obtain GLP-1 and GLP-2 (glucagon-like peptides).

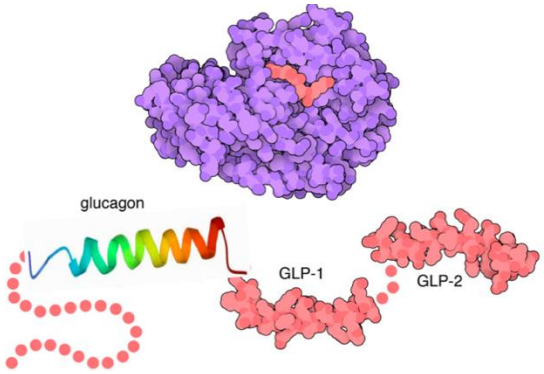


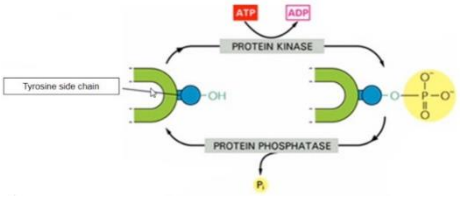
Fig.3, Furin (top) and three hormones created from proglucagon (bottom).

https://pdb101.rcsb.org/motm/184

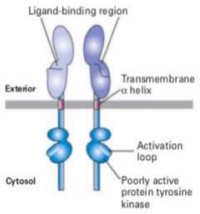
RECEPTORS WITH INTRINSIC ENZYME ACTIVITY

Receptor tyrosine kinases (RTKs), as receptors guanylyl cyclases, are a family of transmembrane receptors with **intrinsic enzyme activity**.

Receptor tyrosine kinases



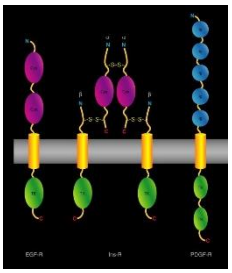
Kinases are enzymes able to catalyze the addition of a phosphoryl gorup (phosphorylation), using a nucleotide tri-phospate as both a source of energy and Pi donor to a free hydroxyl group in residues (such as tyrosine) or to an acceptor molecule.
Enzymes catalyzing the removal of a phosphoryl group (dephosphorylation) are called phospatases.
The phosphate group adds negative charged to he individual amino acid residue and to the whole polypeptide.



All RTKs are single transmembrane proteins composed of three essential modules:

- An extracellular domain containing a ligand-binding site (N-terminus).
- A single hydrophobic transmembrane α-helix.
- An intracellular segment including a domain with protein tyrosine kinase activity (enzyme activity), activated by the ligand (C- terminus).
  N.B. <u>the tyrosin kinase domain is always inactive in absence of the ligand</u>.

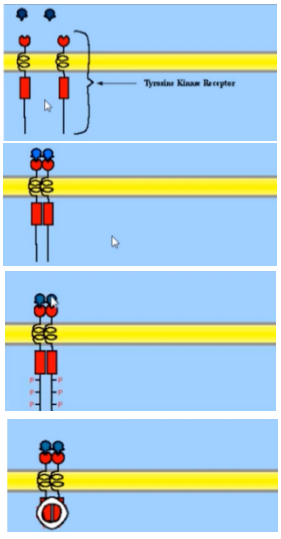
When the protein tyrosine kinase activity is activated by the ligand binding on the extracellular domain of the receptor, ATP is used as a donor and Pi will be attached to the free -OH group on the side chain of Tyr. Since the signal is activated by phosphorylation, we can easily figure out how it can be switched off: dephosphorylation (performed by a protein phosphatase).



Examples of receptors in the RTKs family are:

- **EGF-R**= Epidermal Growth Factor Receptor
- **Ins-R**= Insulin Receptor
- **PDGF-R**= Platelet-Derived Growth Factor Receptor

Insulin, EGF and PDGF are classified as ligands able to bind the RTK family.



Insulin receptor is already a constitutional dimer, whereas EGF and PDGF receptors are monomers, that need to couple with another monomer to form an activated dimer. The dimer formation is strictly triggered by the presence of the **ligand**, that induces the interaction of the intracellular domains of two identical monomers of RTK.

The kinase domains of the two monomers are activated by **trans/auto-phosphorylation**: the intracellular part of the polypeptides (containing **tyr** residues) bind to the binding site of the kinase domain of the adjacent monomer. The phosphoryl group is transferred to the exposed OH group of the tyrosine residues.

At this point, the dimer is **activated**: the kinase domain of the receptor is active and the signal transmitted by the ligand can be propagated into the cell.

So, singal transduction is mediated by receptor activation due to hormone induced dimerization.
Covalent trans/auto-phosphorylation of specific Tyr residues in the intracellular part of the receptor allows signal activation even after hormone dissociation.

The signal can be inhibited by:

- **Dephosphorylation** of the Tyr residues (*PTP* phosphatases); [Tyr residues are stimulatory].
- Additional **phosphorylation** of intracellular residues of serine and threonine, occurring during metabolic pathways involving cyclic-AMP; [Ser and Thr residues are inhibitory]

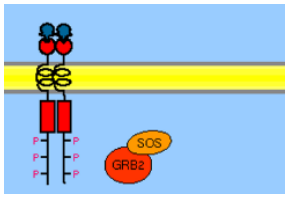
The phosphorylated residues become binding sites for signaling partner proteins (*docking proteins*), also known as **tranducers**, that contain SH2 domains; they also become phosphorilated by the kinase or are activated by conformational changes, and start the intracellular signal transduction. There are two types:

1. Non-enzymatic transducers: GRB2 and ShC.
2. Enzymes: PLC-γ (phospholipase), GAP (Ras-GTPase activator), *c-Src (Tyr-protein kinase), SH-PTP1 and PTP2 (P-protein phosphatase), PI3K (phosphoinositide kinase)* .
   These ones activate their enzymatic activity only when they're phosphorylated.

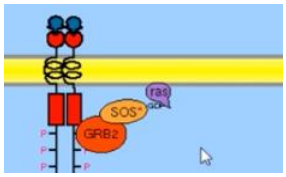
EGF-Receptor:

MAPK cascade (Mitogen Activated Protein Kinases)

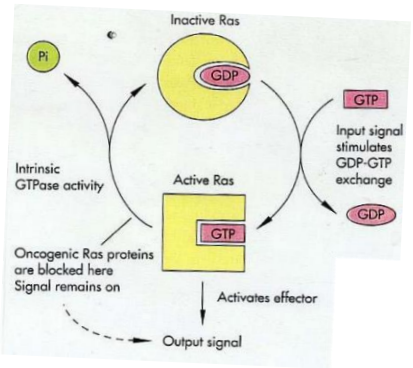
The ligand **EGF** binds to the RTK that is then phosphorylated.
Following this reaction, GRB2 [growth factor receptor binding 2], bound to SOS [son of sevenless], is able to recognize the surface of the phosphorylated tyrosine (note that neither GRB2 nor SOS are enzymes): the surface recognition occurs thanks to the SH2 domain [Src homology 2 domain] of GRB2, which is a docking site for the receptor.



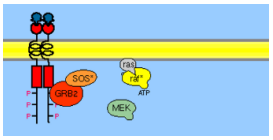
After binding, GRB2 is phosphorylated and leads to the activation of SOS, by conformational changes, resulting into the start of intracellular signal transduction. SOS activation triggers the arrival of Ras [rat sarcoma protein], which is a polypeptide able to interact with SOS. SOS is a guanyl nucleotide exchange factor that promotes GDP/GTP exchange on the small G protein Ras: at first, Ras is bound to **GDP** (inactive form) and after the interaction with SOS it's bound to **GTP** (active form).



- Inactive Ras is an active biosignaling molecule *(able to bind SOS)* that has no enzymatic activity. If SOS is active, an **exchange** of the GDP nucleotide with the GTP nucleotide occurs, and RAS is activated (this reaction is not a phosphorilation).
- Active Ras is not active as signalic molecule, but it has enzymatic activity: it is a GTPase, since it's able to activate an enzymatic reaction by the hydrolysis of GTP.
  The inactivation of RAS occurs thanks to the **release** of the P as inorganic phosphate (it is not attached to anything else, this reaction is not a phosphorilation), so that now RAS is bound to GDP, as shown in the picture. If **RAS-GTP** is not able to **discard the P_i**, the signal could be permanently propagated and this would lead to the arise of some pathological conditions, especially *cancer*.

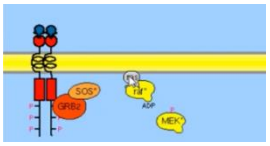


Indeed, Ras is in its active (Ras-GTP) form just for a short amount of time, during which it interacts with **Raf** by adhesions (no phosphorylation). **Raf** is a **cytosolic Ser/Thr protein kinase** able to phosphorylate and activate **Mek**, propagating the signal in the cell.

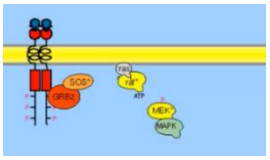


Raf = MAPKKK (kinase of kinase of MAPK)

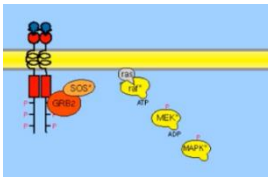
So: RAF-ATP donates one phosphoryl group to MEK —> RAF-ADP;
at the same time, MEK-ADP is the acceptor of the phosphate group: MEK is now bound to ATP and it's active.



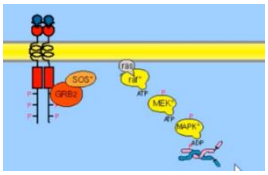
Since **MEK** is also a *Ser/Thr, Tyr protein* **kinase**, it is able to phosphorylate and activate a protein kinase of the **MAPK** [mitogen activated protein kinases] protein family, such as ERK *[extracellular signal-regulated kinase]*.
MEK = MAPKK (kinase of MAPK)



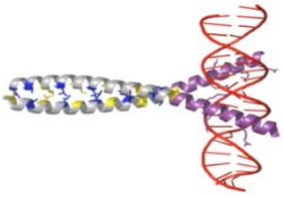
Since the last kinase of this signaling pathway is the MAPK, it is called **mapk cascade**. Starting from the end of the mechanism, MEK is the kinase of MAPK so it's also called **MAPKK** and RAF is the kinase of MEK, so it's also named **MAPKKK**.



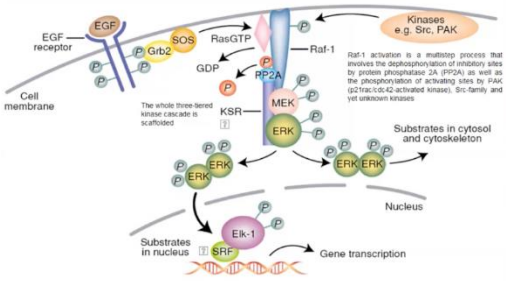
P-MAPK like **ERK** move into the nucleus and find its substrate into the nuclear matrix: it phosphorylates several nuclear transcription factors (such as *Fos*, *June*, *Myc*, *Elk1*), activating them, in order to **regulate genetic expression** and stimulate the synthesis of gene products needed for cell division and proliferation. Indeed, the signaling cascade has been activated by **EGF**.



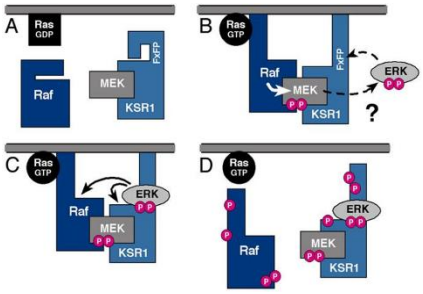
Fos and **Jun** are very short polypeptides that form a **heterodimer** able to interact with the nuclear genome. Their complex is composed by *alpha-helices* that bind together, forming the characteristic structure known as *"Leucine zipper"*. The amino-acid role is directly involved in the **recognition of the DNA molecule**. *(See Lehninger book: chapter 28, page 1136)*



<u>Kinase Suppressor of Ras</u> (KSR)
KSR has a misleading name. It was named after a mutation in the gene for KSR. In the mutation, the gene for KSR was inactivated and the phenotypic effect was the suppression of the effects induced by activated Ras. Thus, it is part of the Ras-Raf-MEK-ERK pathway. It was thought to be a kinase similar to Raf at first, but it's since been found to have no kinase activity; however it has kept the name due to historical reasons.



The organisation and function of the Ras-Raf-MEK-ERK pathway



KSR is a docking site for Raf, ERK and MEK.
(A) In absence of signal (quiescent cells), *KSR is sequestered in the cytosol as part of an inactive multiprotein complex containing MEK.* Upon signal activation KSR rapidly translocate to the plasma membrane, (B) facilitating MEK activation by Raf *[KSR potentiates the signal]*. Then, (C) KSR becomes a docking site for active phosphorylated ERK, that is able to phosphorylate both KSR and Raf (that becomes inactive) *[KSR attenuates the signal*- negative feedback*]*.

6 Lecture (28/04)

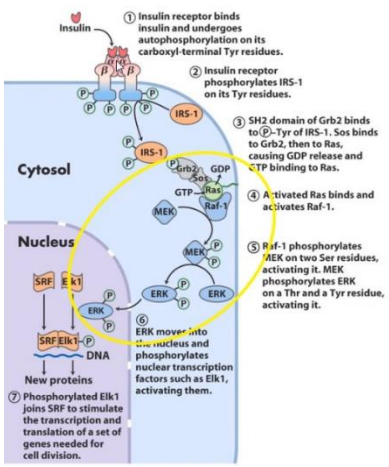
Insulin-Receptor:

Insulin- Receptor: GRB2-Ras-Raf pathway [slow branch]

The insulin receptor is a transmembrane binding protein that exists as a stable dimer.
Note: this is a key difference between it and other RTK hormonal receptors, since those only form dimers upon binding hormone, existing as monomers in the cell membrane.

X-ray crystallography studies have shown that the disulfide-bonded ectodomain of Insulin Receptor has a Λ-shaped structure. It is formed from two α and two β subunits *inter-twinned to form the insulin-binding site in the extra-cellular portion of the receptor:* it can bind two insulin molecules.
Note that insulin does not enter cells, but binds to the α-subunits and initiates a signal that travels a branched pathway (to the nucleus and also to insulin-sensitive enzymes in the cytosol)

1. Signal binds to the receptor and goes into an amplifying cascade, that is, the receival of a single insulin signal can phosphorylate several IRS-1 (on its Tyrosine residue).
    a. The insulin receptor (IR) is trans-auto phosphorylated. The α subunit binds insulin and the β subunit performs the Tyr-kinase activity. Each β subunit can phosphorylate three Tyr residues near the other β subunit (hence the term trans [across subunits] auto [on itself] phosphorylation) which in turn exposes the active site of the receptor, that can phosphorylate Tyr residues in specific proteins, such as IRS.'
    b. Insulin receptor substrate (**IRS**) is an intracellular protein that interacts with the insulin receptor. It also has tyrosine.
2. The Phospho-Tyr terminal of **IRS**, in turn is released in the cytosol where it can bind to the SH2 domain of **Grb2.** Sos binds to Grb2, and then to **Ras**, releasing GDP and binding a GTP to Ras (activating it).
3. Activated Ras binds to and activates **Raf-1**.
4. Raf-1 activates **MEK** (by phosphorylating it on two Serine residues). MEK in turn phosphorylates **ERK** (on a Threonine and a Tyrosine residue) activating it.
5. ERK moves into the nucleus and phosphorylates (activates) transcription factors such as Elk1, which then join SRF to stimulate transcription and translation of several genes needed for cell division.



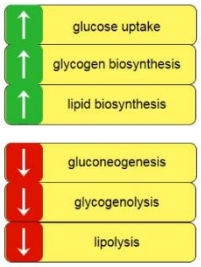
Q&A*:* GRB2 has no enzymatic activity but it is part of a family of GEF, which is a family of allosteric modulators of a protein like Ras and by binding an allosteric molecule they can change the conformation and induce the exchange of the nucleotides. GRB2-SOS is considered like a single complete allosteric protein that can be an intermediate for the signal: GRB2 can recognize the receptor while SOS is able to recognize the partner like Ras. The couple makes the entire full working intermediate.

Curiosity*: while IR is a cell-surface receptor, evidence of its presence in the nucleus (!?) has been unexpectedly reported. More generally, there has been accumulating evidence that several other RTKs that bind ligands at the cell surface can also be found in the nucleus...*

Biological effects of insulin

The insulin receptor (with the signal in the form of insulin) can act in promoting cell proliferation, hexokinase, phosphofructokinase, and pyruvate kinase. These effects, however, can take multiple hours to days to take place, since they require the transcription and translation of genes.

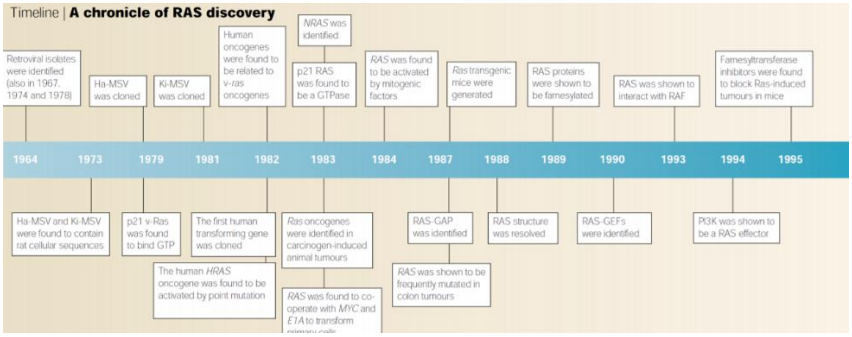
These effects therefore are not the ones responsible for insulin's role in the control of blood glucose levels as it is known that an insulin injection can act in a matter of minutes to alleviate symptoms in diabetic patients. Other biological effects of insulin are expected: it is possible to conclude that Grb2 is not the only protein that associates with phosphorylated IRS-1. *IRS-1 becomes the point of nucleation for a complex of proteins (Grb2, SOS, Ras, Raf, PI3K)*



The image shows the biological outputs of insulin signaling at physiological levels.

Ras – Small G proteins:

Ras is a G protein that can exchange GTP and GDP according to the stimulus coming from the hormone.
The intrinsic GTPase activity of Ras allows the aforementioned signaling pathway to be switched off and or enhanced by some allosteric modulators such as GAP.
Ras stands for Rat Sarcoma Protein because it was discovered many years ago from studying sarcoma in rats.
This is an historical timeline that puts in evidence how many steps science has done in the past years to realize how much Ras is important for cell physiology and cell pathology.



The beginning of Ras research can be traced back to 1964 when scientists observed that a preparation of a murine leukemia virus, taken from a leukemic rat, induced sarcomas in new-born rodents. Retroviruses, later shown to carry Ras oncogenes, were identified.
The virus contained the signal for stimulating the Sarcoma in the control animal, a code which was exactly the code for the Ras protein. In conclusion Ras is a physiological element in signaling but it is an oncogene.

The protein products of the v-ras oncogenes were identified in the 1970s. *Identification of v-Ras proteins (e.g. p21, 21 kDa protein), allowed their functional characterization. They have high affinity for guanine-containing nucleotides and are associated with the inner side of the plasma membrane.*

Doubts regarding the existence of transforming genes in human tumour cells were resolved. Over the following years, many laboratories embarked on a massive search for RAS mutations in human cancer types. Ras is a pathological molecule when it is mutated: just a couple of amino acids conversions are enough for provoking Ras oncogenic function.

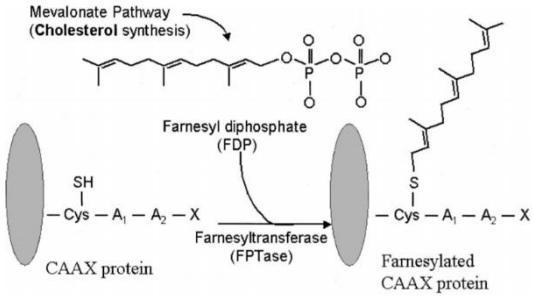
Alteration of Ras can affect several features:

- **Farnesylation**: Ras mutation does not allow Ras to be bound to the plasma membrane.
  Normally, Ras is bound to the membrane through a lipid anchor: a **farnesyl** group on the carboxyl-terminal cysteine, cause the lipid tail for Ras to stay very close to membrane and anchored in the appropriate portion of the cell.

  Note: in general, some lipids covalently attached, can function as hydrophobic anchors for peripheral membrane proteins (e.g. acylation for Ras Gα; Prenylation for Ras Gγ; GPI anchor for acetylcholinesterase). Proteins prenylated by FPTase and GGPTase-I typically undergo two additional processing steps. First, the C-terminal aaX tripeptide is cleaved from the newly prenylated CaaX protein by an endoprotease. This is followed by methylation of the prenylcysteine residue at the new C-terminus by isoprenylcysteine carboxylmethyltransferase. This three-step process increases protein hydrophobicity and often leads to plasma membrane association.

  Farnesylation reaction:

  Farnesyltransferase removes the pyrophosphate group from a phosphorylated tail of isoprenoid units (that is a very energetic molecule): the enzyme takes the energy from the bond cleavage and uses it to create a new covalent bond between the isoprenoid tail *of the previous molecule* and the sulfuryl group of a Cys amino acid present in the target protein, that in this case is Ras.



  Many other target proteins can be farnesylated by receiving this highly hydrophobic isoprenoid tail, so they become able to interact with the phospholipids in the membrane.

  After adding the farnesyl tail, the A1, A2 (*i.e. aliphatic residues*) and X are removed from the protein as a post transcriptional modification and only the farnesyl tail remains. Those post-translation iso-prenylations are a point of interest for scientists: *they are required for the transforming properties of RAS, so* if they are mutated in the cell, Ras cannot interact with the signaling coming from the receptor.

  Farnesyltransferase recognizes CaaX boxes where X = M, S, Q, A, or C, whereas Geranylgeranyltransferase I recognizes CaaX boxes with X = L or E. 1)

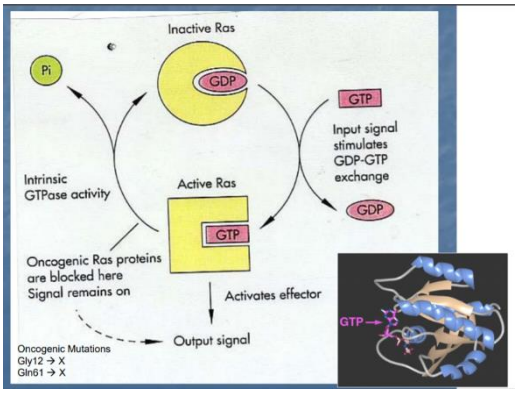
  Farnesylation targets H-Ras and N-Ras (H/N-Ras) to the endoplasmic reticulum (ER), where the post-prenylation processing of the CaaX box (in which 'a' is typically an aliphatic amino acid and the identity of 'X' (the C-terminal residue) determines whether the prenyl group that is added is a farnesyl or a geranylgeranyl isoprenoid) results in a C-terminal farnesyl carboxymethylester (OMe). Farnesylated H/N-Ras can either undergo rapid exchange with other membranes via a non-vesicular pathway or (2) become palmitoylated by a putative protein acyltransferase (PAT) that kinetically traps H/N-Ras on the ER. Dual-lipid-modified H/N-Ras can then traffic to the Golgi (3) and plasma membrane (4) via the vesicle-mediated classic secretory pathway. (5) Once on the plasma membrane, the action of a putative acylprotein thioesterase (APT) removes the palmitoyl group, allowing farnesylated H/N-Ras to undergo rapid non-vesicular exchange (6) with endomembranes including the Golgi and ER. (7) Another putative PAT might trap farnesylated H/N-Ras on the Golgi membrane. The deacylation–reacylation cycle accounts for the dynamic subcellular trafficking of Ras by vesicular and nonvesicular pathways.

- **GTPase activity**:
  Note: in 1983-1984, three groups reported that RAS proteins are GTPases, having an enzymatic activity, thus led to the proposition that RAS proteins are mediators of signal (EGF) transduction across the plasma membrane.

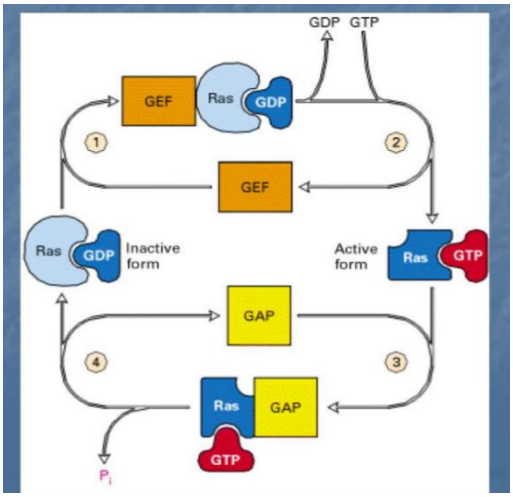
  So, another type of mutation of Ras can affect its GTPase activity: if Ras is not able to work as an enzyme in the appropriate way, its function would alter the signal and become oncogenic. E.g. scientist kept studying how EGF can become oncogenic if Ras is mutated.

- **GAP protein:**
  Note: assays to study the biochemical properties of RAS proteins in an in vivo cellular environment showed that the rate of GTPase activity of normal HRAS proteins is at least 300-fold higher than the activity of their oncogenic counterparts. More importantly, they identified a soluble cytosolic protein as the factor responsible for this stimulation — a GTPase-activating protein (GAP)
  This protein can activate the GTPase activity of Ras: in the presence of GAP, Ras can more easily convert GTP into GDP.

  This is the cycling of Ras into the two forms: the inactive and active forms.
  As already mentioned, Ras becomes oncogenic when is not able to act as an enzyme and to perform the intrinsic GTPase activity. GAP, the allosteric modulator that can modulate this GTPase activity, can be also involved in the possibility that Ras becomes oncogenic.
  If GAP is not present, it is not possible to modulate the enzyme, so that it remains in the GTP bound state.
  A proper mutation of Ras, like substitution of Glycine 12 or Glutamine 61, can provoke a different conformation in Ras and a different GTPase activity.



  GAP is an allosteric modulator that controls the intrinsic GTPase activity of Ras: Ras can release an inorganic phosphorus from GTP and obtain a GDP.
  GEF, guanosine nucleotide exchange factor, is a modulator that can force Ras to change the nucleotide: the inactive form of Ras becomes active by changing GDP and GTP.
  Student: So when GAP acts, GTP loses a phosphate, while when GEF acts there is an exchange?
  Professor: yes, remember that when it is a matter of going into the active form of the signal there is an exchange of nucleotides, while when it is a matter of switching off the signal then GTP has to be enzymatically converted into GDP.



Fred Wittinghofer was the one able to recognize all the specific regions in the Ras structure, which is considered a small G protein.

Ras Structure

(6) Alfred Wittinghofer (MPI) Part 1: GTP-binding Proteins as Molecular Switches - YouTube

Ras Family

Ras family includes many similar proteins that are GTPase enzymes that can sense signals coming from hormone pathways. The conserved 3D structure of the G-domain that is common to the entire superfamily allows these proteins to preserve large structural similarity and common biochemical properties while they recognize their individual binding partners with remarkable affinity and specificity.

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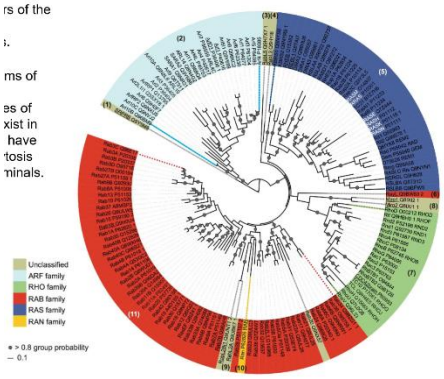


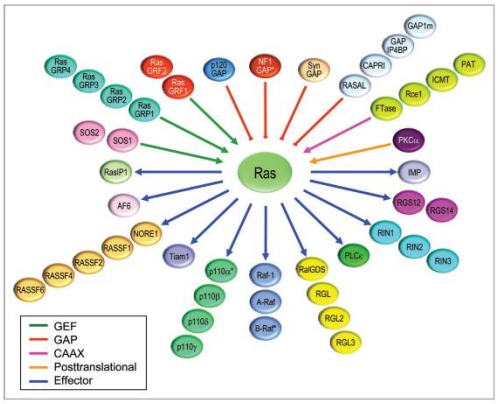
In general, the human oncogenic members of the Ras family regulate cell proliferation, differentiation, morphology, and apoptosis.

Mammalian tissues contain around 30 forms of **Rab** (ras-related proteins in brain), which specifically associate with the various types of membrane vesicles and organelles that exist in cells. Subtypes of Rab, particularly Rab3, have been implicated in the regulation of exocytosis, neurotransmitter release at nerve terminals, and in the development of neuronal cells.

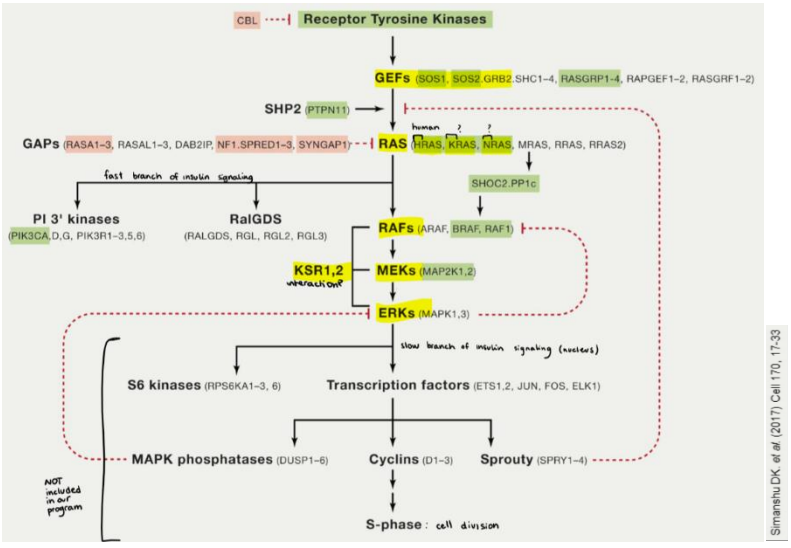
Ras is in the center of many signaling pathways and we can see it clearly in a Ras **interactone**, where there are all the molecules from different pathways that can interact with this GTPase protein.

Examples already mentioned are:

- GAP converts GTP into GDP: it inactivates the signaling role of Ras (i.e. Ras is not active in intermediate pathways).
- FTase (Farnesyl transferase) activates ras
- Ras activates Raf



Exam-like graph:



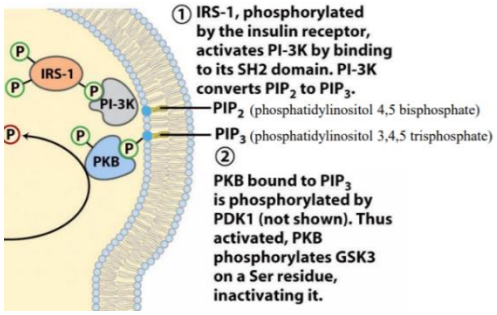
7 Lecture (29/04)

Insulin-Receptor: PI3K pathway [fast branch]

As mentioned in the previous lecture, IRS-1 [insulin receptor substrate] can initiate different branches in the insulin pathway, by interacting with different proteins, such as PI3K. The purpose of this alternative branch is to create a way for insulin to make a signal which is fast enough to induce glycemia dropping in few seconds or minutes.

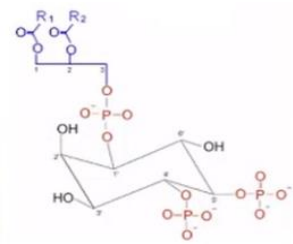
PI3K is a protein (k-kinase), that differs from other proteins because its substrate is not a protein but a phospholipid. In order to understand the network that insulin can stimulate in the cell, at least three nodes should be mentioned : 1st receptor – structure, molecular characteristics and role of the IRS with the receptor, 2nd PI3K node and 3rd AKT/PKB.

1) Phosphorylated **IRS-1** bind to the SH2 domain of **PI3K**, and activates this enzyme through surface interaction.



The substrate of PI3K is a phospholipid in the plasma membrane: **PIP2** (phosphatidylinositol 4,5 bisphosphate). When PIP2 is phosphorylated by PI3K it becomes **PIP3** (phosphatidylinositol 3,4,5 trisphosphate) and the reaction occurs on polar head on cytosolic face of the plasma membrane.

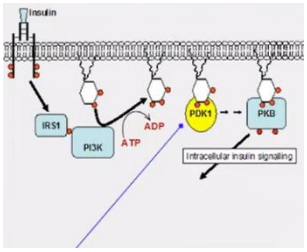
Note on PIP2 structure:



Inositol is the polar head: a hexameric polyalcohol cyclic molecule, with OH attached to each carbon. 4,5 C are phosphorylated ("bisphosphate"); however a third P group is present in position 1: this phosphorous group is present in all phospholipids as it act as a bridge between the glycerol molecule and the head.

Structure of inositol is similar to the one of carbohydrates but inositol doesn't contain any oxygen in the ring, instead there are only six carbon atoms.

Note on mechanism of phosphorylation:



Once PI3K is activated, the substrate PIP2 is phosphorylated on position 3 on inositol to PIP3: the energy and phosphate required are released from hydrolysis of **ATP** to ADP.

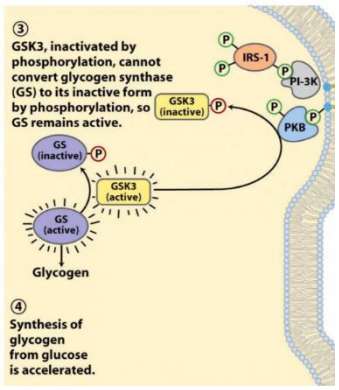
The phospholipids (PIP3) are more polar and become recognizable by second intermediates in the pathway, such as are PKB (protein kinase B type) and PDK1 (*phosphorylated 3-phosphoinositide-dependent kinase*), allowing their interaction.

PIP_3 can be inhibited by PTEN and SHIP (phosphatases), as they convert this phospholipid into another molecule that its signaling partners (PDK1 and PKB) cannot recognize. **PTEN** can convert PIP_3 into $PI(4,5)P_2$ by removing the phosphorous group in position 3 while **SHIP** can convert PIP_3 into $PI(3,4)P_2$ by removing the phosphorous in position 5. Actually $PI(3,4)P_2$ can still interact with PKB but the intensity of the signal is reduced so the phosphorous in position 3 has a central role in the transmission of the signal.

2) PKB (or **Akt**) bound to PIP3 is activated through allosteric modulation, and phosphorylation by **PDK1** (that uses ATP). Thus activated, PKB phosphoryaltes **GSK3** *on a seryne residue, inactivating it.*

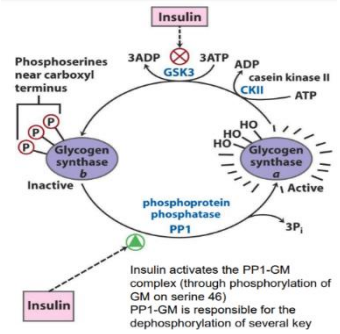
Note: insulin signalling requires lots of ATP (as the process involves many phosphorylations). Remember that insulin is present when there is a high amount of glucose in the blood and in the cell, so ATP molecules are easily available for the different types of metabolic and signaling steps that insulin is going to follow.

3) GSK3 is a protein kinase, that is usually not phosphorylated and active: it phosphorylates its substrate **GS** (**g**lycogen **s**ynthase) inactivating it, so that glycogen is not synthetized.
When **GSK3** becomes phosphorylated (by PKB for example) it becomes inactive: **GS** remains active and is able to produce new molecules of glycogen (and metabolize glucose, by storing it as a source of energy).
Note: GSK3 is present as a modulator only in cells that can produce glycogen.

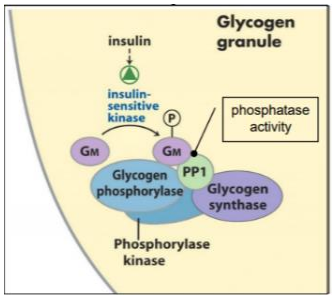


4) Synthesis of glycogen from glucose is accelerated. *Glycogen synthase binds to the growing glycogen chain and adds* UDP-glucose (the actual substrate) *to the 4-hydroxyl group of the glucosyl residue on the non-reducing end of the glycogen chain, forming more α(1→4) bonds in the process.*
GS is phosphorylated in multiple sites: for its inactivation ATP is needed in many copies.
Note that **GS** modulation is a very controlled step in metabolism; its activation is mediated by insulin, that:

- Inactivates inhibitor **kinases** (such as GSK3): GS is not phosphorylated and inhibited anymore.
- Activates phosphoprotein **phosphatase**-PP1: GS is dephosphorylated and activated (i.e. PP1 converts pre-existing inactive GS into active GS by simply removing the phosphorus that was binding the enzyme).



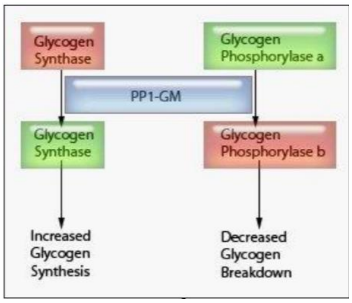
PP1 and GS form a macromolecular complex together with other partners, such as **GM** - a protein factor that can modulate PP1. Particularly, phosphorylated GM can make PP1 more active. The switch of not phosphorylated GM into a phosphorylated GM is stimulated by the "**insulin sensitive kinase**". Insulin does not act on PP1 directly because insulin is outside the cell, but the signaling pathway generated by insulin at one point can induce the phosphorylation of GM. GM can then stimulate PP1, and PP1 can recruit the existing GS molecules that are inactive and make them active again. Note: We may figure out that PKB itself can be the "insulin sensitive kinase" that phosphorylates GM, because active PKB can work on GSK3 and probably can phosphorylate other enzymes such as GM.



Inside the glycogen granules, glycogen is stored with a super assembly of different enzymes: enzymes do not have to diffuse long distances, and all the reaction happen very close. Two of those enzymes are the **glycogen phosphorylase** which catalyzes the opposite process of glycogen synthase – the degradation of glycogen; and **phosphorylase kinase,** that is needed for the proper work of glycogen phosphorylase: these two enzymes are nested one in the other to make the phenomenon of glycogen degradation work.

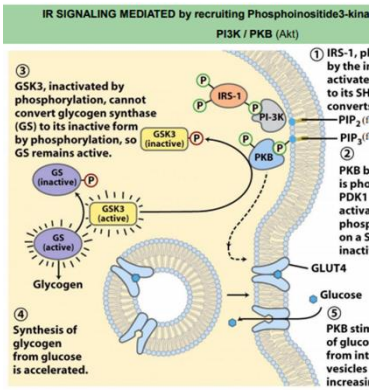
The presence of both the synthesis of glycogen and the degradation of glycogen at the same time would create an energy wasting cycle – a <u>futile cycle</u>. To avoid that, insulin is also in charge of stopping glycogen degradation, so that the new glycogen molecules will stay there and be stored as long as it needed and won't be degraded immediately after being produced.

To summarize the pathway, an inactive GS becomes active thanks to the activity of the PP1-GM group. The PP1-GM must be phosphorylated on the GM subunit to become an active and working complex that can increase glycogen synthesis. At the same time, the same PP1-GM complex with it's phosphatase activity can convert an active glycogen phosphorylase into the inactive form of the enzyme, and that will decrease glycogen breakdown. This is the key step that insulin can regulate: <u>the activation of the PP1-GM complex that can increase glycogen synthesis and decrease glycogen breakdown</u>.



Note: from literature we know that the GM factor is often mutated in patients. Many naturally occurring GM mutations can lead to insulin resistance in some diabetes forms. A patient that cannot store glucose in the form of glycogen has a type of diabetes that is insulin resistance.

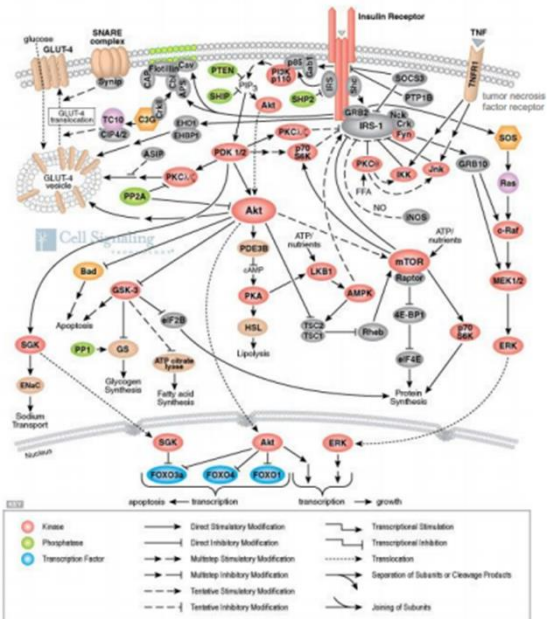
5) Insulin, *through PKB*, induces the fusion of cytoplasmatic vesicles containing GLUT4 with the plasma membrane. Glucose transporter **GLUT4**, is an intermembrane insulin dependent glucose transporter**.**
When GLUT4 is positioned on the plasma membrane the uptake of glucose is 15 times higher than in normal conditions. The activation of GLUT4 is typical of skeletal muscle cells and in the adipose tissue whereas liver exploits the other members of the GLUT family.



In **type-1 diabetes mellitus** (insulin-dependent, juvenile) GLUT4 is not mobilized due to the inability from cells to release insulin so the concentration of glucose in blood.

Exam-like image; recognize:

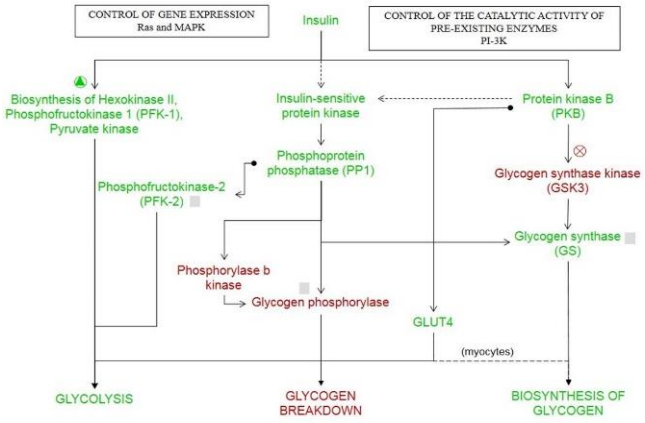
- The insulin receptor;
- **IRS-1** that displays binding sites for numerous signaling partners;
- **GRBB2** from which may start the MAPK cascade or the SOS cascade;
- **PIK3**, which regulates pre-exiting enzymes in the cell;
- **GSK-3**, which activates GS
- **GlUT4**, activated by factors we do not need to remember
- **AKt/PKB**, which are two names for the same protein, PKB (protein-kinase B) emphasizes the kinase activity while Akt owes its name to a stock of animals on which experiment oh these protein were made. In the image we do not find PKB just because the nomenclature Akt is used



High concentration of glucose recap

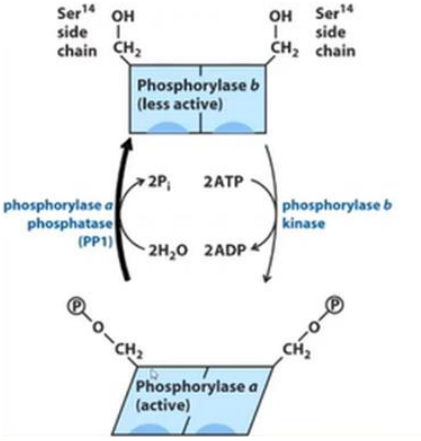
Slow response to high levels of glucose in blood (left): the control of gene expression by **Ras and MAPK** induces the biosynthesis of Hexokinase II, Phosphofructokinase (PFK-1) and Pyruvate kinase, that are key enzymes in Glycolysis.

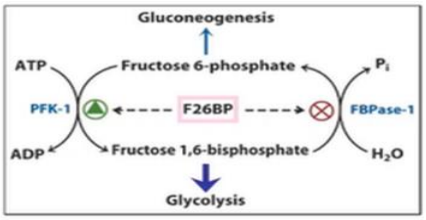
Fast response to high levels of glucose in blood (right): the control of the catalytic activity of pre-existing enzymes;

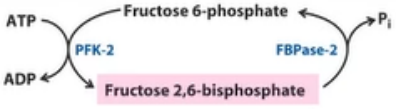


Protein kinase B (PKB) is activated so

- **Glycogen synthase kinase (GSK3)** is inhibited**. Glycogen synthase** remains therefore active, and Glycogen is synthesized in those cells that can produce it like in skeletal muscle and liver (also other cell type can but not considerable quantities). Protein-kinase B can also target some Insulin-sensitive protein kinase, activating **Phosphoprotein phosphatase (PP1)**.
- PP1 can dephosphorylate GS, activating it.
- PP1 also inhibit **Glycogen phosphorylase** (key enzyme for Glycogen breakdown), by dephosphorylating Phosphorylase kinase, which is converted in its less active form (b from). Phosphorylase kinase in its active form (a form) is able to phosphorylate Glycogen phosphorylase, activating it.
- PP1 can also enhance **Phosphofructokinase-2 (PFK-2)**, which phosphorylates Fructose-6-P to Fructose 2,6 bisphosphate, which is a modulator of Glycolysis, in fact it enhances the activity of PFK-1 that converts Fructose 6-phosphate into Fructose 1,6 bisphosphate and inhibit the activity of Fructose-bisphosphatase (FBPase-1), involved in gluconeogenesis.
  Both the steps regulated by Fructose 2,6 bisphosphate are irreversible

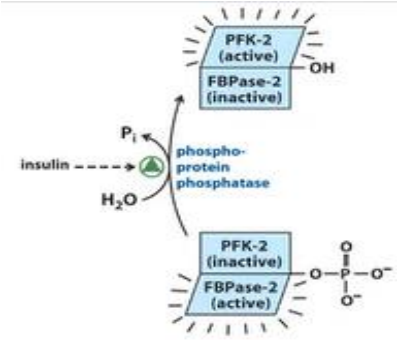






FK2 and FBPase-2 are basically the same protein, they are a tandem enzyme.

Depending on the phosphorylation state of the complex one catalytic site is active and the other is inactive. PFK-2 is active when the complex is de-phosphorylated (insulin action) while FBPase-2 is active when the complex is phosphorylated (glucagon action). PP1 is the phosphatase that regulates the phosphorylation state of the complex.

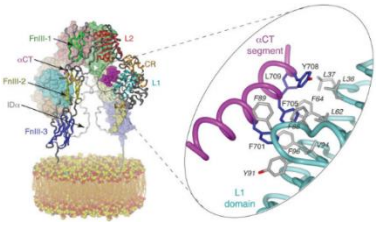


Insulin receptor structure/conformation in detail

The insulin receptor has 2 binding sites for 2 insulin molecules, so one student asked if the presence of only one insulin molecule instead of two would affect the signaling pathway.

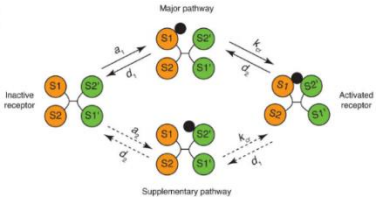
The answer to this question can be found on a 2013 paper by Ward et al.

The title of the article states that "the insulin receptor changes conformation in unforeseen ways depending on ligand binding".



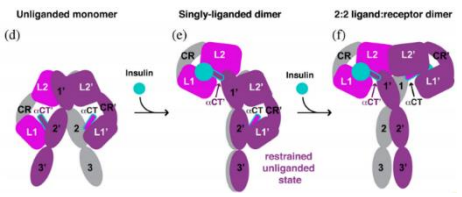
This paper goes into detail on the insulin receptor (IR) ectodomain dimer (external portion of the receptor). The two binding sites for insulin are composed of helices making up a sort of "pocket" in which insulin can fit. These binding sites include the **αCT segment** and the **L1 domain** (respectively fuchsia and light blue in the picture). In each one of the two "pockets", the αCT segment belongs to one monomer of the receptor, whereas the L1 domain belongs to the other monomer of the recpetor.

In other words, there are one L1 and one αCT on the right side and one L1 and one αCT on the left because the two monomers are substantially identical.



In the schematic image on the right, the green represents one monomer and the orange represents the other monomer. When the receptor is empty, all the monomers are in equilibrium with their subunits. When the hormone arrives (black dot in the picture) and binds to S1 (which represents the αCT of one of the monomers) then S2' (which represents the L1 domain of the other monomer) gets closer to it, forming the pocket for the first insulin molecule. As a consequence, the other αCT and L1 of the other monomers (S2 and S1' in the picture) will move away from each other. If another molecule of insulin was available to bind between S2 and S1', these would be too far from each other to form an appropriate pocket for insulin binding.

It is possible to conclude that if one molecule of insulin is already bound to the receptor, the probability of binding of a second insulin molecule will be much lower. This can really influence in the signaling that should start in the cell.



This image on the right is taken from a paper published by Ferguson et al. in 2020. In this paper the same mechanism is explained with a different but very clear picture. L1, αCT and the two monomers are represented in grey and purple. In the image there is an empty receptor (d), that changes conformation upon binding of a first insulin molecule (e) and changes again upon binding of the second insulin molecule (f). Remember that the rearrangement of the receptor happens in the ectodomain (in the external portion). What actually restrains the second insulin from binding isn't the distancing between the unbound L1 and αCT, it is rather the restrainment of the unliganded binding site by the coming together of the purple monomer. In other words, the purple monomer should have some distance between its subunits to properly work, but upon binding of the first insulin molecule it closes on itself and the second insulin molecule can't bind anymore. This restraint requires energy to be broken, only after that the second insulin molecule can bind and there will be a fully bound insulin receptor.

It is not yet known if the insulin receptor works also when it is bound by only one insulin molecule or if it requires both insulin molecules to exert its function. Ward et al., in their paper, suppose that one insulin molecule can induce a conformational change in the ectodomain of the receptor great enough to also change the cytosolic domain, triggering autphosphorylation and the consequent signaling pathway.

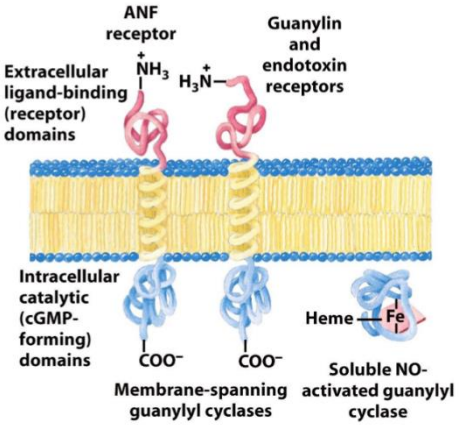
Guanylyl cyclase

N Lecture (06/05)

Guanylyl cyclase receptor is a transmembrane receptor enzyme, which has a catalytic domain in the intracellular matrix.

There are two categories of receptor guanylyl cyclase:

1) Homodimers with a single membrane-spanning segment (α helix) in each monomer, an extracellular amino ligand-binding domain, and a carboxyl intracellular catalytic domain (cGMP forming domain). These receptors are present as monomers, but form dimers upon hormone binding to the extracellular domain: the two extracellular domains twist relative to each other (*about an axis roughly perpendicular to the membrane surface*), forming a kind of coiled coil that will force the cytoplasmic domain to change the *geometry and orientation* (change in the quaternary structure).
   [An example is ANF/ANP Atrial Natriuretic Factor/Peptide].
2) Soluble heme-containing enzymes (intracellular) – kind of an exception in the family of receptor enzyme.

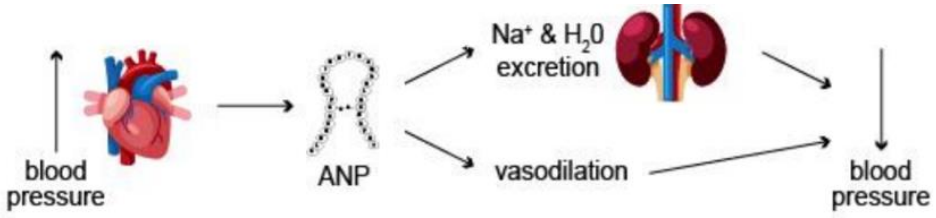


Main examples of ligands that bind receptor guanylyl cyclase:

- **ANF/ANP –** small peptide secreted from the atrium that travels through the blood (hormone-like) and targets renal collecting ducts and vascular smooth muscle tissue.
- **Guanylin and endotoxin** (produced in intestinal epithelial cells) **–** signaling pathway widely used by bacteria.
- **NO –** acts as a signaling molecule over short/medium distance and targets smooth muscle and blood vessels. It can diffuse through the membrane and bind to cytoplasmic receptors.

Atrial Natriuretic Peptide

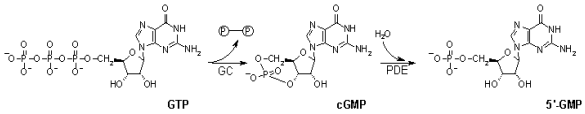
ANP is a critically important regulator of sodium and water balance, blood volume, and arterial blood pressure. It is synthesized (28 amino acids in length, a loop created by a disulfide bond) and secreted by cardiac muscle cells in response to the expansion of atrial walls due to abnormally high circulating blood volume (volume sensors which respond to increased stretching of the atrial wall). ANP stimulates renal excretion of sodium and water (natriuresis) and acts as a potent vasodilator (of the afferent arteriole of glomerulus). The combination of ANP actions effectively decreases blood volume, blood pressure, cardiac output, *and is a counter-regulator of the renin-angiotensin-aldosterone system.* Pharmacological modulation of ANP is currently under investigation and synthetic homologues of ANP are being assessed for the treatment of hypertension and acute heart failure.



ANP-induced action in medullary collecting duct cells:

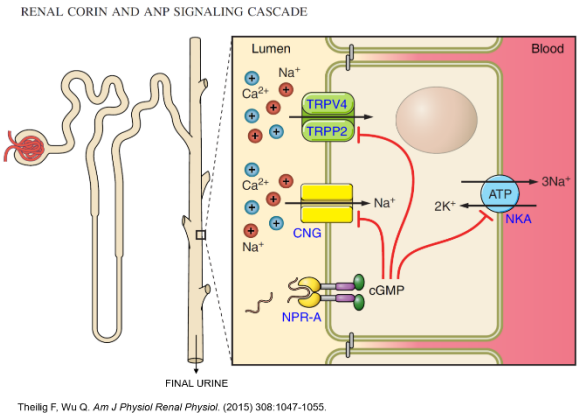
The epithelial cells of the collecting duct reabsorb ions like Na+ and Ca2+, but they also contain receptors for ANP, called **NPR-A**.

These receptors recognize the peptide and generate the signal inside the cell via secondary messenger cGMP: guanylyl cyclase converts GTP to the cGMP (guanosine 3',5' – cyclic monophosphate)



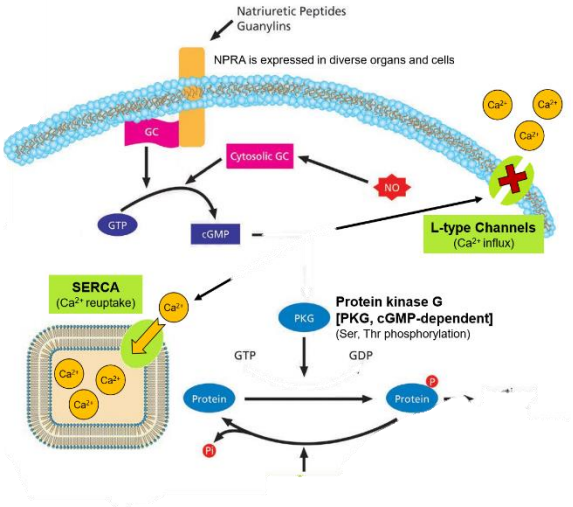
At this point, cGMP will inhibit:

- Ion channels in the apical portion of the epithelial cell. In this way, ion concentration will decrease because instead of being absorbed, the ions will be secreted with urine, and for osmotic reasons the volume of blood will decrease. Those ions channels include cyclic nucleotide-gated channels (**CNG**) and the heteromeric channel of transient receptor potential V4 and P2 (**TRPV4, TRPP2**).
- Na-K-ATPase (NKA) in the basolateral portion.



Note: outputs created by cGMP differs upon tissue; *it causes relaxation/vasodilation to increase blood flow (heart-blood vessels), changes in ion transport and water retention driven by osmotic pressure (kidney and intestine).*

cGMP, *produced by guanylyl cyclase*, acts as an allosteric modulator of **PKG** (protein kinase G) that can phosphorylate Ser and Thr residues of the target protein, taking a phosphate group from GTP. Depending on the type of protein that is being phosphorylated the outcome could be control over cardiac vasculature, smooth muscle relaxation and blood pressure regulation, *cellular growth, sensory transductions, neural plasticity and memory*. Phosphatase can switch the signal off.



cGMP also affects **Ca2+ carriers**, *reducing the forcefulness of contraction.* Specifically it:

- inhibits the action of L-type Ca2+ channels that regulates Calcium influx (so that Ca does not enter)
- enhances SERCA channel that is responsible for compartmentalization of the Ca ions into the sarcoplasmic reticulum.

Net result: Calcium concentration decreases in the ECM.

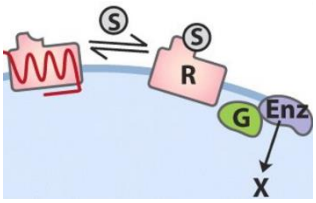
To stop the signal of cGMP, it needs to be converted from the cyclic molecule into a linear one via the action of **phosphodiesterase**. The conversion of 5'-GMP to GTP is highly endergonic and must be coupled with highly exergonic reaction. If PDE is inhibited, usually by exogenous molecules like drugs, cGMP will transmit the signal. Drugs that inhibit PDE include Viagra (inhibits PDE5, penile tissue specific), Cialis and caffeine (non-selective inhibitor).

SERPENTINE RECEPTORS

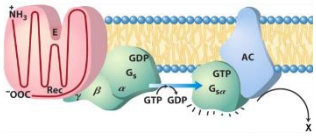
8 Lecture (30/04)

Serpentine receptors are a family of large monomeric **transmembrane** receptors also known as **7TMRs** (7 transmembrane receptors, as they contain seven transmembrane helices) or **GPCRs** (G protein coupled receptors). The most common G protein-coupled receptor are sensory receptors.

Serpentine receptors are **metabotropic receptors** since they don't have a direct enzymatic activity, but they use signal **transduction mechanisms** (G proteins) to alter the metabolism of the cell.



These receptors are activated upon ligand binding (S) on the extracellular domain, and transduce the signal on the cytosolic domain via coupling to an intracellular G protein (Guanosine-binding proteins; i.e. featuring a GTP-binding domain). G proteins act as a shuttle transducing the signal coming from the receptor to the effector enzyme (or, in some cases an ion channel), which will generate an intracellular second messenger (X) able to amplify the hormone signal.



Thus, the signal transduction mechanism of serpentine receptors is defined by three components:

1. The plasma membrane receptor.
2. The protein that binds guanosine nucleotides (G protein).
3. The effector enzyme that is regulated by the activated G-protein.

Hormones that bind to this family of receptors are:

- Epinephrine (adrenergic β receptors)
- Acetylcholine (muscarinic receptor; not nicotinic receptors)
- Glucagon
- Follicle-stimulating hormone (FSH)
- Luteinizing hormone (LH)
- Thyroid-stimulating hormone (TSH)
- *Odorizing molecules and flavors*

Robert Lefkowitz and Brian Kobilka were awarded the 2012 Nobel Prize in Chemistry "for studies of G-protein–coupled receptors" (link to Robert Lefkowitz' Nobel lecture)

Heterotrimeric G proteins

Heterotrimeric G proteins are much larger than the monomeric G protein Ras and feature three subunits **(α, β, γ).** Keep in mind that both α and γ subunits are anchored to the plasma membrane and that Gα binds GDP in the inactive form . The main differences between different types of G protein are usually found in the α subunit.

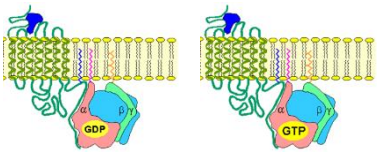
The main types of heterotrimeric G proteins are:

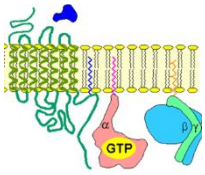
- **G_s** increases the activity of adenylyl cyclase
- **G_i** inhibits the activity of adenylyl cyclase
- **G_q** increases the activity of phospholipase c
- G_t increases the activity of cGMP phosphodiesterase (PDE)
- G_{olf} increases the activity of adenylyl cyclase, similar to Gs but specific for odorizing molecules

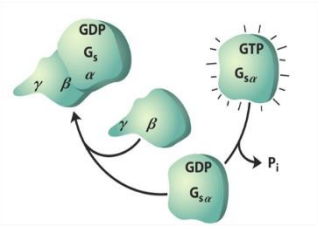
Both Ras and heterotrimeric G proteins are **able to bind Guanosine nucleotides**. Ras resembles the α subunit of the heterotrimeric G proteins, thus everything explained before about Ras can also be applied to the latter. For instance, both Gα[5] and Ras are attached to the plasma membrane by acylation (while Gγ is anchored to the plasma membrane by prenylation).
Note: G-proteins in general, can interact with the plasma membrane not as external proteins, but linked with the double phospholipid layer of the plasma membrane. This characteristic allows them to be located in specific compartments of cells, which are needed for their function.

General mechanism

1. Hormone binding triggers a conformational change in the polypeptide receptor, specifically in the C-terminus (cytosolic domain)
2. The G protein (the α subunit specifically) can recognize a docking site on the hormone-receptor complex.
   Upon binding, Gα will displace **GDP** in favor of **GTP**.
3. When Gα bind to GTP, the G protein is activated and the β and γ subunits dissociate from the α subunit.
4. Activated Gα can move in the plane of the membrane (since it is anchored to it) towards nearby effectors which can both be membrane enzymes or ion channels.
5. Gα's GTPase activity, allows a *biological* **switch off**.
   Gα+GTP is hydrolyzed in Gα+GDP (the inactive form) thanks to the intrinsic GTPase activity and the β and γ subunits will associate again with the α one, *so the whole protein is again available to interact with the hormone-bound receptor.*
   Some regulatory factors can determine how quickly the GTPase activity hydrolyses GTP







Gα subunits

$G\alpha_S$

GαS stimulates ("s" stands for stimulatory) **adenylyl cyclase**, it is associated with the receptors of the following hormones: epinephrine, glucagon, PTH, LH, ACTH.

$G\alpha_i$

Gαi inhibits ("i" stands for inhibitory) **adenylyl cyclase** *(lowering the level of cAMP in the cell),* it is activated by the receptor for: somatostatin and acetylcholine (muscarinic M2, M4)

$G\alpha_q$

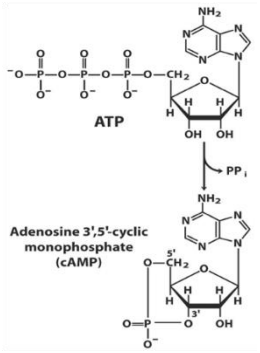
Gαq activates **phospholipase C (PLC)** which generates the following second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). It is activated by the receptor for: vasopressin, TSH, angiotensin II and acetylcholine (muscarinic M1, M3, M5)

$G\alpha_t$

Gαt is the molecule responsible for generating a signal in the rods of the retina in response to light ("t" stands for transducing).
It also triggers the breakdown of cyclic GMP (cGMP) by Phosphodiesterase (PDE)

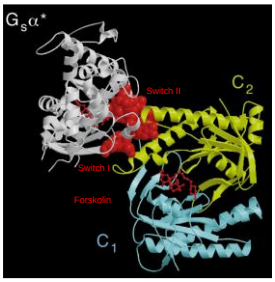
Gαs – Gαi : cAMP second messenger

Synthesis of cAMP:



Adenylyl cyclase is the effector enzyme of Gαs, Gαi and Gαolf. It is an integral transmembrane protein composed of a single polypeptide, but the enzyme domain is located on the inner face.

It can catalyze the synthesis of cyclic AMP (cAMP) starting from ATP as a substrate. The reaction requires the removal of the pyrophosphate (PPi) from ATP and the remaining phosphate will become a "bridge" between C3 and C5 in the ribose.



Gα has two "arms" called switch I and switch II which are able, if they are in the right position, to interact with adenylyl cyclase. Switch I and II have different conformations in Gα's active and inactive form: different residues in both "arms" interact with the last P group from GTP7, thus the conformational change enables active Gα to bind to adenylyl cyclase. Adenylyl cyclase may have different allosteric regulators, such as Gα itself, but also smaller molecules such as **forskolin**, which can regulate the catalytic activity of the enzyme even when Gα is not bound. Forskolin is a diterpene compound, active component of mint roots (more information on virtuale).

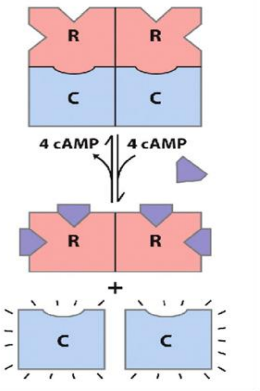
Forskolin has multifaceted pharmacological effects that have been linked to its role as an activator of adenylate cyclase. It has been suggested to activate tmACs by inducing dimerization and/or active site rearrangements. It appears to be well indicated in conditions, such as eczema (atopic dermatitis), asthma, psoriasis, cardiovascular disorders, and hypertension and other conditions where the decreased intracellular cAMP level is believed to be a major factor in the development of the disease process.

Effects of cAMP:

cAMP is a second messenger that has many different effects:

- Interacts with ion channels
- Modulates gene transcription
- Modulates cytoplasmic proteins (allosteric regulation) such as in protein kinase A (PKA)

cAMP can also induce the following physiological effects: bronchodilation, prevention of platelet aggregation, activation of olfactory cells, positive inotropic action in the heart, lowering of blood pressure, anti-glaucoma effect, counteracting the inflammatory status.



Protein kinase A (PKA) is a tetramer formed by two regulatory subunits (R) and two catalytic subunits (C) which are bound together only in the inactive form. Four cAMP molecules bind to the R subunits activating the tetramer. Upon cAMP binding the C subunits will dissociate, exposing the binding site for substrates. PKA phosphorylate various target proteins showing the consensus sequence:

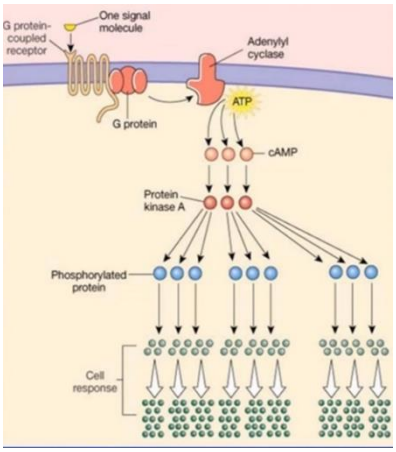
(X-Arg-(Arg/Lys)-X-(**Ser/Thr**)-B).

Note: Ser/Thr are the phosphorylation site

This is the reason why PKA is able to phosphorylate many different proteins.

9 Lecture (04/05)

Signal amplification



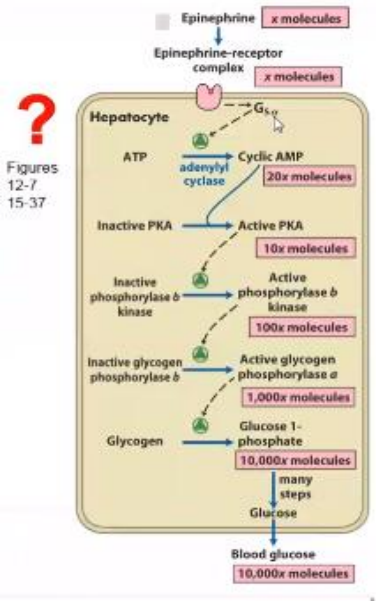
Upon ligand binding, the G-coupled receptor is activated and transduces the signal via G proteins, *that, depending on their α subunit,* can target different proteins: G*αs* proteins can activate Adenylyl Cyclase, that is able to produce cAMP in multiple copies (amplification of the signal).

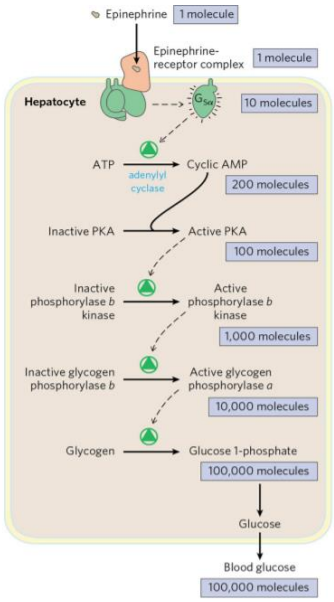
Once it is synthesized, cAMP can interact with soluble enzymes in the cytoplasm, such as PKA (Protein Kinase A).

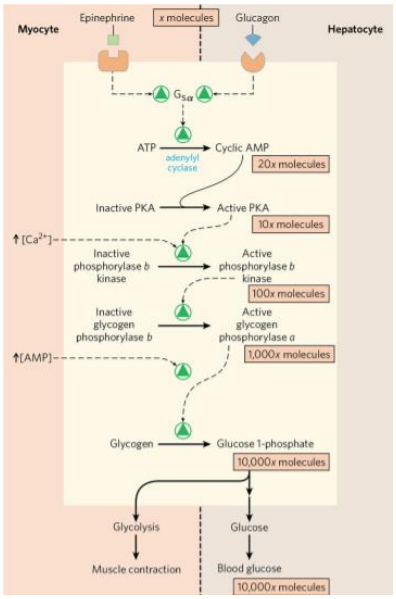
PKA has many substrates, and as shown in the picture, the signal is amplified. This occurs not just because PKA phosphorylates several copies of the same protein, but also because it phosphorylates different types of proteins. Since most of the substrates are enzymes, their products will increase in the cell cytoplasm and amplify the response to the hormone signal coming from the external environment.

In response to acute stress, **G proteins** are involved in the physiological preparation of the body for "fight or flight" a metabolic status featured by highly energetic reactions. *Epinephrine (through the serpentine β adrenergic receptors) triggers a phosphorylation cascade in hepatocytes, skeletal muscle cells and adipocytes, which mobilizes the energy generating machinery. Time elaps from stimulus to glucose release into the bloodstream is only few seconds (fight-or-flight response).*

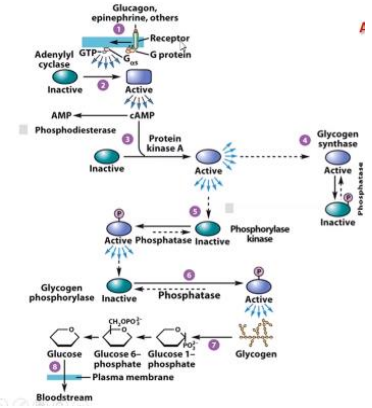
The images below shows the key role of signal amplification in this process:
Epinephrine reaches the receptor in 1:1 stoichiometry, but inside the cell the stimulated G-α protein targets Adenylyl Cyclase and many copies of each product are synthesized (amplification). At the end, around 10.000 molecules of Glucose are produced in the blood starting from 1 hormone.
? Find the difference between the 2nd and 3rd images.







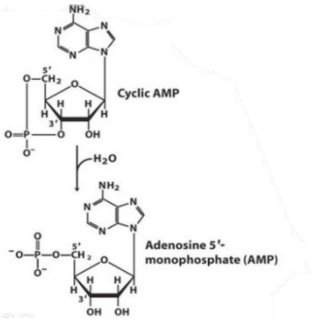
Pathway inhibition



The image shows a schematic representation of glycogen synthesis and breakdown pathways, both regulated by glucagon.

All pathways need to have control points where it is possible to modulate the response by switching off the signal.

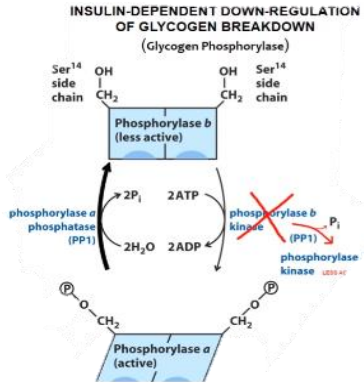
The **first control point** of this pathway is at the level of **Cyclic AMP**. Since cAMP is required for the activation of the pathway, its removal switches the whole system off.

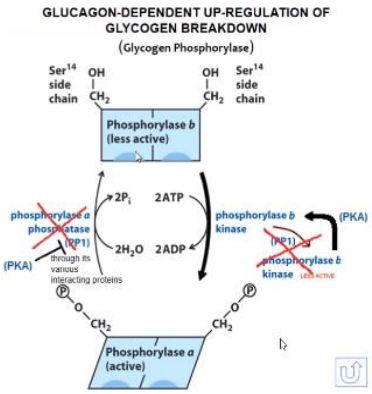


cAMP is removed by phosphodiesterase.
These enzymes catalyse the hydrolysis of the ester bond that allows cyclisation of the molecule, transforming cAMP into AMP.
It is known that caffeine can rise cAMP levels by mediating PDE inhibition, so that the signal for cAMP is not switched off.

Thus, this is the first control point in a long chain of events that characterize the epinephrine or glucagon signaling through the $G\alpha_s$ protein.

The **second control point** is in the glycogen breakdown pathway, at the level of glycogen phosphorylase.
The pathway shown on the right has already been described in the insulin dependent down-regulation of glycogen breakdown (first image). In that case it was a downregulation, but now it is an upregulation, as glucagon induces an increase of glycemia (second image). The enzyme that is regulated is the same for both pathways, but it is regulated in the opposite way. It is possible to make a comparison between the two pathways and find the differences.

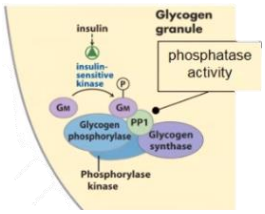




Glycogen Phosphorylase is active when it is in the phosphorylated form, while less active when dephosphorylated. Thus, to have an active and phosphorylated form of glycogen phosphorylase that is able to break glycogen, the enzyme phosphorylase kinase is required. Phosphorylase kinase can be in the active or inactive form as well. To remove its less active form, and have more copies of the active one:

- PKA (kinase regulated by glucagon hormone) is activated: it phosphorylates and activates phosphorylase kinase.
  So: PKA activates phosphorylase kinase, that phosphorylates glycogen phosphorylase that is needed for glycogen breakdown.
  Note: PKA sets the stage for the shutdown of glycogen degradation by adding a phosphoryl group to the α subunit of phosphorylase kinase after first phosphorylating the β subunit. This addition of a phosphoryl group renders the enzyme a better substrate for dephosphorylation and consequent inactivation by PP1: it is an internal stop that makes the phosphorylase kinase stop working.
- PP1 activity has to be inhibited (next page)

At the same time PKA is activated, any possible futile cycle must be eliminated: the active glycogen phosphorylase must not be converted back to the less active form. Since PP1 is the enzyme needed for glycogen phosphorylase inhibition, glucagon must remove PP1 by the action of PKA (which phosphorylates PP1). However, this is not a direct phosphorylation of PKA on PP1, but there are other proteins phosphorylated by PKA that can modulate PP1.

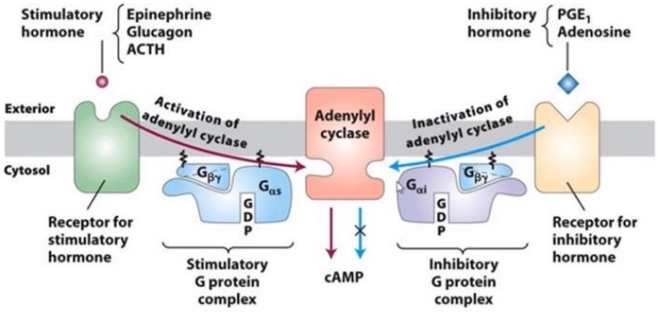


In the picture on the right (mentioned in the insulin pathway) PP1 is modulated by GM (interacting protein factor able to stimulate PP1 activation). Similarly, PKA can phosphorylate an X factor different from GM. This factor, that is close to PP1, will inhibit it.

Another way to create a switch off signal for G protein dependent signaling pathway can be through a mechanism of inactivation by several **hormone antagonists.**

There are some types of G proteins, such as $G\alpha_s$ that can stimulate Adenylyl cyclase. However, there are other types ($G\alpha_i$) that will use AC as a target for inhibition . $G\alpha_i$ comes from its own receptor that is bound by a specific hormone.

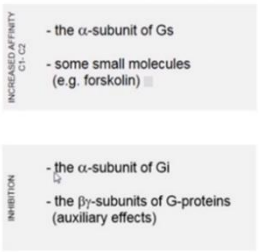
In the image below, it is possible to see from the left the stimulatory hormone going through $G\alpha_s$. However, if the signal needs to be switched off after a while, more hormones coming from the blood inhibit the same enzyme and stop cAMP production.



Some example of hormones involved in this $G\alpha_i$ pathway are prostaglandins (E1), adenosine, somatostatin (produced by δ cells in the pancreas) and acetylcholine (M2 and M4 receptors).

This is a **hormone antagonist derived inactivation of G protein in cells**.

The image on the right shows the regulation of cyclase activity by different effectors; in particular, the enzyme can be inhibited by:



- **$G\alpha_i$** (instead of **$G\alpha_s$**): the interaction with C1 and C2 is slightly different, and the active enzyme site of AC is affected (inhibition of the enzyme).
- **β** and **γ** subunits represent a possible inhibition step in this phenomenon. They have an auxiliary effect; they can induce the time off for the signal that the corresponding α subunit created.

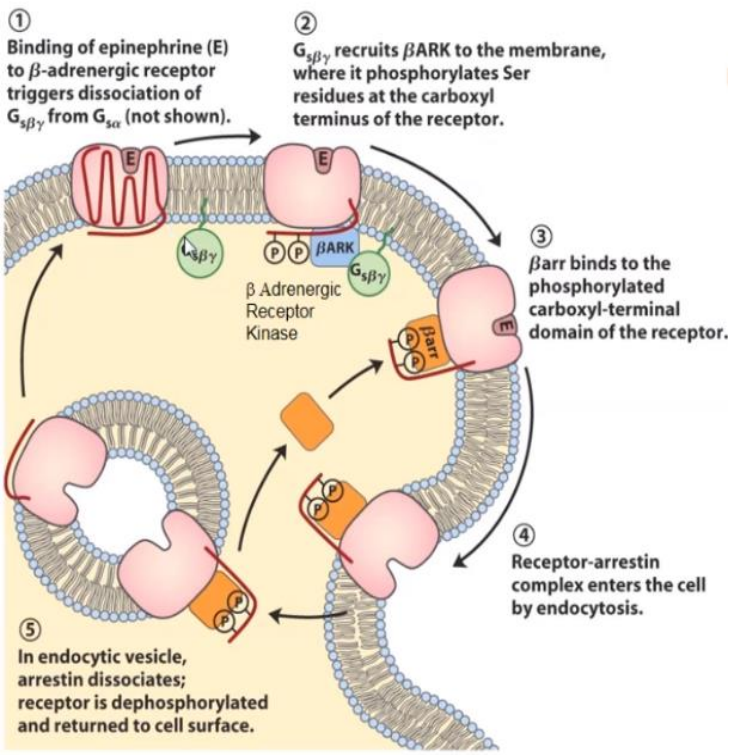
Q: How do accessory β-γ subunits inhibit adenylyl cyclase? Does the process induce AC compartmentalization or is it a form of allosteric regulation?

A: I am not sure whether β-γ subunits can stimulate AC internalization, but they may interact with AC through surface recognition (in a different position from the site of recognition for Gα): after this interaction, some kinases may be recruited to phosphorylate specific residues of AC and make the enzyme less active.

An example of off-switch by β and γ subunits is the desensitization of the **β-adrenergic receptor in the continued presence of epinephrine** due to auxiliary effect of **β** and **γ** subunits.

1. Regular activation of the receptor leads to the Gα stimulation of Adenylyl cyclase. However, the picture shows only the fate of β and γ proteins, that diffuse on the surface of the membrane without going very far from the receptor since their target is the receptor itself.

2. β and γ attract β-ARK protein (β-adrenergic receptor kinase). This is a protein named according to this pathway; however, there are many other proteins with the same function but in different pathways. β and γ displace it in a proper position so that β-ARK phosphorylates the carboxyl terminal tail of the receptor (carboxyl terminus of the 7-transmembrane receptor).



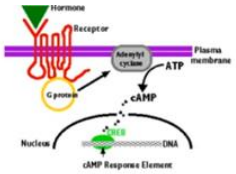
3. The phosphorylation becomes a signal for many other steps in the cell. In particular it becomes a docking site for β-arr. It is a protein that can interact with the phosphorous residue in the phosphorylated receptor.

4. By binding to the receptor they trigger an endocytosis process. The β-arr interacts with the cytoskeleton in the cell and they are included in vesicles so that the entire receptor is stored in vesicles and disappears from the surface. Thus, the cell become insensitive to the signal, even when the hormone is still in the extra-cellular environment. In some specific cases, the receptor is endocytosed with the hormone bound: this is a way to make the hormone enter the lumen of the vesicle in the target cell.

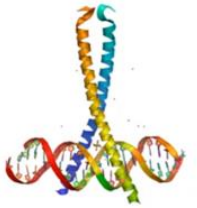
5. After a while, the enzymes in the cell remove the phosphorous from the carboxyl terminal of the receptor and the latter is regenerated in its basic form. Thanks to other regulatory pathways, the entire vesicle fuses again with the plasma membrane and releases the receptor ready for a new hormone to arrive.

This is a way to make the entire cell desensitized to the signal for a fairly long time. It is important when dealing with the threshold of sensitivity to hormones. Some cells become insensitive to hormones as they have the lag time due to internalisation of the receptor from the cell surface.

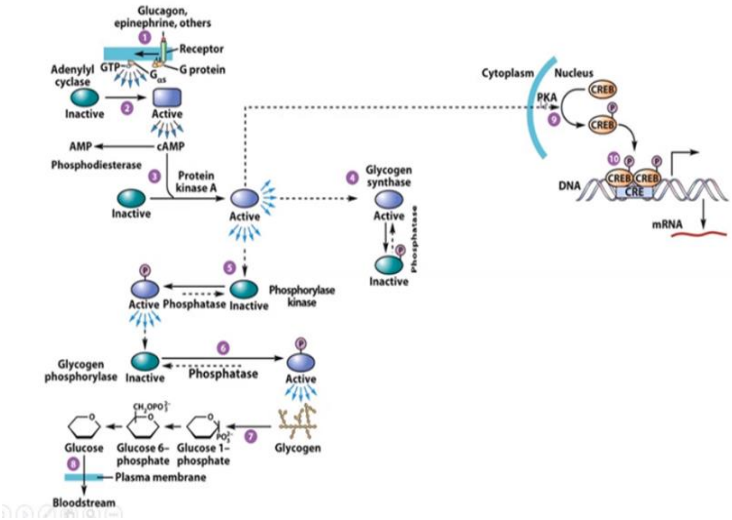
cAMP-dependent gene transcription



G protein coupled receptors are metabotropic: they alter metabolism by altering pre-existing enzymes in the cell. However, there is a way for these receptors to affect DNA metabolism/genome expression. This occurs because cAMP can modulate indirectly CREB (c-AMP response element binding protein).
Since CREB is a DNA binding protein, it will interact with specific recognition sequences in the promoter of the gene (5' TGACGTCA 3') and change gene transcription (either stimulation or inhibition). This mechanism has been found in many physiological conditions, like spermatogenesis, circadian rhythm, and memory.
DNA binding of CREB is mediated by a specific leucin zipper domain (alpha helix).


The DNA binding of CREB is mediated via its basic leucine zipper domain (bZIP domain).

It is possible to observe a third branch in the following scheme; as shown, PKA is thought to phosphorylate CREB.



Q: Is Ca also allosterically activating CREB?
A: for what I know, when PKA phosphorylates CREB, calcium is not needed. However, once CREB has been phosphorylated by PKA, it might need other factors in order to interact with DNA: one of these proteins could require calcium to function properly.

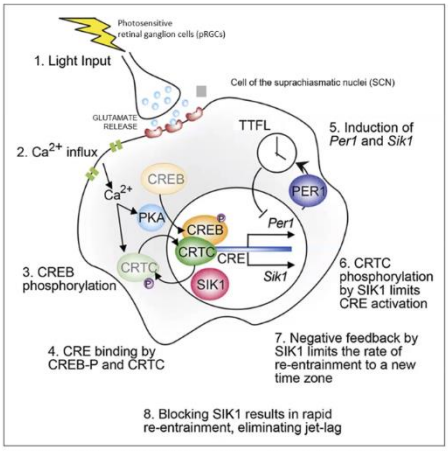
Q: Is cAMP also allosterically activating CREB?
cAMP modulates PKA allosterically, then PKA can phosphorylate CREB (that is not an enzyme, but a protein that can recognize DNA).

Note: CREB is involved in the resetting of the **circadian rhythms**, in the way we sense the light and understand whether it is day or night: light-induced phase shifts are accompanied by rapid phosphorylation of CREB at Ser133 and Ser142.

In the image, neurotransmitter glutamate is released in the synaptic cleft between specific retinal ganglion cells and binds to both ionotropic receptors and metabotropic receptors.

Retinal photoreceptors entrain the circadian system to the solar day through a transcriptional translational-feedback loop (**TTFL**).

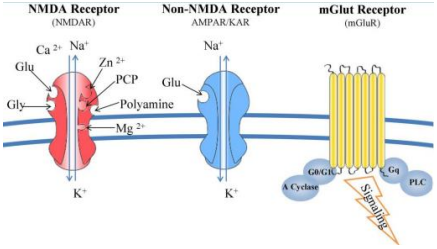
This permits SCN-neurons to readily fire action potentials during the day (up-state) and make them less likely to spike at night (down-state). In most mammals, behavioral shifts are limited to approximately 1 hr (one time zone) per day. Thus, entrainment is a gradual process requiring repeated shifting stimuli over multiple days.



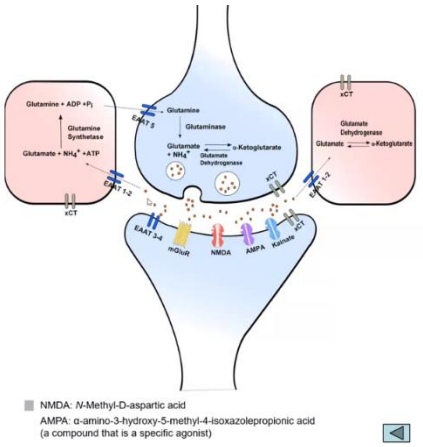
SIK1, salt inducible kinase 1; CRTC1, CREB-regulated transcription coactivator 1; TTFL, transcription-translation feedback loop; PER1, *period* circadian protein homolog 1

It is possible to distinguish:

- Ionotropic glutamate receptors:
  - **NMDA receptors**, also called N-methyl aspartic acid receptor. *NMDA form channels that are more permeable to Ca2+ than other ions.*
    The formula of NMDA is very similar to aspartic acid, except for the methyl group bound to the nitrogen. The receptor takes the name not from the biological signal but from the chemical used to study these protein receptors and how they affect physiology of cell.
  - **Non-NMDA receptors**. Kainate and AMPA receptors *are more permeable to Na+ and K+.*
    AMPA is similar to glutamate (recognize the carboxylic group, amino group, the a carbon and a lateral chain).



- **Metabotrobic glutamate receptors:** *there are eight different types of these receptors. Thet are G-protein coupled receptors targeted to their associated ion channels via a second messenger cascade.*
  - *mGluR-1 and -5 → Gq → PKC-dependent pathways potentiating the NMDA responses → larger quantities of Ca2+ ions can be moved*
  - *Other mGluRs → Gi → PKA-dependent pathways → cAMP decrease*

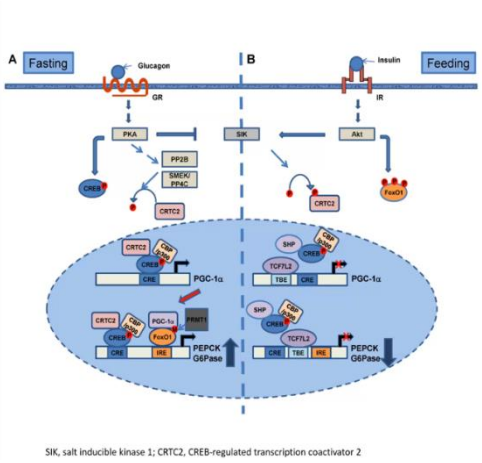


The image is a good representation of integrated pathways of signaling and disease: physiology (nerve synapses), cell signaling (NMDA receptor), and metabolic biochemistry (cell role of glutamate in the neutralization of ammonia). GLUTAMATE MODULATION | XVIVO Scientific Animation

Image: description of how energy metabolism is related to circadian rhythm. The temporal signals of fasting and feeding hormones (glucagon and insulin) regulate the signaling cascade of the CREB/CRTC2 transcriptional complex.

- *During fasting:* Glucagon, after interaction with its receptor, activates PKA. *Glucagon activates CRTC2 activity through its dephosphorylation by cAMP-mediated signaling, resulting in:*
  - nuclear localization of CRTC2
  - increased association with CREB *on the CRE sites of the promoters of gluconeogenic genes.*

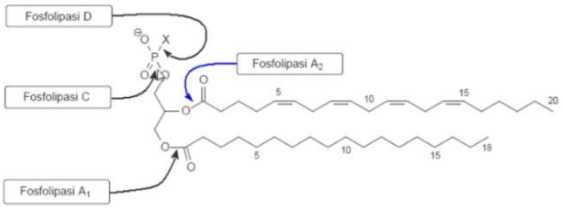
- *During feeding:* Insulin, after interaction with its receptor, activates AKT (also called PKB). *Insulin inactivates this coactivator by promoting its phosphorylation and nuclear export.*



Note: it is possible to observe also PP2B, CREB and other nuclear factors involved in the pathway (e.g. FOXO).

$G\alpha_q$: DAG and IP3 as second messangers

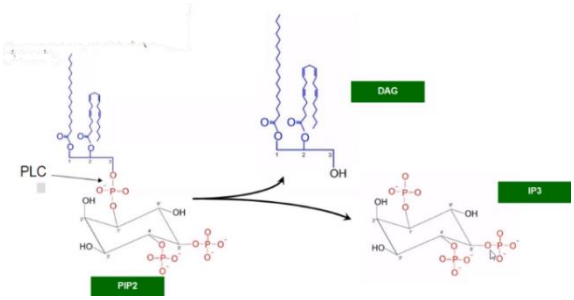
$G\alpha_q$ subfamily has PLC as a target (phosphoinositide-phospholipase C).



Phospholipase C is a membrane enzyme that, as shown by the image on the right, can break the covalent bond between phosphorous and oxygen, so that the entire polar head will be released.

PLC cleaves this type of covalent bond in **PIP2** phospholipid. PIP2 was mentioned in the fast branch of insulin signaling. In this situation there is another enzyme that has PIP2 as a substrate.
PLC can break the phospholipid into two portions, generating the second messengers:

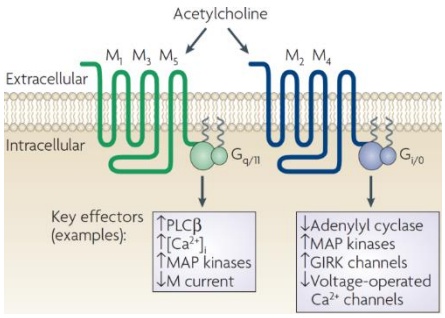
- **DAG** (diacylglycerol): glycerol and two acyl chains
- **IP3** (inositol 1,4,5-triphosphate): it is not a phospholipid (while PIP3 is) and it is a free soluble molecule, corresponding to inositol alcoholic group. Moreover, IP3 has three phosphorous groups, while PIP3 has four P groups bound to the inositol. PIP3 is obtained by the addition of a phosphorous group to position 3 of PIP2.



IP3 and DAG become second messengers in the new signaling pathway that $G\alpha_q$ type heterotrimeric protein has stimulated. This comes from several types of hormones that can stimulate $G\alpha_q$ proteins, such as acetylcholine (muscarinic receptor M1, M3, M5), vasopressin, thyroid-stimulating hormone (TSH) and angiotensin.

Note that acetylcholine can act in different ways on our bodies depending on the type of receptors it binds to. In fact, acetylcholine can bind to many receptors, differently from most signal molecules which only bind one receptor:

- Nicotinic receptors (not shown in the image);
- Muscarinic receptors with odd number of M which stimulate **Gq** proteins.
  M1, M3 and M5 mAChRs preferentially couple to G-proteins of the Gq/G11 family.
- Muscarinic receptors with even numbers of M which stimulate **Gi** proteins.
  The M2 and M4 muscarinic acetylcholine receptors (mAChRs) selectively activate Gi/G0-type G-proteins.The



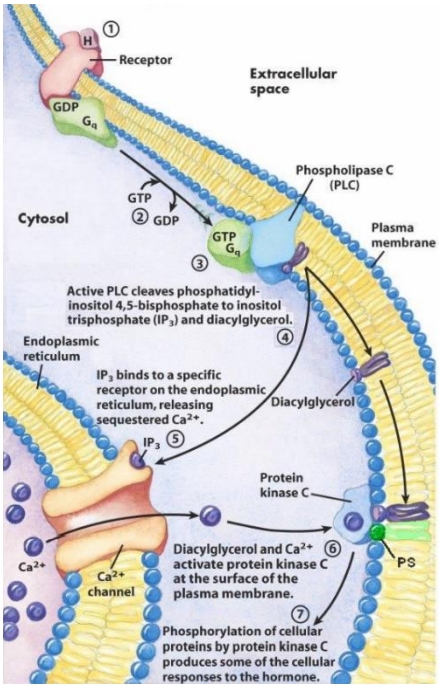
The effects of Gq and Gi proteins are briefly listed in the image. More in detail: M3 causes increase in smooth-muscle contractility and glandular secretion, M2 causes decrease in heart rate.

Not all cells will present all 3 categories of receptors; they will most likely predominantly express only one type of receptor. They can all be found in the central nervous system. M1, M4 and M5 are predominantly expressed in the CNS. M2 and M3 are widely distributed both in the CNS and in peripheral tissues that are innervated by parasympathetic nerves.

What is the final destiny and target of the second messengers DAG and IP3?

Phospholipase C (PLC) cleaves PIP2 in two portions:

- the diacylglycerol portion, which will still be very hydrophobic because of the acyl chains and will remain in the membrane.
- the polar head cut at the level of the phosphorous group. It is highly hydrophilic thanks to the presence of many phosphorous (which have a negative charge at neutral pH) and is therefore released into the cytoplasm.



The released polar head corresponds to **IP3** which is soluble in the cytoplasm and can diffuse until it reaches its target. The target is a protein channel of the ER: IP3 is the modulator of a ligand-dependent protein channel which will open only when IP3 binds to it. Channels are a way to connect two separate compartments of the cell and are normally transmembrane proteins. Be careful: this channel isn't found on the plasma membrane, it is found on the endoplasmic reticulum!

When this channel is opened, the Ca2+ ions originally stored in the endplasmic reticulum will pass through it (remember that Ca2+ is also stored in mitochondria, but they aren't involved in this specific pathway). Ca2+ ions will be forced by the concentration gradient to move from the ER to the cytosol.

In normal conditions, the cell will have low Ca2+ concentrations in the cytosol. The cell will need an increased concentration of cytosolic calcium in case, for example, of muscle contraction in muscle cells or insulin release in β pancreatic cells. In this particular case the examples just mentioned aren't the purpose of the increase in cytosolic calcium concentration.

In this case, calcium, an allosteric modulator, is used for the activation of **protein kinase C** (calcium-dependent protein kinase). For the full activation of protein kinase C, however, other elements are required: the diacylglycerol **DAG** (still present in the membrane after PIP2 cleavage) acts as allosteric modulator; and a side *acidic* phospholipd called phosphatidylserine (PS).

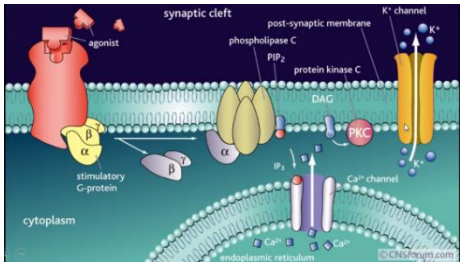
> *Note*: PKC is the *major* receptor for *phorbol* esters, *a class of powerful tumor promoters. Phorbol* esters mimic DAG and cause a more massive and prolonged activation than physiological stimuli. Plants that contain phorbol esters are often poisonous.

To sum up, DAG, PS and calcium will act together to fully activate protein kinase C. This process happens on the inner surface of the plasma membrane; the soluble pre-existing protein kinase C and calcium must reach the membrane.

Protein kinase C will then go on to phosphorylate its substrates. In praticular it can control a potassium (K+) channel in the plasma membrane, particularly important for signal transmission in neurons.

Schematic sum up:

1) Phospholipase C cleaves PIP2 phospholipid into diacylglycerol and IP3
2) IP3 activates Ca2+ channel in the ER membrane
3) Ca2+ diffuses into the cytosol
4) Ca2+, diacylglycerol and phosphatidylserine activate PKC
5) Protein kinase C is now active and can phopsphorilate its substrates (among which a K+ channel).

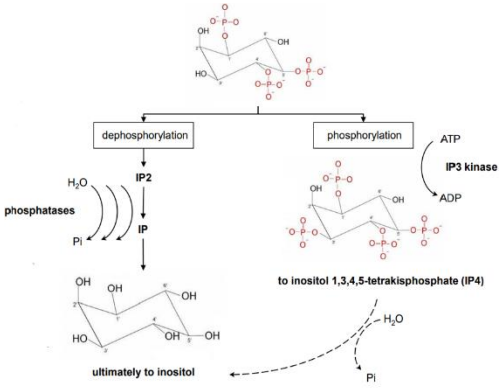


Q: Does PKC remain on the membrane after it has been activated?
A: Probably it will remain close to the membrane as one of its substrates (K^+ channel) is in the membrane; so there would be no reason for it to move far away from the membrane.
Q: Will DAG release PKC?
It is unlikely because there are 2 phospholipids anchored to PKC: in order to release it, both phospholipids should be detached; and, even if the interactions between them are not covalent, this process is not that easy

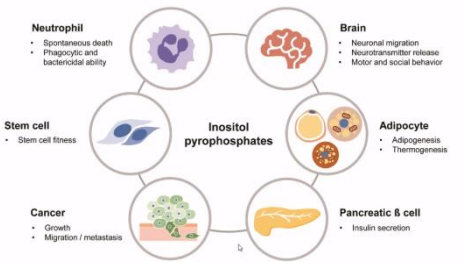
Pathway inhibition

This signaling pathway can be turned off by the removal of IP3 from the cell so that the ligand-dependent Ca2+ channel on the ER won't be able to open. However, IP3 can't just be "removed", it's rather transformed:

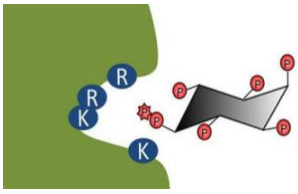
- It is converted to **IP2** by dephosphorylation. The binding site of the ligand-dependent channel won't recognize IP2. By further removal of the other phosphate groups by phosphatases, IP2 will be converted to IP and ultimately to *inositol*, which doesn't have any phosphorous groups.
- It is converted to **IP4** by phosphorylation (by IP3 kinase). IP4 won't be recognized by the ligand-dependent calcium channel. This reaction requires ATP.
  Phosphatases then remove phosphorous from IP4. IP3 has phosphorous in positions 1,4 and 5 as in the original phospolipid.
  If phosphorous begins to be removed from IP4 in one of these positions (1,4 or 5), the resulting molecule will be an IP3 which won't be recognized by the channel because P will be in different positions than originally.



Check: 1 paper on this topic, and 1 on the discovery of the conversion of IP3 to pyrophosphate IP molecules (PP-IPs). This conversion (still under study) would allow the inhibition of the pathway by the addition of a phosphorous next to one of the existing phosphorous group, creating a pyrophosphorous group (two P linked together). These PP-IPs have been found to participate in a wide range of metabolic processes: they are versatile signaling molecules with a variety of biological roles in cell/organs and in pathological conditions such as cancer.



Pre-existing proteins in the cell have lateral chains (in the image a protein with K=lysine and R=arginine) which can receive a phosphorous group donated by the PP-IP. It is possible to deduce that the formation of PP-IP to remove IP3 from the cell not only inhibits the IP3 patwhay, but can induce the start of a new signal.

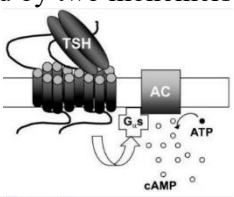


During the metabolic biochemistry module we will be taught how lipids are biosynthetised in cells. Another way to stop the signal is to recreate the initial PIP2 phospholipid by the union of DAG and inositol, but this is a very slow pathway which cannot be used as the major mechanism to stop the signal.

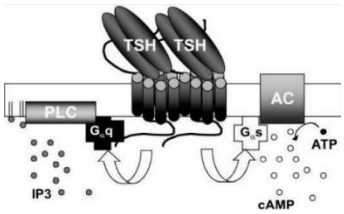
Thyrotropin (TSH) receptor

When talking about G protein mediated singnals many alternatives and exceptions exist.

One example of a more complicated mechanism can be visualized in this image. *Adenilyl cyclase* (AC) converts ATP to cyclic AMP (cAMP). The *Gα stimulatory factor* (Gαs), located next to AC, is important for production of cAMP. The receptor that generates the Gαs protein is a dimer formed by two monomers with 7 transmembrane helices each (so it has a total of 14 transmembrane helices). This is the receptor for the *thyroid stimulating hormone*/*thyrotropin* (TSH). Since the receptor is a dimer, the hormone can bind either in a monomeric or in a dimeric form:



- If only 1 molecule of TSH binds, Gαs will be stimulated to work
- If 2 molecules of TSH bind to the receptor, both the Gαs and the Gαq molecule will be stimulated. As a consequence, two separate signaling pathways will start: the one through the adenylyl cyclase (AC) and the through phospholipase C (PLC) and IP3 second messenger.



This complicated intracellular response will obviously affect metabolism.

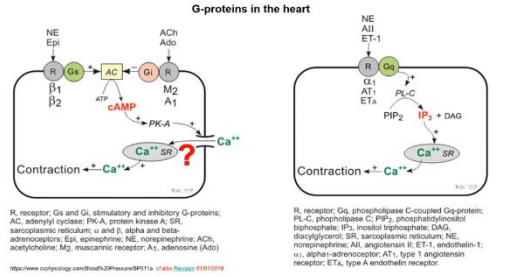
G-proteins in physiological process

In the heart

G proteins are extremely important in signaling in muscle cells, especially in the heart.

Read and explain the image: contraction of the heart can be regulated by calcium that is released from the sarcoplasmic reticulum in the heart can lead to a contraction, as mentioned in previous lectures. Its release can be regulated by the action of:

- Epinephrine (left image): *it binds to a serpentine receptor, Gs protein dissociates and activate AC that synthetize* cAMP. *cAMP activates PKA which targets Ca channel.*
- Other hormones: *bind to a serpentine receptor, Gq protein dissociates and activate PLC that produces DAG and* IP3 as a second messenger. IP3 is the major actor on Calcium protein channel in sarcoplasmic reticulum.



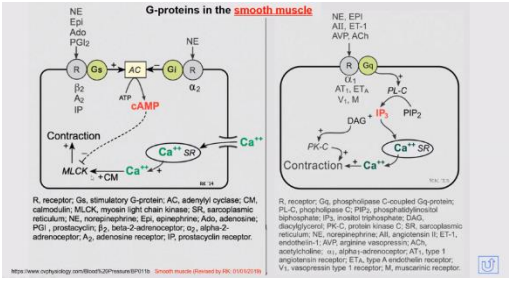
R, receptor; Gs and Gi, stimulatory and inhibitory G-proteins; AC, adenylyl cyclase; PK-A, protein kinase A; SR, sarcoplasmic reticulum; α and β, alpha and beta-adrenoceptors; Epi, epinephrine; NE, norepinephrine; ACh, acetylcholine; M_2, muscarinic receptor; A_1, adenosine (Ado)

R, receptor; Gq, phospholipase C-coupled Gq-protein; PL-C, phospholipase C; PIP_2, phosphatidylinositol biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; SR, sarcoplasmic reticulum; NE, norepinephrine; AII, angiotensin II; ET-1, endothelin-1; α_1, alpha1-adrenoceptor; AT_1, type 1 angiotensin receptor; ET_A, type A endothelin receptor.

In the Smooth muscle

Something (unknown) can regulate calcium flux: when Ca increases it binds to calmodulin and activates MLCK kinase protein (*check histology notes*), *iniating contraction*. cyclic-AMP, *produced by AC in response to Epinephrine binding*, can inhibit MLCK.

The second image shows the increase of Ca levels due to IP3 activity



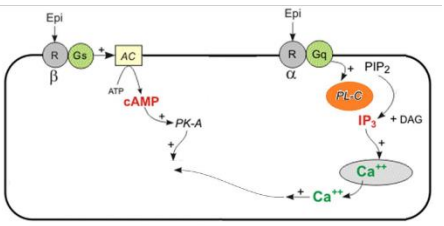
R, receptor; Gs, stimulatory G-protein; AC, adenylyl cyclase; CM, calmodulin; MLCK, myosin light chain kinase; SR, sarcoplasmic reticulum; NE, norepinephrine; Epi, epinephrine; Ado, adenosine; PGI , prostacyclin; β_2, beta-2-adrenoceptor; α_2, alpha-2-adrenoceptor; A_2, adenosine receptor; IP, prostacyclin receptor.

R, receptor; Gq, phospholipase C-coupled Gq-protein; PL-C, phospholipase C; PIP_2, phosphatidylinositol biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; PK-C, protein kinase C; SR, sarcoplasmic reticulum; NE, norepinephrine; AII, angiotensin II; ET-1, endothelin-1; AVP, arginine vasopressin; ACh, acetylcholine; α_1, alpha1-adrenoceptor; AT_1, type 1 angiotensin receptor; ET_A, type A endothelin receptor; V_1, vasopressin type 1 receptor; M, muscarinic receptor.

In the Liver

Protein kinase A phosphorylates the β subunit of phosphorylase kinase, which subsequently activates glycogen phosphorylase.

The signal-transduction processes in the liver are more complex than those in muscle. Epinephrine can also elicit glycogen degradation in the liver. However, in addition to binding to the β-adrenergic receptor, it binds to the 7TM α-adrenergic receptor, which then activates phospholipase C and, hence, initiates the *phosphoinositide cascade* (Section 15.2). The consequent rise in the level of inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from endoplasmic reticulum stores. Recall that the δ subunit of phosphorylase kinase is the Ca^{2+} sensor calmodulin. Binding of Ca^{2+} to calmodulin leads to a partial activation of phosphorylase kinase. Stimulation by both glucagon and epinephrine leads to maximal mobilization of liver glycogen.



R, receptor; Gq, phospholipase C-coupled Gq-protein; Gs, stimulatory G-protein PL-C, phopholipase C; PIP_2, phosphatidylinositol; Epi, epinephrine; biphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol; PK-A, protein kinase A

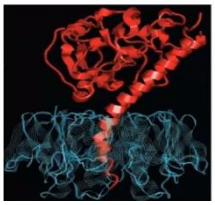
Microbes

Certain microbes cause disease by disrupting the G-protein signaling pathways. Bacteria that are pathogenic to humans can release poisons that interrupt specific G-protein-linked receptor function, leading to illnesses such as cholera and pertussis.

Cholera Toxin

The water-born cholera bacterium, Vibrio cholerae is a toxin that can secrete different types of toxins, which are able to affect G-protein signaling pathway in the target host. These toxins binds to both the epithelial and nervous cells of the small intestine.

There is an abrupt onset of symptoms after 6 hours to 5 days incubation, including severe diarrhea (can reach a rate of 1 L/hr).



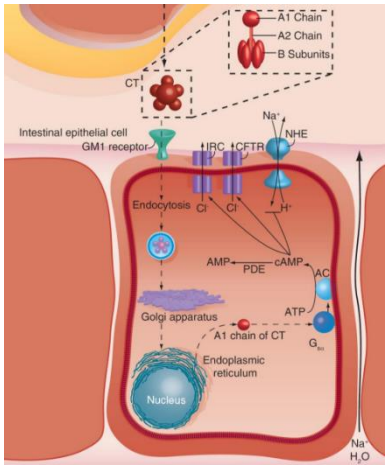
The image shows the protein structure of protein toxin, with its subunits A and B.

- Toxin A-subunit (*CTA1*) is transported to the cytosol and induces intoxication by affecting the activity of adenylate cyclase
- Toxin B-subunit is the effector of the cell, responsible for binding to the cell.

At this point (action of Cholera toxin):

- Toxin will be elaborated by the host cell: *the* CTx-GM1 complex *is trafficked retrograde from the plasma membrane to early endosomes*, the Golgi, and finally to the endoplasmic reticulum (ER) where the A1 chain of the toxin is able to utilize the ER-associated degradation pathway to enter the cytosol.
- The A1-subunit of the toxin activates the heterotrimeric G-protein, Gαs.
- The modified Gαs loses its GTPase activity and remains constitutively active in its GTP-bound state, causing a continuous stimulation of AC

  Note: GTPase activity is decreased by changing the structure of GαS polypeptide: specific domains are modulated in such a way that they cannot work properly and cannot catalyze the release of the phosphorous group form GTP. Examples of possible modifications are:
  1. Phosphorylation [covalent modification]. In this case, A1 should be a kinase to phosphorylate GaS, but it is not.
  2. Allosteric modulation [non-covalent modification]; *this regulation has two main requirements*: the substrate needs a binding site for modulator, and the modulator must be well positioned in the binding site in order to change the 3D shape of enzyme.
- The loss of GTPase activity will make the Gαs protein stay active, so that Adenylyl Cyclase is constantly activated and produces cyclic-AMP.



E.g. Effects of cAMP.

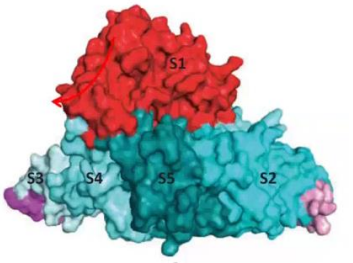
CFTRs channels *in enterocytes*: cAMP regulates the channel and changes the flux of Cl so the amount of chloride that is secreted is too high compared to physiological levels. Since cAMP is never switched off, all the Chloride diffusing outside the cell will cause water to follow due to osmotic reasons causing dehydration and leading to cystic fibrosis. (cAMP can regulate the CFTR channel, specifically enhancing the outflow of chloride. If cAMP is excessively produced, CFTR is constantly active, creating a non-physiological situation).

This results in large losses of intestinal fluids and potentially fatal dehydration as a result.

Pertussis Toxin

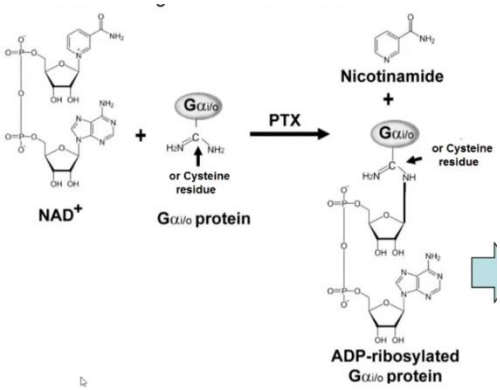
Bordetella pertussis is the bacterial agent of pertussis or whooping cough; it is a toxin which is induced to enter in the target human cell by the bacteria.

The toxin is composed of 6 subunits (AB5):



- *Active subunit (S1)*: will induce the molecular alteration. *This part is covalently linked with the rest of the molecule*
- *B subunits* are adhibited to the intrusion in the cell, they attach the toxin to the target cell (both toxins have to be internalized in the cytoplasm). *They bind glycoconjugate molecules on mammalian cell surfaces.*
- Once the protein is *endocytosed* and metabolised in the host cell, *retrograde transport pathway makes it access the cytoplasm*. The A-subunit modifies a cysteine residue near the C-terminus of the a-subunit of Gi proteins in virtually all mammalian cells, thus inhibiting activation of these Gi proteins by their G protein-coupled receptors (GPCR).
- The inactivation of Gi occurs due to ADP-ribosylation.

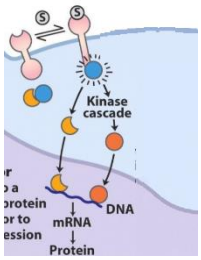
  The ADP-ribosylation requires NAD+ and a target protein (Gi in this case) as substrates. PTX (Pertussis ToXin) enzyme breaks the bond between Carbon and the Nitrogen: it catalyses the removal of nicotinamide from NAD+ and attaches the remaining ADP-ribosyl group to a lateral residue in the target protein (depending on the catalytic site of the toxin: pertussis toxyin attaches it to cysteine, *while cholera toxin attaches it to arginine*). This way the protein substrate will change structure, losing the functions.



- After the alteration, Gαi protein will be inhibited, adenylyl cyclase won't be inhibited by Gαi so cAMP will be overproduced.
- The result is the same as the previous toxin, but the cell type is different since the Bordetella pertussis is specific for the respiratory system. The effect is the same (excess of cAMP), with consequences in lungs in ion channels, very important in that type of epithelium. *(reduced vascular barrier integrity and edema in the lung)*

RECEPTORS WITH NO INTRINSIC ENZYME ACTIVITY

N Lecture (06/05)



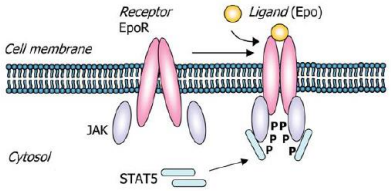
Receptors with no intrinsic enzyme activity are transmembrane proteins, that, upon hormone binding, recruit a pre-existing enzyme from the cytosol. In many cases, the pre-existing enzyme in the cytosol is a protein kinase. The enzyme will then bind to the intracellular domain of the activated receptor to form a complete active enzyme receptor. Receptors with no intrinsic enzyme activity activate (directly or through a cascade of MAPK) transcription factors that modify gene expression.

Example of ligands:

- Cytokine (Interleukin, IL-2, -3, -6).
- Hematopoietin (Erythropoietin), which can regulate the production of blood cells.
- GH (GrowthHormone).
- Prolactin is involved in the signal for the production of breast milk.

These hormones send a signal to the nucleus and to the genome in order to synthesize new proteins needed for cell division or for production of specific molecules that need to be secreted (e.g. prolactin).

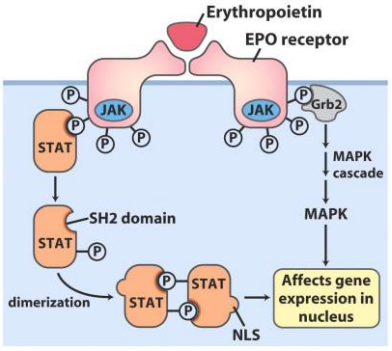
Jak-Stat pathway



Binding of the hormone (*EPO*) *promotes dimerization of the receptor so that* the cytoplasmic part of the transmembrane receptor becomes able to recruit kinase JAK (Janus Kinase) from the cytosol. When JAK is free in the cytosol it is a non-active enzyme but when it is recruited and assembled with the transmembrane receptor (through hydrophobic interactions) it becomes an active kinase, that can phosphorylate:

- JAK present on the other monomer: auto-trans phosphorylation at tyrosine residues *on the cytoplasmic tail* results in full activation of the receptor.
- STAT[1] protein: *STAT's SH2 domain interacts with JAK's P-Tyr motifs.* STAT is also a substrate for the kinase activity of the newly assembled receptor and STAT itself will be phosphorylated on a single tyrosine residue.

Note: While JAK will stay bound to the enzyme as long as the ligand is present, STAT is a regular substrate: it will be released as a phosphorylated substrate.



Once released as a free soluble product in the cytosol, STAT will form a **homodimer**: two molecules of phosphorylated STAT will bind together, through their SH2 domains and P-Tyr.

A nuclear localization signal (NLS) *allows STAT to enter the nucleus through nuclear pore complexes (highly regulated process) and* affect gene expression.

Note: when there is a receptor with many phosphor-tyrosine, it may be recognized by other signalling intermediates from the cytosol, such as GRB2 that can easily bind the JAK-STAT phosphorylated receptor and induce the MAPK cascade, aimed at the regulation of transcription factors which will affect gene expression in the nucleus.

In summary, the activated STATs dissociate from the receptor and dimerize to become competent for nuclear translocation and DNA binding (GAS enhancers, palindromic consensus sequences).

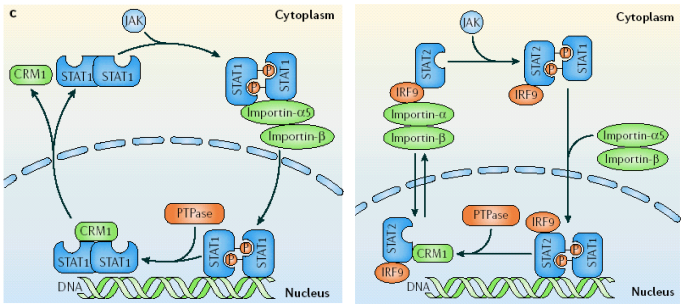
The Inner Dynamics of the Cell on Vimeo

[1] In the scheme, STAT5 isomer is present. This is because there are many different STAT types.

Importance of the cycling of STAT from the cytoplasm to the nucleus and vice versa:

An advanced description of the mechanism focuses on the fact that phosphorylated STAT protein will be dephosphorylated by phosphatases in the nucleus when the signal stops. When **dephosphorylated**, STAT will be allowed to exit the nucleus.

In addition, researchers found that dephosphorylated STAT can even enter the nucleus with a different role.

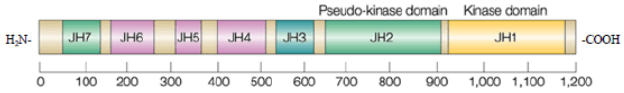


The regulation of the nuclear trafficking of STAT-family members is diverse. Some STAT proteins constitutively shuttle between the nucleus and the cytoplasm, whereas others require tyrosine phosphorylation for nuclear localization. In either case, the regulation of nuclear trafficking can provide a target for therapeutic intervention. - nri1885.indd (unibo.it)

JAKs (JAK1 to JAK5 and TYK2) constitute a family of the very large superfamily of cytosolic tyrosine kinase proteins and almost 40 cytokine receptors perform signalling through combinations of various JAK and STAT. Many types of hormones can interact with these receptors.

- An example is **EPO** (erythropoietin), which is the hormone inducing haematopoiesis. The specific cells targeted by this physiological hormone signal are red blood cells and thus, EPO leads to the production of red blood cells through a JAK-STAT signalling.
- **Interferons** (IFNs) are a family of immunoregulatory cytokines with important roles in anti-viral and anti-tumor responses. The biological significance of STAT2 in type I IFN signaling is apparent in STAT2 deficient mice. These mice are vulnerable to viral infection and host immune response is compromised.

JAK



JAK molecule can be considered as a relatively small single polypeptide of 1200 amino acids. It is divided of several domains, called JH domains. **JH1** is most important; it is the **kinase** domain and the catalytically active domain. Thus, this is the part where the binding site for the substrate will be located.

The other domains are needed for regulatory purposes, for recognition of the receptor and regulation of JAK activity.

- JH3-JH7 [*FERM domain*] mediate association with the proline-rich membrane proximal domain of receptors. This is because the surface recognition between JAK and the transmembrane receptor is a hydrophobic interaction thanks to the proline-rich transmembrane domain.

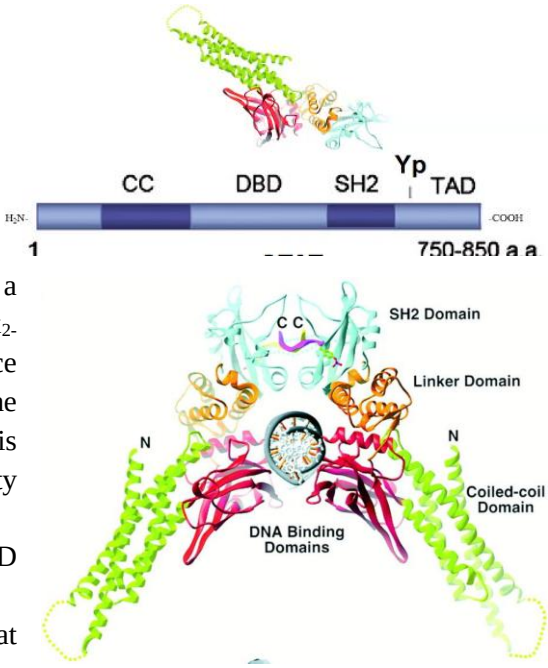
The name is taken from the two-faced Roman god of doorways, Janus, because the JAKs possess two near-identical phosphate-transferring domains.

- One domain (**JH1**) exhibits the kinase activity
- One domain (**JH2**, also called the pseudo-kinase domain) negatively regulates the kinase activity of the first. As a result, it is a way to have a **switch-off** signal (internal clock) inside the molecule.

STAT

On the other hand, STAT is a protein composed of 750-850 amino acids.

There are different domains:



- **DBD** stands for DNA binding domain, and it is a specific 3D conformation of a polypeptide that has to match with a nucleic acid molecule, like DNA.
  In this structure, there is a β-barrel conformation and a binding motif in the dimer which recognizes a palindromic sequence in the DNA (sequence TTC x_{2-4}GAA), common to the gamma activated sequence enhancers. Also, the dissociation constant (K_d) is in the order of nano molar, which means that the binding is specific but not very stable. A low K_d means high affinity between STAT and the genome.
  Each DBD will have a scissor-like structure in the 3D conformation.
- **SH2** domain has been discussed above. Remember that these domains match to form a connection.
- **CC** domain in the amino terminus of the protein, or coiled coil domain, is composed by 4 -helices forming an *80 Å* protrusion. It presents a hydrophilic surface composed by the 4 helices and this is region that can be modulated by other protein factors so that STAT can participate in forming supramolecular aggregates with other signalling molecules. In particular, the IRF9 regulatory interacting protein can be mentioned as an example. This region is also implicated in pore recognition and nuclear transport.
- **TAD** domain, on the carboxyl domain of the protein stands for transcriptional activation domain. Its role is activation of STAT: it contains a single tyrosine residue, indicated with the letter **Y**, that becomes Yp (phosphor-tyrosine) after activation by the JAK receptor.

Dangers of EPO

Synthetic blood boosters are a genetically engineered version of the natural hormone erythropoietin (EPO) found in the body. The synthetic version works the same way as natural EPO. It is generally used to treat anaemia associated diseases such as diabetes and kidney disease.

However, athletes use synthetic EPO to enhance their performance. When administered intravenously, it travels to the bone marrow where it interacts with EPO receptors to stimulate the production of red blood cells from bone marrow stem cells. The increase in the number of red blood cells delivers more oxygen to all cells in the body, especially muscle cells.

By taking synthetic EPO, athletes can boost their red blood cell count by stressing their cells and are able to supply their muscles with oxygen for a longer amount of time. EPO essentially gives the athlete more fuel for their muscles. This leads to increased performance and an unfair advantage over competitors.

Endurance athletes who use EPO can increase the oxygen carrying capacity of their red blood cells by as much as 7 to 10 %!

However, if synthetic EPO is present in the blood, all the JAK-STAT receptors will recognize the molecule and induce cell division. This uncontrolled cell division puts the athlete at a greater risk for cancer, mainly leukaemia.

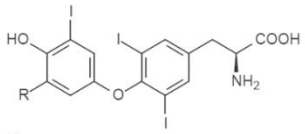
THYROID HORMONES

12 Lecture (07/05)

Thyroid hormones are synthetized by the thyroid gland and are critical regulatory molecules with important roles in vertebrate physiology and development, including fetal and post-natal nervous system development (cretinism) and the maintenance of adult brain function (severe intellectual defects and defects in fine motor skills).

These hormones bind to nuclear receptors.

Thyroglobulin, a protein precursor with numerous enzymatically fused residues of iodinated-Tyr, accumulates in the thyroid follicle (colloid). The two thyroid hormones are released as a result of proteolytic processes (stimulated by TSH).

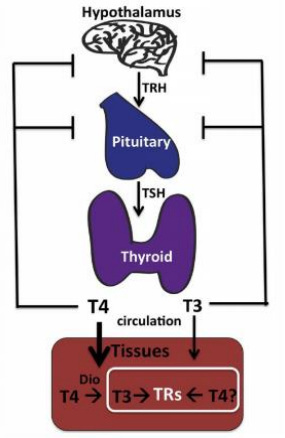


3,5,3'-Triiodotironina (T_3) R = H

Tiroxina (T_4) R = I

Note: Triiodothyronine (T3) and Thyroxine (T4) are the two hormones. The difference is in the R, so T3 has 3 Iodide (I) , while T4 has 4 Iodide.

The hypothalamus senses low TH in the circulation and responds by stimulating synthesis and secretion of ***TRH****, which in turn circulates and stimulates synthesis and secretion of* ***TSH*** *by the pituitary. Circulating TSH then increases THs production by the thyroid and ultimately in the circulation.* The thyroid gland makes both T4 and T3, although T4 predominates. Tissue-specific selenoprotein deiodinases ("DIO") are expressed in peripheral tissues such as brain astrocytes to increase local concentrations of T3 from circulating T4: when T4 and T3 reach peripheral organs (majorly the Liver) T4 is converted in T3 form (by removal of an I), that is the active form of the thyroid hormone. The cellular plasma membrane is not significantly permeable to T3 and T4; both molecules enter and exit cells through specific transporters (MCT8 - monocarboxylate transporter). This transmembrane protein is also in thyroid glands cells and allow the exit from it to reach the blood. The hormones are synthetized in the lumen and exit from it thanks to MCT8.



For the Formation of this hormone, there are some carriers:

-**NIS**: Ion carriers for Iodide (I-), such as NIS (Symport active transporter of second type Na+/I-) to synthetize T3 and T4

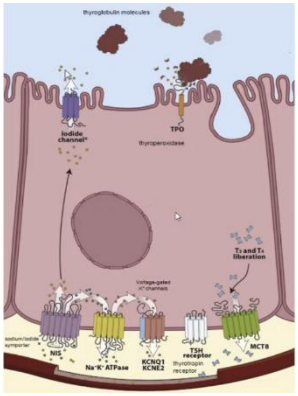
-**Iodide Channel**: diffuse the ion I (passive transport) in the lumen

-**Thyroglobulin molecules** (polypeptide) produced by the glands, are in the lumen and contain the amino acids residue to form the Hormones T3, T4.

-**The receptor** (G protein type): it has an important role in order to regulate both NIS and production of thyroglobulin.

-**TPO (thyroperoxidase):** apical transmembrane enzyme (for synthesis of T3 and T4) conjugates the I atom with the lateral chain of Tyr in polypeptide. In order to make this covalent bond the molecule needs peroxide H2O2

- **MCT8**: to allow to T3/T4 to go out from the cell.

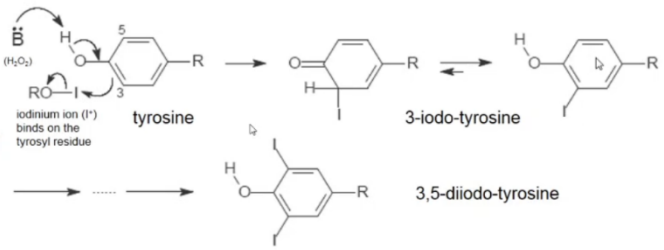


Iodide enters the follicular cell through **NIS**, and it reaches the lumen inside the glands passing through **Iodide channel** (passive transport). The Iodide ions and Thyroglobulin (provided by cells) with peroxide and **TPO** form the T4 molecule. Then T3 and T4 are released in the cytoplasm of the cell leave it through **MCT8**.

Formation of Thyroglobulin

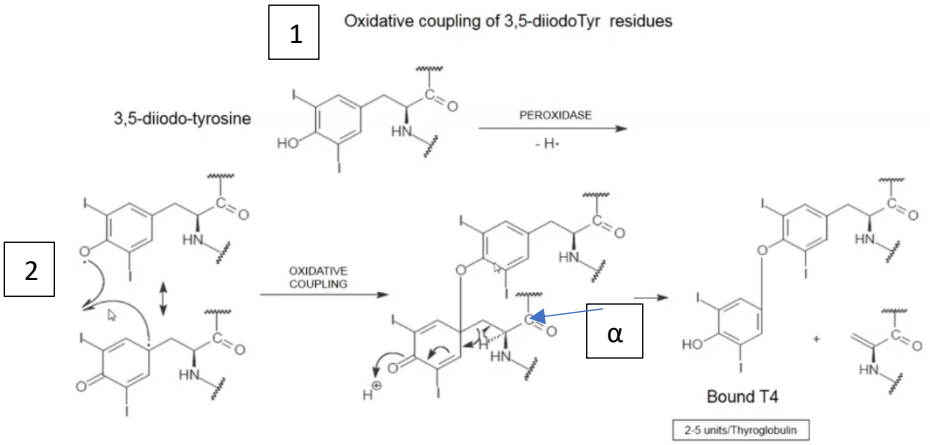
Note: no need to remember the formula and reaction

Hypoiodite (RO _ I) reacts with Tyr (66 residues in humans) and becomes incorporated in the molecule of thyroglobuli



Binding Iodide in the lateral chain, it can be positioned in the 3° and 5°.

Reaction between Thyroglobulin, TPO and Peroxide

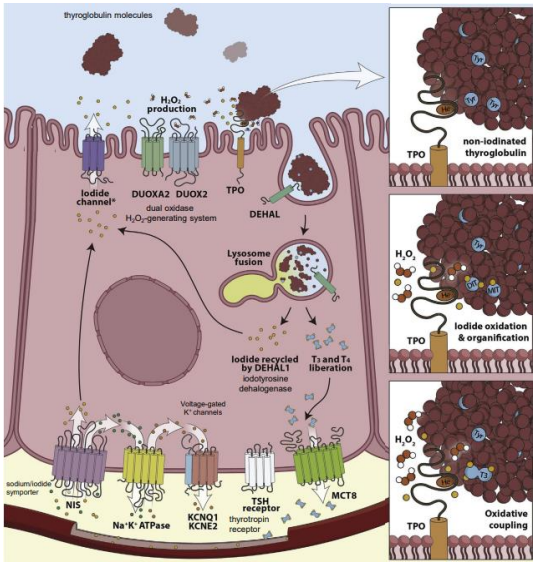


1 – Formation of **3,5-diiodo-tyrosine**

2&3 - Two ions **3,5-diiodo-tyrosine** are matched together with oxidative coupling (enzymatic catalase), to erlang lateral chain by the connection of two lateral chain of amino acid residue.

4 – The α-Carbon is linked with peptide bond, but this bond is broken, so it has been obtained the T4

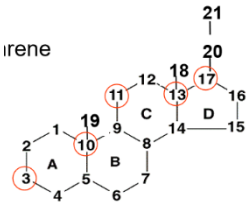
After T4 and T3 can go out from the cell (MCT8)



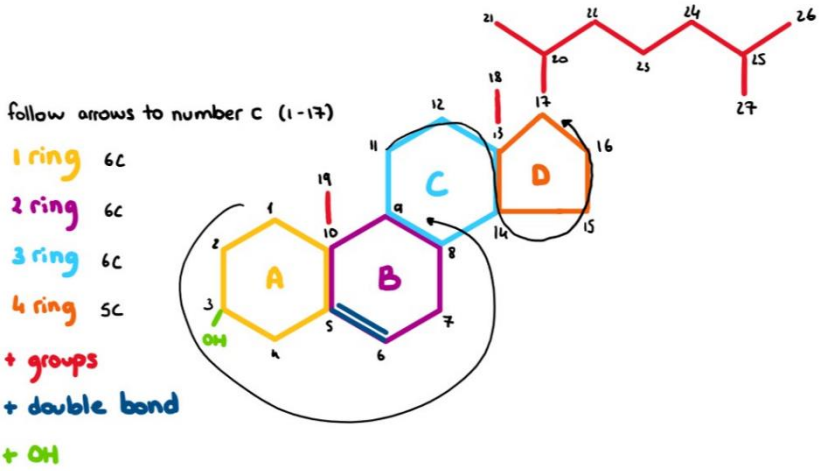
CHOLESTEROL

13 Lecture (07/05)

Steroid hormone's structure is derived from cyclopentane perhydro phenanthrene - Sterane - (composed of 17C and 4 rings). These hormones are not storable. They are produced and released in the circulation. Since liposoluble, S.H. require protein carriers in order to circulate in the plasma: albumin (low affinity) and α globulin also protect S.H. *against inactivating metabolism while travelling towards target tissues.*

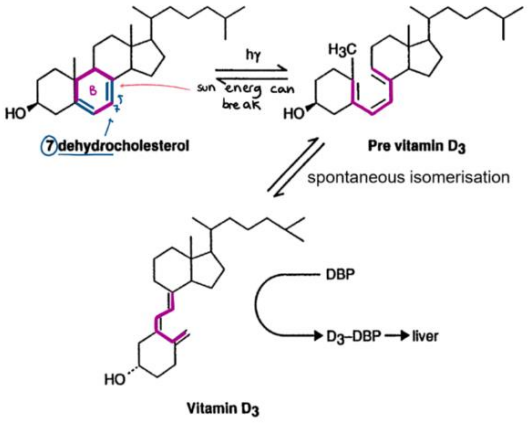


How to draw cholesterol molecule:



Cholesterol can be converted to:

- Vitamin D: at the level of the skin, after the exposure to UV light, the non-enzymatic conversion of cholesterol to previtamin D3 occurs through the breakage of ring B; then Vitamin D is formed (it is a signaling molecule)
  - Cholesterol is converted to 7 dehydrocholesterol: a dehydrogenation reaction occurs at carbon 7 and a double bond is formed
  - Sun energy breaks the unstable B ring present in 7 dehydrocholesterol, forming Pre-vitamin D3
  - A spontaneous isomerization transforms Pre vitamin D3 into vitamin D3, that is transported to the liver *bound to the DBP (vitamin D binding protein)*



- Bile acids: Cholesterol can produce bile acids, such as cholic acid that is involved in the digestive process. These *compounds* have more OH groups than cholesterol, thus they are more hydrophobic
- Progesteron and steroid hormones

Conversion to bile acids and sex hormones will be described in the next pages

Byosynthesis of steroid hormones

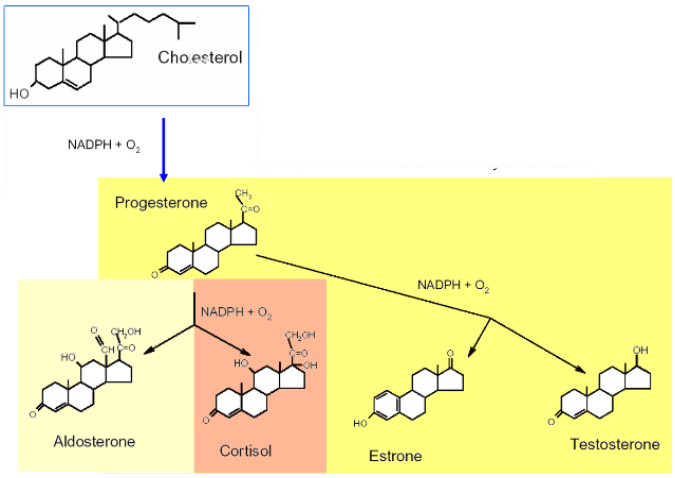
Cholesterol can be converted to the intermediate pregnenolone, that can be converted into progesterone. Both Pregnolone and Progesterone can give rise to steroid hormones.

In humans, steroid hormones derive from cholesterol, but they lack the alkyl chain in C17. Steroids are more polar than cholesterol: they have more O due to **oxidation** [mixed function oxygenases (monooxygenases), NADPH, O2 , cit. P-450, adrenodoxin].

These hormones are effective at very low concentrations (in small quantities), and, for this reason, their production consumes relatively little cholesterol.

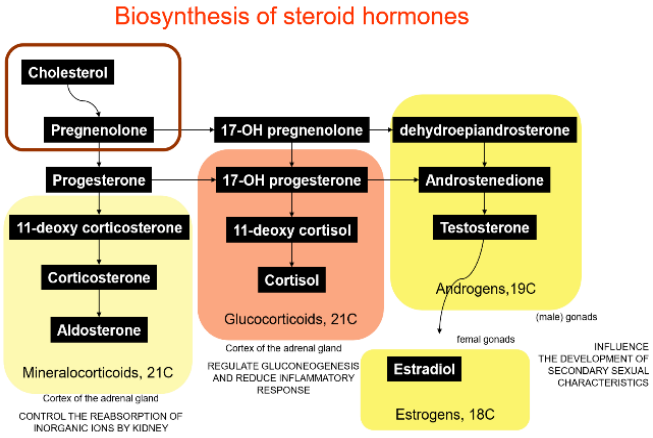
The main types of steroid hormones are:

- **Mineralocorticoids** (such as aldosterone): they regulate reabsorption of ions (*Na, Cl, HCO_3*) in the kidney
- **Glucocorticoids** (such as cortisol): they affect carbohydrate and *protein* metabolism (*regulate gluconeogenesis*); *suppress immune response, inflammation and allergic responses*
- **Sex hormones** (such as testosterone and estradiol): *they influence secondary sexual characteristics; and they regulate female reproductive cycle*



Note that specific tissues, express specific genes that are involved in the formation of specific enzymes: for this reason, different types of cells produce different types of steroid hormones. Mutations or other alterations in gene expression, may affect the production of steroid hormones and result in pathological conditions. In particular:

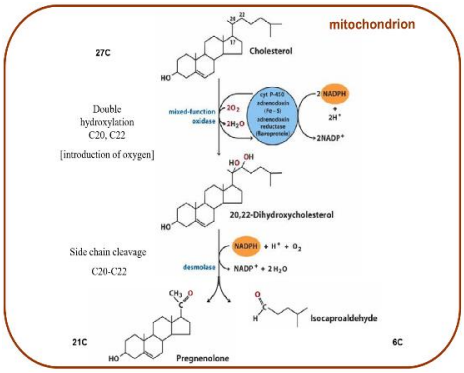
- **Mineralochorticoids** are produced in the cortex of the adrenal gland
  [They have **21 C**: Cholesterol loses 6C]
- **Glucocorticoids** are produced by the cortex of the adrenal gland
  [They have **21 C**: Cholesterol loses 6C]
- **Sex hormones**:
  - In males: androgens (male sex hormones) are produced by the male gonads
    [They have **19 C**: Cholesterol loses 8C]
  - In females: androgens (male sex hormones) are produced by the female gonads; then these hormones are converted into estradiol (female sex hormones)
    [They have **18 C**: Cholesterol loses 9C]



14 Lecture (28/05)

Prognenolone

There is a previous step to create steroid hormones, that is rate limiting and hormonally regulated: the transfer of cholesterol from the outer to the inner mitochondrial membrane, where the P450scc enzyme is located. At this point, cholesterol undergoes:



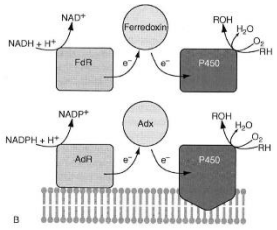
1. Double hydroxylation C20, C22

[introduction of oxygen]: starting from 2 oxygen molecules, 2 oxygen atoms are reduced to water, and the remaining 2 are incorporated into oxidized cholesterol (at the level of C20 and C22) forming <u>20,22-Dihydroxycholesterol</u>. The reaction is catalyzed by mono-oxygenase (i.e. a multienzymatic apparatus which transfers electrons from NADPH to O_2).

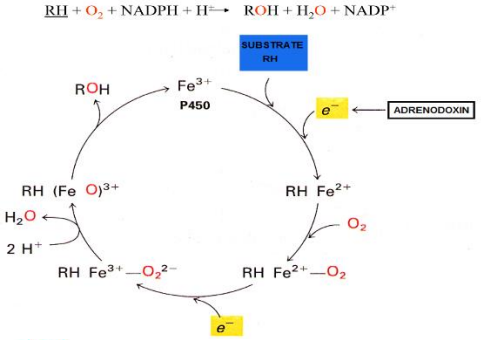
Note: the previous image refers to this enzyme as mixed-function oxidase. However, oxidase usually catalyze reactions in which oxygen atoms are involved but not present in the major product. Since in this case, these atoms are present in the major product, it is better to refer to this enzyme as mono-oxigenase ("mono" as, for each cycle, only 1 of the oxygen atoms of O_2 is incorporated into the organic product, the other being reduced to H_2O).

Super-family of NAD(P)H dependent monooxygenases, contain:

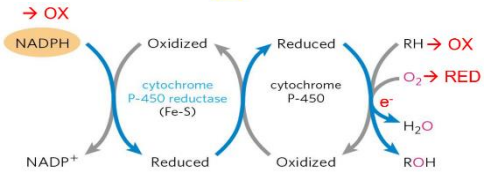
- Cytochrome P-450 (peptide with *b-type* heme group; the name derives from its characteristic ability to absorb light, as 450 is one of the peak of wavelength);
- Adrenodoxin/ferredoxin (intermediate acceptor of electrons, containing Fe-S groups that can carry 1 e^- at a time);
- Adrenodoxin/ferredoxin reductase (flavoproteins, containing FAD)



<u>Mechanism of action</u>: substrate (RH) and the electron carried by adrenodoxin (e^-) enter the same binding site of P450 (with Fe^{3+}); Fe is reduced to Fe^{2+} by the e^-. O_2 also enters the same binding site and is the final acceptor of the e^-: Fe is re-oxidized to Fe^{3+} while O_2 is reduced to a very reactive specie in the pocket (it receives both e^- from Fe and from the external environment). This reactive specie reacts with H^+ to form water, *and part of the oxygen is incorporated into the substrate, that is released.*



Various hydroxylases are involved in the synthesis of the steroid hormones. Nomenclature indicates the site of hydroxylation (e.g. 17a-hydroxylase introduces a hydroxyl group to carbon 17) in addition to being identified as P450 class enzymes (e.g. the 17a-hydroxylase is also identified as P450c17, currently CYP17).

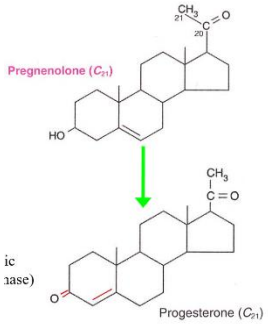


2. Cleavage C20-C22:

20,22-Dihydroxycholesterol is very unstable, so the bond between 20-22 is broken, and electrons are released to reduce oxygen to water (while NADPH is oxidized to NADP). The reaction is catalyzed by desmolase (i.e. any enzyme that catalyzes the addition or removal of some chemical group to or form a substrate withouth hydrolysis; various enzymes that break or form carbon-to-carbon bonds in a molecule) and results in the formation of two compounds: <u>isocaproaldehyde</u> (the alyphatic chain of cholesterol; an aldehyde; 6C); and <u>Pregnenolone</u> (21C)

In a steroidogenic cell, the nascent 21-C pregnenolone has only two primary fates:
1. Progesterone: can be secreted or form corticosteroids (mineralocorticoids and glucocorticoids)
2. 17-OH Pregnenolone: can form sex hormones

Progesterone



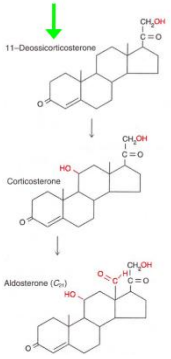
Pregnenolone can be converted to progesterone through:

- NAD^+-dependent **oxidation** of the hydroxil group in C3 (alcohol), that becomes a carbonyl group (keton) [dehydrogenase]
- **Isomerization** C=C (Δ^5-isomerase): the double bond that was in position 5-6, is moved to position 4-5

Progesterone has two main faith:

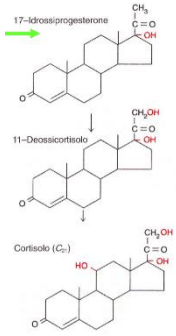
1.1 Corticosteroids

1.1.1 Mineralocorticoids (21C)



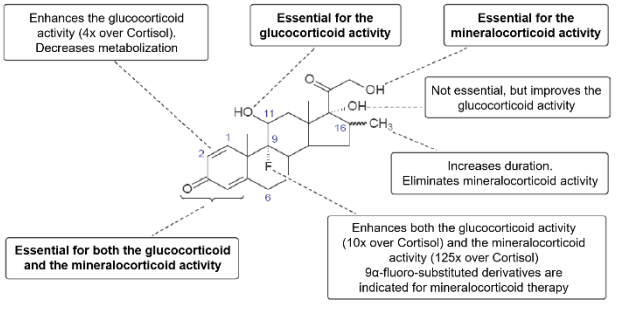
- 21-hydroxylase catalyzes hydroxylation in position 21: progesterone becomes 11-deoxy corticosterone.
  Then, in mitochondria:
- 11-hydroxylase catalyzes hydroxylation in position 11: progesterone becomes corticosterone.
- 18-hydroxylase catalyzes hydroxylation in position 18; 18-hydroxysteroid oxidase oxidizes the OH in position 18 that becomes a carbonyl group (aldehyde). Aldosterone is formed.

1.1.2 Glucocorticoids (21C)



- 17-hydroxylase catalyzes hydroxylation in position 17: progesterone becomes 17-OH progesterone.
- 21-hydroxylase catalyzes hydroxylation in position 21: 17-OH progesterone becomes 11-deoxy cortisol.
- 11-hydroxylase catalyzes hydroxylation in position 11: 11-deoxy cortisol becomes cortisol.

Structure function relations:
OH in position **11** is essential for the activity of glucocorticoids, while some mineralocorticoids may miss this step and still work.
OH in position **21** is essential fot the activity of mineralocorticoids
Carbonyl group in position 3 and isomerized bond in position 5, are both necessary for the formation of progesterone, thus essential for both the glucocorticoid and the mineralocorticoid activity



1.2 Secretion

Progesterone can be secreted by corpus luteus during pregnancy, and act as an hormone itself

17-OH Pregnenolone

17-hydroxylase catalyzes hydroxylation of pregnenologne in position 17.

2. 1 Sex hormones

2.1.1 Androgens (19 C)

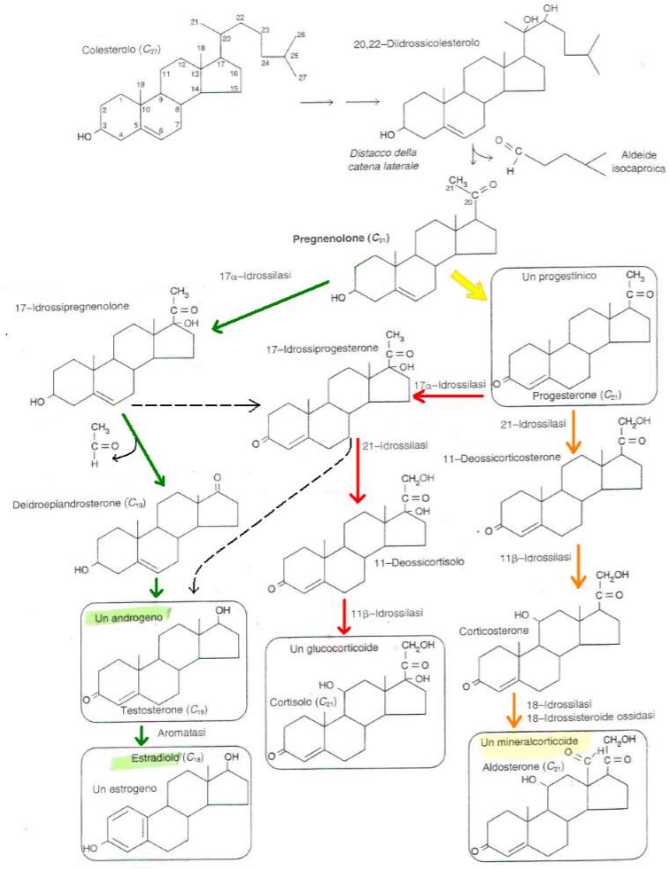
- 17-20,21 desmolase catalyzes the cleavage of C-C bond (17-20), with release of 2 C atoms (20 and 21), forming ***dehydroepiandrosterone***.
- Dehydrogenase catalyzes oxidation of the alcholic group in C3, that becomes a ketone. ***Androstenedione*** *is formed*
- Dehydrogenase catalyzes the reduction of the keton group in C17 that becomes an hydrocyl group
- Δ5-isomerase isomerizes the C=C bond in position 5.
  At this point, testosterone is formed (containing 19C)

2.1.2 Estrogens (18 C)

- Cleavage of C-C bond (10-19), releases the methyl group in C19: 1 C atom is lost. Subsequently, the A ring is aromatizated by aromatase (becomes phenol). Estradiol is formed.

All the described hydroxylation and oxidation reactions in steroid hormone byosynthesis are exergonic and unidirectional.

Summary



Rassegna della biosintesi degli ormoni steroidei. *Tutte le reazioni di idrossilazione o ossidazione coinvolgono ossigeno molecolare, citocromo P-450 e NADPH.*

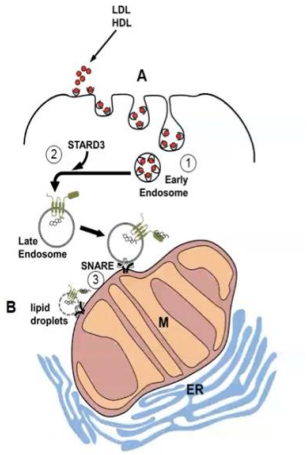
15 Lecture (31/05)

Flux of cholesterol

For all cells that do not produce cholesterol the pathway is the following:

A) Cholesterol is mainly transported to these cells through LDL and HDL. These lipoproteins are recognized on the surface of the cell and their content is internalized through **endocytosis**.
1. Once inside the cell, *early* endosomes initiate cholesterol transport to mitochondria *to stimulate steroid hormone synthesis.*
2. STARD proteins (i.e. StAR, steroidogenic acute regulatory protein) coat the surface of the endosomes for them to be labeled to go towards the mitochondria. The incorporation of STARD3 onto early endosomes promotes maturation into late endosomes and facilitates the transport of cholesterol from the outer to the inner mitochondrial membrane.

B) Another possibility for the transport of cholesterol to the mitochondria is through **lipid droplets**.



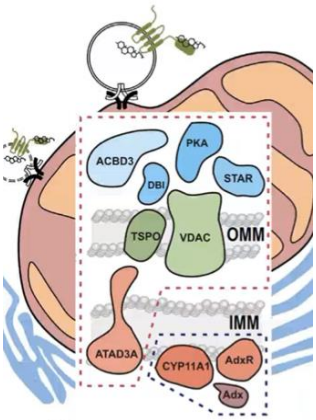
3. In both ways A and B, the late endosome and the lipid droplets anchor to the mitochondria through SNARE complexes. SNARE proteins (present also in synapsis of neuronal cells) allow the fusion of the vesicle with the organelle's membrane.

Multiple functions of syncytiotrophoblast mitochondria - PubMed

Once located in the outer mitochondrial membrane, cholesterol must continue its transit to the inner mitochondrial membrane by simple diffusion, reach cytochrome P450scc, and be transformed into pregnenolone. The amount of cholesterol in mitochondrial membranes is low, suggesting a fast transformation of the sterol into pregnenolone.

During the research done by Midzak and Papadopoulos, it was discovered that many proteins are needed to allow the transfer from the outer membrane to the inner membrane: the Mitochondrial Contact Sites are domains where inner (IMM) and outer membranes (OMM) are in close proximity, allowing exchange between them. MCS are established and maintained in durable or transient states by different proteins and enzymes such as VDAC (Voltage-dependent anion channel), ATPase-ATD3, TSPO (translocator protein), ANT (adenine nucleotide translocator), IP3R (ER-resident inositol triphosphate receptor), σ1 (chaperone), Mfn1 and Mfn2 (mitofusine).

Transduceosome (cholesterol import machinery), contains cytoplasmic, OMM and IMM proteins and some matrix proteins. They are all independent proteins that constitute subunits of a **supercomplex**, that is assembled in response to specific hormonal stimulation, and transduce to mitochondria the resultant cAMP signal for cholesterol import.
IMM metabolon (CYP11A1, FDX, and FDXR) metabolizes cholesterol to pregnenolone, the precursor to all other steroids.



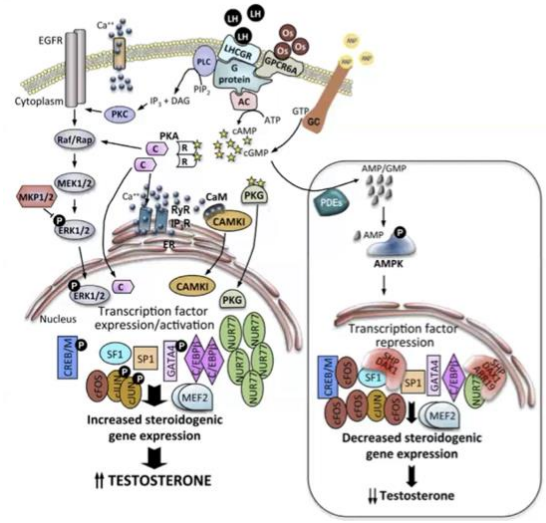
PKA → controlled by cAMP and thus interconnected with other pathways;
VDAC (Voltage - gated Anion Channel)
CYP11A1 monooxygenase (the modern name of a monooxygenase enzyme is "CYP" + the number of the carbon on which it acts);
AdxR (Adrenodoxin Reductase)/FdxR;
Adx (Adrenodoxin)/Fdx.
In addition to those cited above, other molecules indirectly regulate the biosynthesis of steroid hormones:
G-proteins: control the serpentine receptor GCPR, but also the formation of cAMP due to their influence on adenylate cyclase;
PIP2, **IP3**;
PKA and **PKG** act on the expression of TFs, influencing transcription regulation. Even the **MAPK cascade** is influenced by the transport of cholesterol.

Turning off the mechanism

Schematic representation of the mechanisms involved in LH-mediated steroid production in Leydig cells within the male gonad

Kinases are involved in the activation of several transcription factors that induce the expression of genes involved in steroid hormone synthesis. These stimulatory pathways are inhibited via phosphatases.

To prevent overstimulation, when too much cAMP or cGMP are present in the cell, phosphodiesterase enzymes turn these cyclic structures into AMP and GMP respectively, which are the allosteric modulators of AMPK (and probably GMPK). The result is the phosphorylation of some transcription factors that **suppress** transcription. *C-FOS and DAX 1 are some of those suppressors that can be modulated to reduce the synthesis of testosterone.*

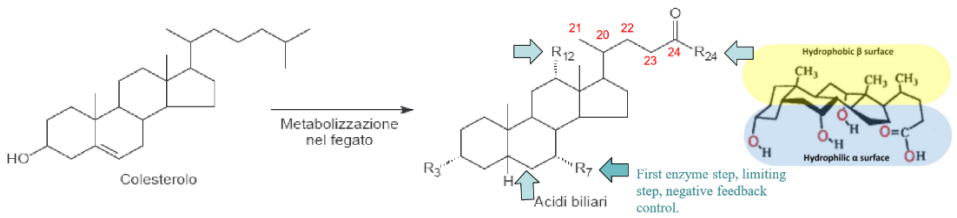


Byosynthesis of bile acids

In the liver, cholesterol (27 C) undergoes 17 enzyme steps to become bile acids (24C);

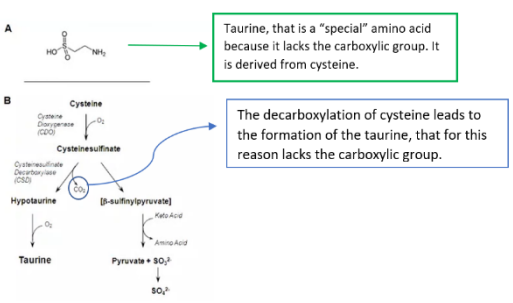
- The 4 rings remain the same, while the tail is substrate for a decrease in the n. of carbon atoms.
- Cis-junctions are added and they give the molecule a specific **3D** conformation: the hydrophobic components are located on one side, while the hydrophilic components are on the other side.
  Note: because of this structure, a sort of "sandwich" is formed where lipids and water are separated by bile acids: for this reason, the bile (made also of bile acids) acts as detergent during lipid digestion
- The acidic properties of these molecules are due to the presence of the carbonyl group in position **24**
- Modification at the level of **R7** is the first enzyme step, it is the limiting step and has a negative feedback control over the process (image on the right).

Acidi biliari	R_3	R_7	R_{12}	R_{24}
Colico	OH	OH	OH	OH
Chenodesossicolico	OH	OH	H	OH
Desossicolico	OH	H	OH	OH
Litocolico	O-SO_3H o OH	H	H	OH
Ursodesossicolico	OH	—OH	H	OH

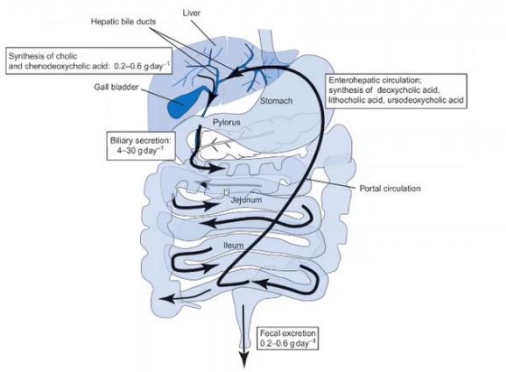


Bile acids are usually **conjugated**, which means that the terminal portion, that is negatively charged and highly reactive, can form a covalent bond with another molecule, forming the equivalent salt of the acid. For this reason the bile acids are also called bile salts. They are often linked to:

- Glycine (A)
- Taurine (B): it derives from the decarboxylation of cystein (since it does not have the carboxyl group, it is not an amino acid).



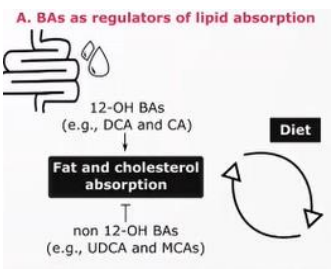
The primary bile acids are produced in the liver (0.2-0.6 grams) and can be released in the lumen of the GI tract and converted into **secondary bile acids.** The conversion from primary to secondary bile acids is catalysed by **bacteria** present in the intestine. This is a clear example of how humans can interact with the bacteria present in their organism. The bacteria present in the gastrointestinal tract usually remove the hydroxyl group on position number **7** and can also remove the conjugation with glycine or taurine. The secondary bile acids will be reabsorbed by the human body, so they work as a hormone signal from the bacterial flora to the human cells (most of the bile that is produced is reabsorbed).

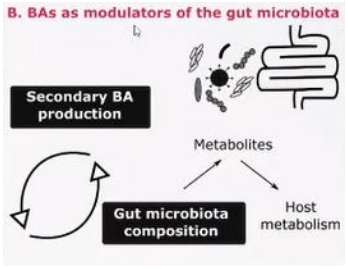


Summary of the **main functions** of BAs (bile acids):

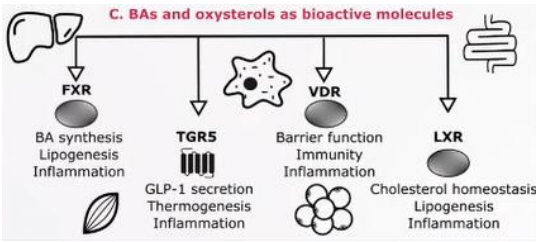
A) **Control of lipid assimilation**, with 12-OH BAs promoting fat and cholesterol absorption

B) **Mutual relationship** between the **gut microbiota** and **BAs**: BAs regulate the proliferation, maturation and the composition of the intestinal bacteria while the gut microbiota generates secondary BAs





C) BAs and oxysterols are considered as **signalling molecules** since they can interact with a panel of receptors distributed in the whole body and be involved in inflammatory and metabolic disorders.

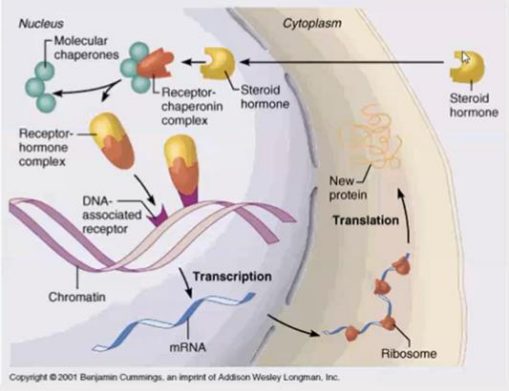


It's important to understand that oxysterols can influence cell signalling and therefore gene transcription in human cells.

FXR, farnesoid X receptor;
LXR, liver oxysterol X receptor;
TGR5, Takeda G-protein coupled receptor 5 (glucagon-like peptide 1 secretion);
VDR, vitamin D receptor.

NUCLEAR RECEPTORS

Steroid hormones are hydrophobic signaling molecules, that can cross the plasma membrane and bind receptors inside the nucleus to regulate the expression of specific genes, affecting protein synthesis.



Many of these receptors, upon hormone stimulation, become prone to **dimerization** (i.e. they form dimers with another receptor). There is a high degree of specificity for both the interaction between hormone and receptor and DNA and receptor i.e. a certain receptor will interact only with a certain DNA region or gene. The area of the DNA that interacts with the receptor is called **HRE** (hormone response element). HREs are closely related to gene promoters which in turn are able to regulate the transcription of their corresponding gene. *The bound hormone-receptor complex modulates gene expression by altering the rate at which adjagent genes are transcribed and translated into proteins.*

Note: the receptor is active only if bound to the hormone; so it can affect gene expression only if linked to its ligand.

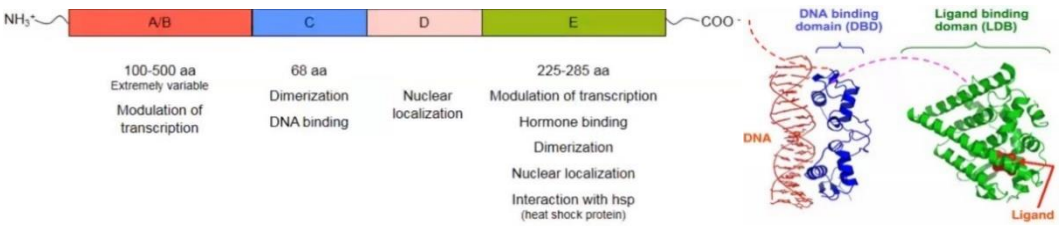
Main types of nuclear receptors based on their hormones

- **Steroid** [type I]: Glucocorticoid. (GR-receptor); Mineralocorticoids. (MR-receptor); Progesterone. (PR-receptor); Aldosterone. (AR-receptor); Estrogen. (ER-receptor)
- **Thyroid** hormone [Type II]
- **Retinoids** [Type II] *(RAR, RXR)*

Structure

Lipophilic hormone receptors are single polypeptides, with one amino (NH3+) and one carboxyl (COO-) terminus. In this polypeptide chain, there are at least five different domains (A/B; C; D; E), each of which has dedicated functions.

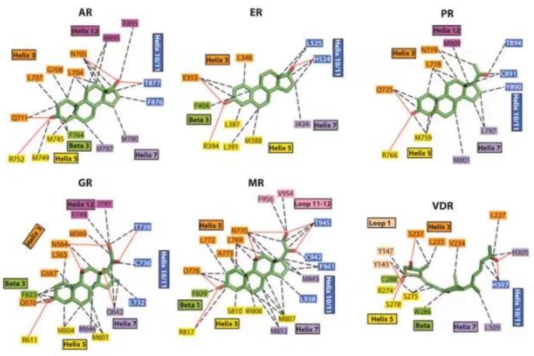
1. Ligand binding domain (LBD): it is found at the carboxyl terminus and it is the domain where the ligand usually binds. There are several (*nearly 12*) α helices that form a pocket for hormone binding. *It is usually 200 to 300 amino acids long and it is typically involved in the dimerization process; indeed, it is able to sense the presence of the hormone, which has to be in place before the beginning of this conformational change, which will allow the monomers to interact. It has the ability to induce the cytoplasmic receptors to move to the nucleus.*
2. DNA binding domain (DBD): it is the region of the polypeptide chain that interacts with DNA. Note that the DNA binding domain is not characterised by a leucine zipper domain, but by **Zn-fingers.**
3. Amino-terminal domain: has various functions; it is extremely variable.
4. Intermediate domains (D): have regulatory functions.



Interactions

Hormone - receptor

In the image, different types of steroid hormones are shown. All of them have four rings, including an oxygen molecule and have either a hydroxyl or a carbonyl group on **C3**. In particular, this last region is the part of the steroid hormone that will sit inside the pocket of the receptor: this happens because, in this region, the oxygen polar residue of the hormone will recognize the amino acids of the receptor, forming hydrogen bonds. In some of the residues shown, it is also possible to find arginine (R, positively charged amino acid), that is typically involved in this polar interaction.

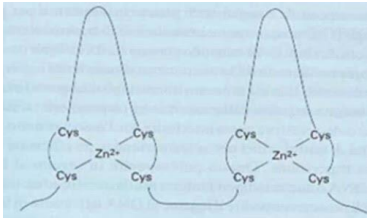


Note that the interaction between the hormone and the receptor is peculiar because of the association of one hydrophobic molecule (the steroid hormone) and a hydrophilic one (the receptor, which is a protein).

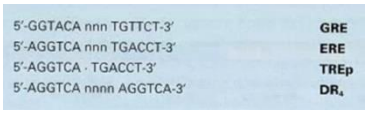
DNA – receptor

The DNA binding domain of the receptor has the function of forming a specific interaction with the DNA, due to the presence of the so-called **Zn-fingers** on its C-domain.

Zinc fingers normally interact with the HREs (hormone response elements), which are usually located in the promoter region in the DNA: their association happens thanks to their ability to recognize a specific nucleotide sequence in the DNA. Clearly, there are many different consensus sequences, according to the different target genes of the hormones.These consensus sequences are formed by six base pairs and are generally palindromic:



- *Palindromic with three spacing nucleotides (n): type 1*
- *Palindromic without spacing nucleotides: type 2*
- *Repeated, non-palindromic sequences: type 3*



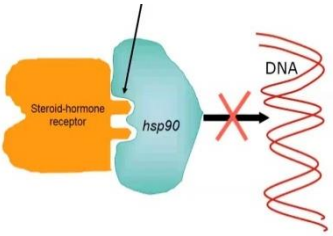
Note that each molecule of the receptor dimer may interact with half palindromic (or direct repeated) sequence.

Heat shock proteins (HSPs)

In the absence of the hormone, the steroid hormone receptor interacts with **HSP**, which have the function to protect the Zn-finger portions of the DNA binding domain of the receptor, and, consequently, it does not allow its interaction with the DNA. They are extremely important in the signaling induced by any hormone which works through nuclear receptors.

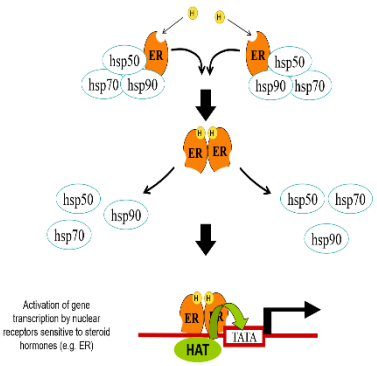
Note that many kinds of HSPs exist, each of which is characterized by a different identification number

As a consequence, as long as the LBD is not occupied by the hormone, the receptor cannot interact with the DNA, due to the presence of the heat shock protein.



Mechanism of interaction (complete animation)

E.g. Estrogen Receptor (ER, but it could be any steroid hormone receptor) has a ligand-binding domain waiting for the estrogen to get in the cell: as long as the hormone is absent, the receptor is not able to interact with any DNA molecule because of the presence of clusters of heat shock proteins of different kind, which are covering the DBD in the receptor.



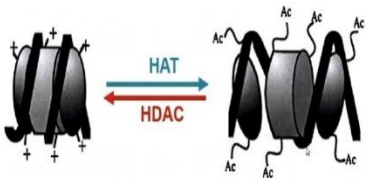
When the hormone reaches the cell, it induces a conformational change of the receptor: thus, the heat shock proteins are released from the DBD surface on the receptor, which is now free to interact with the DNA, at the level of its appropriate response element sequence.

In the end, an active steroid receptor with its hormone bound has finally reached the DNA (red line).

It is important to remember that not any of those receptors is an enzyme: indeed, steroid receptors are adaptor proteins, that recognize the DNA and become docking places for the enzymes that are needed for the DNA metabolism, eventually allowing modifications in specific regions of DNA (e.g. TATA boxes).

The activity of HATs is stimulated by steroid hormones through their receptors:

- Histone acetyltransferase (HAT) are enzymes that catalyze the transfer of acetyl groups to the histones, to loosen the histone-DNA and the nucleosome-nucleosome interaction.
- Histones deacetylase (HDAC) are enzymes that have an opposite functions as they can catalyze the removal of acetyl groups from histones and restore the compactness of the nucleosome, switching off this signaling pathway.



Outputs

Steorid hormone receptors promote the synthesis of different kinds of proteins: this largely depends on the target tissue, the type of hormone, and other various elements.

- In **liver** cells: steroid hormone receptors have been shown to increase the level of activity of glycogen synthase and tyrosine- and alanine-transaminase, increasing their synthesis by as much as 150%. The response takes about 24 hours to become effective and enables cells to perform protein synthesis.
- **Aldosterone**: regulates the permeability to Na+ (absorption) in renal tubules by regulating the presence of protein carriers for those ions and enhances the synthesis of proteins of the mucosal barrier in the intestine, which also regulate the permeability to Na+.
- **Estradiol**: in the corpus luteum; it upregulates the production of the progesterone receptor, which in turn increases the control of progesterone over protein synthesis. In the absence of fertilization, hormone levels drop → protein synthesis, controlled by estradiol and progesterone, ceases → cells die.
- **Estrogens**: control cell growth; these hormones are essential for development during puberty, but they are also necessary in the course of adult life. In particular, estrogens are important in bone growth; in fact, low levels of estrogens can induce osteoporosis. Estrogen can enhance pathological cell growth and can eventually cause cancer worsening: indeed, in many cases, cancer is characterized by alterations in the hormonal signaling. *Tamoxifen: acts as an estrogen antagonist that competes for the binding site on the estrogen receptor → the tamoxifen-receptor complex has little or no effect on gene expression → in some types of breast cancer, it slows or stops the growth of cancerous cells.*

Switch off

Receptor degradation

Receptor degradation limits both duration and intensity of the hormonal response, by returning the system to its homeostatic baseline, where it is ready for subsequent rounds of activation, after a little time.
This switch off mechanism, requires a protein to be removed from the extracellular environment, and this process, besides being slow, would cause a waste of energy.

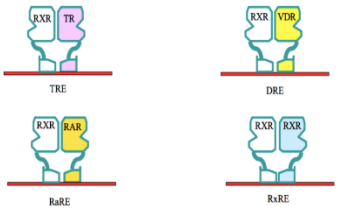
Ligand inactivation

Some **cytochrome P450** enzymes can also take center stage in ligand inactivation and clearance: indeed, they can physically modify the hormone which has just entered the cell before it could find its receptor.
The most common kind of physical modification would be the hydroxylation, which consists in the addition of hydroxyl groups. For example, addition of a hydroxyl group on C5, would result in the formation of a different molecule, that would not recognize the pocket in the ligand-binding domain of the receptor
It is the so called "make it and break it" process, because these cytochromes "make" the hormones but also "break" them preventing their proper functioning by the addition of OH groups

Dimerization

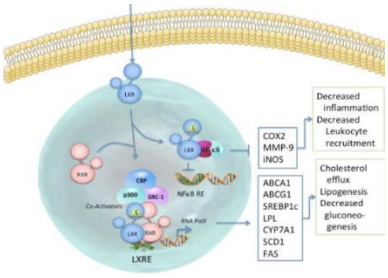
In many cases hormone-receptor complexes form dimers before interacting with DNA and they can be:

- Homodimers: 2 identical copies of the receptor,
- Heterodimers: 2 different copies of the receptor.
  E.g. a glucocorticoid receptor could bind with a retinoid receptor with its hormones.
  These heterodimers are very important at physiological level: in order to have the effect of the ligand of the first monomer, also the hormones of the second monomer need to arrive to the target cell at the same moment, because the dimer has to form. Thus, different glands need to be coordinated to produce their own hormones at the same time.



There are 48 nuclear receptor family members in the human genome and for some of these receptors the genomic sequence is known, but in many cases the ligand is not known (the receptors whose ligand is not known are referred to as **orphan receptors**; once the proper hormone is discovered they become **adopted orphans)**.

PPAR receptors, involved in peroxisome proliferation, are very important drug targets because peroxisomes are involved in xenobiotics modulation. Fatty acids are endogenous ligands for PPARS. They are steroids or lipophilic type receptors that are able to interact with DNA.



RXR receptors have been recently discovered.

Liver X receptor (**LXR**) is not a human molecule, but a bacterial molecule coming to human cells. The oxysterols synthetic agonist, created artificially, is recognised as natural oxysterol molecule coming from bacteria. Oxysterols nuclear receptors can interfere with inflammation status and they can be involved in cholesterol efflux and gluconeogenesis.

Exeption: not all of these receptors are intracellular, or located in the nucleus. Recently, membrane-bound receptors for glucocorticoids have been discovered. So, a new area of research is now investigating on these glucocorticoid receptors that can interact with the hormone before the hormone itself gets into the cell. These types of receptors are G-protein-coupled receptors, they often come from research about oxysterol. Their roles are still unknown.

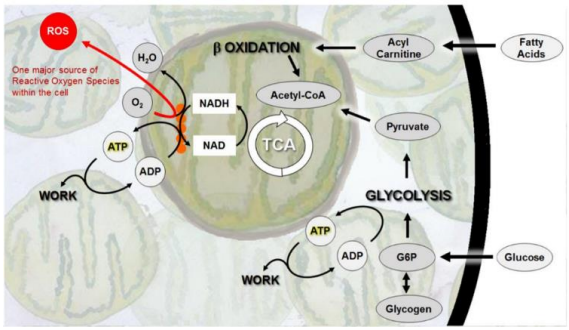
DEATH SIGNALING: CELL STRESS AND MITOCHONDRIA

16 Lecture (01/06)

Mitochondria play an essential role in:

- The energetic metabolism
- The generation of steroid hormones. *These hormones are, in turn, able to modulate mitochondrial activities (as* mitochondria can also sense the presence of hormones).
- Cell maintenance: mitochondira can make the difference between life and death for the cell *because they are the main source of energy within the cell*. They can sense the changes in the environment and react positively to those changes, letting the cell survives, otherwise the cell will die.

In this scheme the major pathways in mitochondria, studied in metabolic biochemistry, are summed up.



Is possible to observe what are the relationships that mitochondria have with the cytoplasm, with the extracellular environment or between other mitochondria of the same cell. Some molecules of the metabolism of fatty acids and carbohydrates point to mitochondria, such as acyl carnitine and pyruvate. 2 major molecules enter inside mitochondria and take part in the TCA cycle and β-oxidation: oxygen (necessary for oxidative phosphorylation and not only) and ATP/ADP (provides energy needed for cell work). At the same time, the presence of oxygen inside mitochondria can be very dangerous because it can be a very reactive molecule (ROS), if electrons, donated to this molecule, aren't properly controlled by the enzymes that can catalyze reactions with oxygen.

ROS

ROS can be produced by some enzymes in the respiratoy chain:

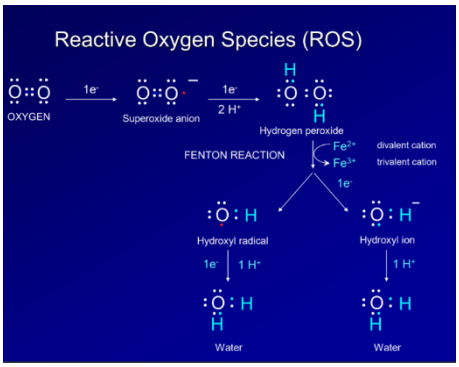
- Complex V (ATP synthase) does <u>not</u> produce ROS, since it doesn't receive any electrons, but it allows the passage of protons.
- Complex IV (cytochrome oxidase) does <u>not</u> release ROS. It has the binding site for oxygen, and 4 electrons are needed for the complete oxidation of oxygen to water. If the complex receives only 1 electron, it doesn't release ROS, but it keeps the electron bound to its binding site until the other 3 electrons arrive and water is released.
- **Complex I** and **Complex III** (sometimes also **Complex II**) are considered the <u>main sources of ROS</u>, since they don't have a physiological binding site for oxygen, but they have electrons flowing in their redox center. If the electrons are stopped, they will not find their natural acceptor (Complex I → Coenzyme Q; Complex II → Coenzyme Q, Complex III → cytochrome C) but they will find an alternative acceptor, which is **oxygen**. These proteins don't have a proper binding site for oxygen thus the binding is very unstable: one electron is sufficient to cause the oxygen to easily move away from the complex, creating free ROS in the environment. All the **dehydrogenases** of the respiratory chain are able to produce ROS, if they can't donate the electrons to their physiological acceptor.

Some impairments should occur for those complexes that produce many ROS but the recent idea in science is that ROS are not only dangerous molecules: if our cells are able to produce ROS it means that they are needed for life. The problem is in the unbalance.

The role of ROS in physiological conditions

Since ROS are produced by the cells, they aren't dangerous under certain concentrations, and they have a specific role in the physiology of the cell.

If molecular oxygen, composed of two atoms, receives one additional electron, the molecule is able to host it in a dedicated molecular orbital.

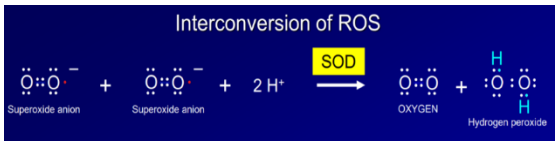


- The addition of a single electron will generate a **superoxide anion**, that is a free radical as it has an unpaired electron: it is more likely to accept other electrons (becomes eager of electrons) in order to stabilize the first one and at least have a couple of additional electrons on the same orbital.
- The addition of a second electron will form **hydrogen peroxide**. This molecule is not a free radical but it is still a ROS, as it reacts with ions of iron bivalent cation (Fe^{2+}),
- Iron donates another electron to hydrogen peroxide becoming a trivalent cation (Fe^{3+}), while hydrogen peroxide dissociates, forming **hydroxyl radical** and **hydroxyl ion** [study the difference].
- To stabilize ROS, they are converted into **water**.

This is the first way the cell has to keep ROS under control and balance.

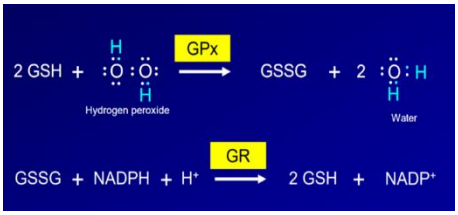
Interconversions

Cells have evolved a chemical way in order to counteract and neutralize the effects of ROS if they increase in concentrations. Thanks to **enzymes** involved, interconversions of ROS happen very fast.



- 2 **superoxide anions** can react and form
  - ➔ molecular oxygen
  - ➔ hydrogen peroxide

  The enzyme system that can facilitate this chemical reaction is SOD (Superoxide dismutase enzyme).

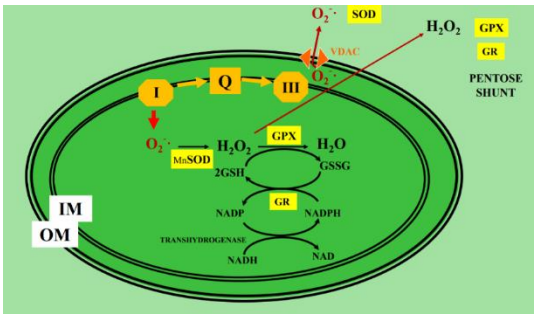


- In presence of **GSH** (i.e., the reduced form of glutathione with a sulfhydryl group), **hydrogen peroxide** can be converted into water. This reaction can be catalyzed by GPx, Glutathione peroxidase. Once that the reduced form of glutathione is oxidized, the cell needs to regenerate the GSH form through GR, glutathione reductase.

All of those systems are the antioxidant systems that a cell can have.

Some of those enzymes are located inside mitochondria, in the same space where ROS are created.

In some cases there are isomeric forms of the same enzymes in the cytosol, so also in the cytosol there are enzymes that can use molecular oxygens and produce ROS (*think about some examples*). In this picture are illustrated two similar antioxidant systems inside mitochondrion and outside. ROS produced by mitochondria can also exit the organelle and affect the cell structure in the cytoplasm.



Signalling role

ROS can leave mitochondria because they can work as signaling molecules: they can be used as a signal pathway to tell other parts of the body that something wrong is happening inside the mitochondria.
Specificity in signalling is achieved through the non-covalent binding of a ligand to its conjugate receptor by complementarity of macromolecular shapes. By contrast, ROS operate in signalling through oxidation of specific thiol groups of target proteins involved in signal transduction pathways. ROS molecular recognition occurs at the atomic and not at the macromolecular level. Moreover, ROS can diffuse through the plasma membrane, so their **receptors** will not be transmembrane proteins, but **intracellular** ones.

Various levels of ROS will induce different cellular responses:

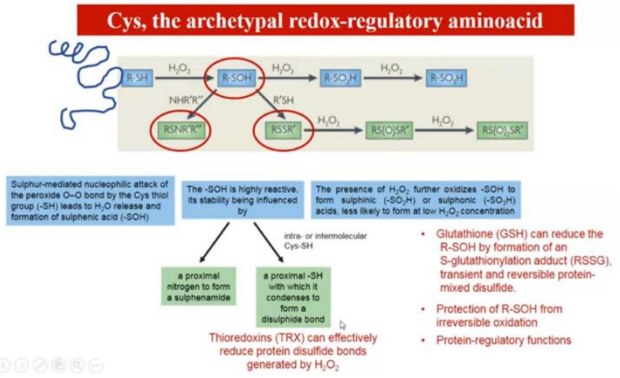
- Low: physiological signaling → low amounts are enough to trigger signals transduction
- High: affecting proliferation of the cell.
- Very high: senescence, apoptosis are triggered, in order to remove the cell from the organism.

There are several receptors for ROS, one of the most common is the protein tyrosine phosphatases (intermediate step that regulates carbohydrates metabolism and signaling): *ROS can promote intracellular signaling by the oxidative inactivation of protein tyrosine phosphatases downstream of growth factor and insulin-mediated receptor activation.*

Interaction of ROS with proteins: how the target protein can sense the presence of a ROS signal

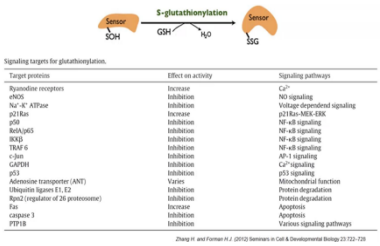
In the image there is a protein with a cysteine residue (R-SH), an amino acid that can transfer the signal to ROS. The interaction of the SH group with H_2O_2 occurs by a sulphur-mediated nucleophilic attack of the peroxide O–O bond releasing a H_2O molecule as a product. So, SH will be oxidized to SOH which is extremely reactive. From now on there can be different pathways:

1. SOH can react with a proximal **nitrogen** to form a sulphenamide
2. SOH can react with a proximal **-SH group** with which it condenses to form a disulphide bond.



These 2 are the **physiological pathways**, but in a situation in which there is an abundance of ROS, -SOH will be further oxidized forming other forms of sulphur oxidation and this pathway is irreversible (which is bad for cell signaling).

Glutathione (GSH) can be a very important partner in this process.
It can react with the partially oxidized protein intermediate (R-SOH) and reduce it, forming an S-glutathionylation adduct (RSSG), transient and reversible protein-mixed disulfide.
Glutathionylated protein are the ones that can go further in the signaling pathway. In the table there is a list of protein that can be glutathionylated. (E.g. Ras, eNOS)



Thioredoxins are proteins also important (present in the cytoplasm), as they can counteract the effect of hydrogen peroxide – they have an antioxidant function.

This picture shows how the pathways involving ROS have a cyclic nature:

- H_2O_2 is produced by the mitochondrion, then it exits the organelle to go in the cytoplasm where, due to redox status of the cell, it can alter some protein structures.
- The altered proteins can be components of signaling pathways, like PI3K or MAPKs, or they can be transcription factors like Jun or Nrf2 or others.
- Altering of these signals will go back as a negative feedback to the mitochondrion itself, in order to regulate and switch off the signal.
- If these systems are in equilibrium, the cell will live. If equilibrium in these arrows is shifted, the cell will be destined to death.

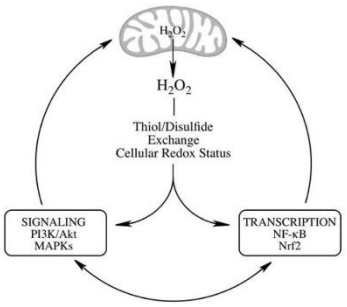
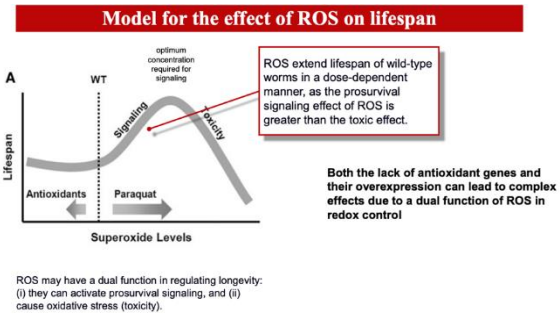


FIG. 1. Regulatory device encompassing the coordinated interactions of mitochondrial function and redox-sensitive signaling and transcription. PI3K, phosphatidylinositol 3-kinase; Akt, Protein kinase B; MAPK, mitogen-activated protein kinases; Nrf2, nuclear factor erythroid 2-related factor.

To understand which is the border between life and death, the scheme below can be used. It has been demonstrated that by changing the level of superoxide in the cell, a double effect can be obtained.

- From the starting point, which is represented by the dotted line, is shown that an increase in the amount of superoxide level (e.g. due to addition or paraquat) will correspond to an increase in the lifespan because it acts as a positive signal. When the peak of the curve is reached, i.e., the optimum concentration required for signaling, the best physiological effect for the cell is obtained. By increasing too much the level of superoxide, it will become toxic, and lifespan will decrease.



- On the other hand, also if there are too many scavengers (antioxidants) there will still be a decrease in the lifespan because cell metabolism is impacted.

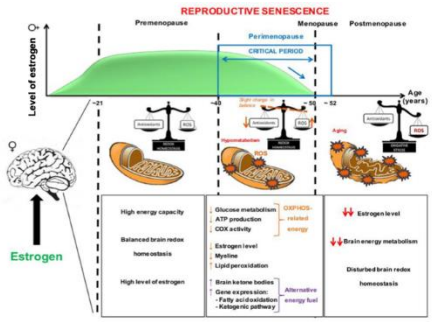
The nature of the signaling pathways that may contribute to ROS signaling remains largely unknown.
In eukaryotes, ROS-mediated signaling has been adapted for purposes other than oxidative stress defense: for example, the molecules oxidized by ROS can become active transcriptional factors (which could induce expression of antioxidant response genes). Such signaling appears to regulate normal growth and development, so not only cell death-related mechanisms.
Dysregulation of ROS homeostasis and ROS signaling have been linked to the development of various age-related diseases such as diabetes, cancer and neurodegeneration.
Impaired mitochondrial function has been implicated in the aging process in a number of different ways, and this is not only related to issues regarding ATP synthesis but also because of the production of ROS which happens in this organelle.

The image shows the relation between the age of an individual and the equilibrium between antioxidants and ROS. A young individual (20-40years) is in the healthy condition. After that age, the body starts aging and it changes the equilibrium between antioxidants and ROS induced by hormones. *E.g.* an experiment has been done on ROS induced by estrogen level.

When the compensatory system (antioxidant defenses) of the cell is exhausted, metabolic impairments may occur, such as glucose hypometabolism and mitochondrial respiration → a decrease of ATP levels

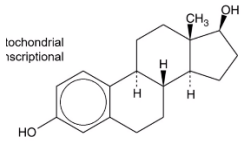
In the "critical period" when a fast drop of estrogen levels occurs, women are more vulnerable and more likely to develop age-related brain disorders such as Alzheimer's disease (AD).

In some cases, estrogen replacement therapies might be beneficial before women reach a critical threshold of cellular damage during perimenopause and early after menopause. ROS, reactive oxygen species.

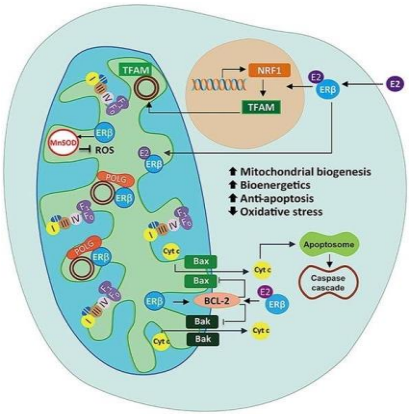


Steroid Hormones and the ETC

17β-estradiol present in estrogens can affect mitochondrial energy production by transcriptional regulation of ETC (electron transfer chain).



Once <u>estrogen</u> (E2) binds to its intracellular receptor (ERβ) and enters the **nucleus**, it can activate transcription factor A (<u>TFAM</u>), which will leave the nucleus and enter the **mitochondrion** where it will induce expression of mtDNA-encoded <u>ETC subunits.</u>



- Sex hormones impact brain organization and function at critical developmental periods
- Pharmacological evidences describing the enhancement of brain mitochondrial metabolism following systemic supplementation with sex steroids
- Age-induced decrease in sexual steroid production could contribute to brain mitochondrial decay
- Uncover biological mechanisms that contribute to sex and individual differences in the manifestations of mental disorders

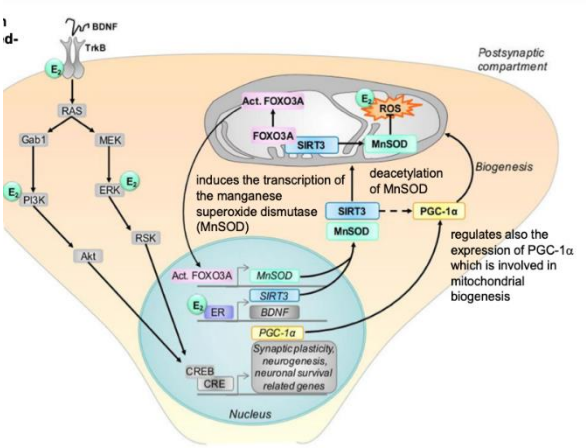
Also Bax and Bcl2 are sensitive to estrogens.

So, in the end the consequences of aging on mitochondrial metabolism will likely differ between males and females.

It has been proven that the reduction of estrogen in blood levels during menopause is much more abrupt than that in testosterone levels during andropause (testosterone can affect mitochondria as well).

Estrogens can affect the RAS-MAPK cascade and the alternative PI3K pathway for insulin

Model of possible interactions between estrogen, BDNF (Estrogen-brain-derived neurotrophic-factor) and SIRT3.
BDNF protein binds to its tyrosine kinase B receptor (TrkB) which stimulates signaling pathways, including the extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K) pathways.
This also leads to the activation of CREB (cAMP response element-binding protein) and transcription of genes, including PGC-1a (peroxisome proliferator-activated receptor gamma coactivator 1-alpha). Estrogen (E2) activates similar signaling pathways as BDNF, and, by binding its receptor (ER), modulates the gene expression of BDNF, TrkB and SIRT3.



E2 designates where estrogen may interact with the BDNF and SIRT3 pathways. BDNF: brain derived neurotrophic factor, CRE, cAMP response elements, PGC-1a, proliferator-activated receptor gamma coactivator 1-alpha, SIRT3, sirtuin 3.