Cellular and Molecular Neurobiology

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January 18, 2016

Todo list

Contents

Chapter 1

Lesson 1: Cellular organization of the NS

Dr. Enrico Tongiorgi. Morphology and function aspects of neurons and molecular details.

Exam: Written + oral: true-false questionary. The questions are subdivided lesson by lesson. Oral exam is optional, focused on the lessons which I failed. Book: Alberts, Fundamental Neuroscience.

In the CNS (spinal chord, brain and retina):

- Gray matter: nerve cell bodies.
- White matter: axons.
- Neuropil: areas in which axons and dendrites synapse.
- Nuclei: regions in the brain which contain a collection of nerve cell bodies. If they go left to right →fasciculi, peduncule. The contrary: lemnisci, tracts.

In the PNS (sensory and autonomic ganglia) :

- Ganglia: collection of nerve cell bodies.
- Nerves: collection of axons.

In the human brain we find 1 billion of million of cells, that are neuron and glial cells. These cells express the largest number of protein in the human body.

1.1 History of neuroscience

Aristotel believed that the mind was in the heart, because heartbeat changes following emotions. Hyppocrates considered that it was in the brain. In particular, he was proposing the theory of four fluids or *humours*:

1. Sanguis or blood, produced by the heart
2. Choler or yellow bile, produced by the liver
3. Melancholia or black bile, produced by the spleen
4. Phlegma or phlegm, produced by the brain

Later, Galen tried to explain the functions of the brain elaborating the dottrine of fluids. In Baghdad there was a school for the development of psychology and physiology: there was the first mental hospital in the world. They were divided the mental illness into two part: the medical part and the psychological part[1]. *Ibn Sina* is the father of Neuroscience, in particular of the cognitive functions (logic, memory etc). He was the first to question the Galen theory of fluids. In 1867 they started to understand that brain contains different functions.

1882: all body tissues are formed by cells (cell theory). The brain was still a mystery!

1906: thanks to the possibility to fix tissues, Camillo Golgi found out the *Black reaction*, a method in which golden and silver were used (fist nobel in neuroscience).

1950: selective silver staining.

1970: antibody staining, fluorescent markers.

Golgi staining Potassium chromate and silver nitrate. Nerve cells, instead of working individually, act as a network. He was saying that neurons form a continuous network, but that is not correct. Anastomotic means sincitiuum (sincizio). Golgi believed that nervous cells are a sincitiuum.

1.1.1 The Neuron doctrine from Santiago Ramon y Cajal

Neurons are cells. Each neuron is an individual entity anatomically, embriologically and functionally. They form a network, but they are separated to

[1]Musical therapy was already in use at that time

each other. Neurons have also a polarity (visual observation, but he couldn't demonstrate it). Recurrence of similar structures.

Structure of neuron. Spines: specializations of dendrites.

The morphological polarization underlies the functional polarization. There are some peculiarities, some recurrent aspects:

- All neurons have dendrites, soma and axon
- They might have different shapes

So they observed that there are *sensory neurons* (CNS, except for the cell body), *interneurons* (90% of the neurons in the brain) and *motor neurons*.

1.1.2 Structure of a neuron

Cell body = soma. It may be smaller or larger and this helps classifying neurons. Single, central and prominent nucleolus (the site of production of proteins, meaning that the cell is producing a lot of proteins). The cytoskeleton is very ramified to keep the shape. We need RER (rough endoplasmic reticulum), ribosomes and Golgi apparatus (a lot!). We can see tiger dots (isole tigroidi).

Dendrites: from 1 to 10, shorter than the axon, branched.

Axon: just 1, it starts from the axon hillock (small hill, colletto) and ends with a ramification (synapses). It may have the myelin. Excitatory synapses are made between axon and dendrites, inhibitory with the soma.

Comparison with somatic cells

Not so many cells have these strange, long ramifications (over a meter in some cases). In somatic cells, like epithelial cells of the gut, there is an apical membrane and a basolateral membrane. There are proteins and receptors that are exclusive for the apical part and others for the basolateral part. Is there also this membrane diversification in the neuron, or there is a mix in terms of proteins? They used viruses that produce different proteins: injected, in the gut some proteins were going up and some down. In neuron cells, some were going in the axon and some in the cell bodies $\longrightarrow$membrane polarization also in neurons. That is non true in C. Elegans, in which these proteins are mixed.

Microtubules orientation determines the polarity of neurons.

Epithelial cells have a polarity because the apical surface looks into a lumen. The lateral surface has tight junctions. In neuron, somatic and axon

membrane are separated by the axon hillock. The axon hillock is a molecularly distinct domain: it contains protein and channels that are important to starting the action potential.

Anterior Horn Motor Neuron (corna anteriori). Nissl stains (it's a colorant) colors areas which are rich in RNA.

The similar structures that Cajal was talking about, neurons, can be classified in:

- Multipolar neuron: most common, multiple dendrites and one axon
- Bipolar: one dendrite/one axon. Common in retina, ear, olfactory (is the typical sensory neuron
- Unipolar neuron: typical sensory neuron: out of the cell body one single process comes out, a half has the function of dendrites (but is not a dendrites) and one works as an axon. Morphologically are both axons, but not functionally (pseudounipolar). Cell bodies are collected in the ganglia $\longrightarrow$PNS
- Anaxonic neuron: only in the retina

The mammalian brain contains many multipolar neuron, while in invertebrate there are may unipolar neurons. Morphology is strictly linked to function. In mammalian brain we have to integrate lots of informations $\longrightarrow$that's why we have lots of multipolar neurons, to *integrate informations*. Fish or invertebrate have very simple reflexes, they don't have to integrate many information. Neuronal somata are collected in rind or ganglia in invertebrates, while in vertebrate are grouped in nuclei in the CNS. Invertebrate lack myeline, because the axons are short and because they have simple reflexes and neurons are smaller, bigger and unipolar: they compensate the lack of speed that vertebrate would have if their neurons lack myelin.

Summer school in SISSA sul sistema nervoso degli invertebrati!!!!!

Multipolar neurons are very different: the classification of Santiago Ramon y Cajal is too simple. In the cerebral cortex, there are different layers that host different kind of neurons.

In the Hippocampus, we find *pyramidal neuron*.

Dendritic Spines are 10-40000 each neuron. In retina, there are lots of type of cells, like *Starbust*. For the classification, the largest difference is in the dendrites.

Morphometric analysis: analysis of dimensions, shapes etc changes in dendrites.

Characteristic arborization patterns

So far we have considered neurons as flat, but they are 3D structures. The newest classifications comprehends this observation. The stellate neurons can be flat or occupy a sphere like a sea urchin or a half-spherical distribution. It depends on what kind of information you want to collect. We can have also a laminar radiation: cell body can be inside or outside; neurons can occupy cylindrical structures, biconical radiation or fan radiation, uniq for the pyramidal cells.

The functional meaning is: bipolar neurons collect informations only by few neuron. If ramifications are a lot, like in Purkinje fibers, the neuron collect information from every neuron in that area.

Chapter 2

Lesson 2: Neuroglia

6 types:

- Astrocytes
- Microglia
- Ependimal cells
- Radial glia
- Oligodendrocytes
- Schwann cells

They are protecting the NS because they sequestrate the ions in excess, they give metabolic support. Astrocytes have contact with neurons and capillaries.

2.1 Functions

Astrocytes have a physical support, trophic, signaling, homeostasis function. They are also star-shaped and symmetrical.

Microglia is the resident immune system: these are small cells, mesodermally derived. Microglia is very small and difficult to detect.

Oligodendrocytes are the cells that create myelin in CNS, they can collect many neurons; Schwann cells are in the PNS and can collect just 1 neuron.

A typical features of astrocytes is the stellar shape with spines and enlargement called feet. The processes contact the vessels.

2.2 Gliogenesis

The making of the glia. It starts from the *neuroepithelial stem cells*[1], that will generate neurons or glial cells. Thanks to the presence of specific factors, PDGF and FGF2, there is a commitment of these cells, which will become *multipotent* cells. They are now committed to generate a glial precursor. After further interaction with others growth factors, there are 2 pathways that lead to the formation of the precursors of glial cells:

- The type 2 astrocyte (from the pathway with PDGF and CNTF) and oligodendtrocytes (with inly CNTF) are the final stage of maturation
- The type-1 astrocyte (from the pathway with CNTF and EGF) has no processes and can generate the adult neural stem cells

2.2.1 Microglia

They behave like macrophages. Their origin is not from neuroepithelial stem cells. CNS is not completely isolated from immune reactions. They can be marked with lots of molecules. They can contribute to several disease, in particular those who concern inflammation in the brain.

Microglia is important in most pathologies in the CNS, because its activation is an attempt of the brain to react to possible damages. It can eat red blood cells after a stroke, because red blood cells contains ions.

The microgliogenesis occurs in the *bone marrow* (midollo osseo), from the monocyte line: during the development they can pass through the blood vessels inside the brain[2], where they find their position and differentiate to a typical microglia. They need to have an ameboid behavior. During the development, the cells continue to migrate to expand even in the new area, and they also proliferate (but they need to stop at a certain moment). The activation depend on the secretion of ATP from the neurons and the M-CSF and GM-CSF from the astrocytes (they are also present in the rest of the body). Also many toxic factors can activate the microglial cells, and also some of the neurotrophic molecules.

If you eliminate glial cells from the brain with irradiation of the brain during development, death is the result.

[1] pluripotent cell

[2] Those who migrate outside becomes macrophage

2.2.2 Ependimal cells

Epithelial-like structure, cube cells. They are located in the outer surface of the brain, under the meningi, and in the spinal cord canal They are also present on the surface of the choroid plexus[3]. They are ciliated, with adherence junctions, and express the glial markers. They have the intermediate filaments; they can extend cytoplasmic processes into the brain parenchyma. NCS derives from astrocytes.

2.2.3 Radial glia

It has some radial structures that go from the lumen of the spinal cord (the neural tube in embryogenesis) to the surface of the NS during the embryogenesis. In the central zone of the lumen the newborn neurons are created from the neuroepithelial precursor cells: the neuroblasts migrate along the radial glia. The radial glia produces a matrix and adhesion proteins to help the neuroblasts moving.

The radial glia is not only present during the development, but persist in the cerebellum, where is called *Berman glia*, and in the retina, where is called *Muller cells* and is found with a marker...

2.2.4 Astrocytes

They are called star cells, the most numerous cell type in the brain. Important for the migration of neurons during development, for the blood-brain barrier (induction and mantainance). There are 2 types of astrocytes:

- Protoplasmic: pedunculated, are typically located in they gray matter. They are in contact with blood vessels
- Fibrous: in the white matter

They produces neurotrophic factors, they buffer excess of ions and transmitter in the extracellular space, endocyting it. Then, they car or destroy these substances for neuron to recycling, or they can ...? They protect neurons from the excitoxicity, that can cause neuron degeneration. They release specific neurotransmitters only secrete by glial cells.

Astrocytes can contact basically *every* cell component in the brain! They can control every function of the brain. Just like the microglia, they can divide when stimulated: dangerous if a cell come in a tumoral behaviour. There are probabilly different pattern of activation of these cells.

[3]cells that produce the liquor

2.3 Blood-brain barrier

The end feet of the astrocytes form the cover of the blood vessels. The barrier has been discovered in 1885. The continuous basement membrane coats the vessels.This barrier is also present close to the ependimal cells, on the surface of the brain: it is flat and cover perfectly the brain, and it is called *the glia limitans*.

What is the blood-brain barrier?

A structural and functional barrier that prevents the entrance of molecules in the brain, selectively. Formed by BMEC (brain microvascular endothelial cells), astrocyte end feet and pericytes, that are surmounted by the astrocytes and circondano the vessel. It is essential for normal function of CNS because it manteins the neural environment in and out of the brain. It is not present in the ipothalamus. It regulates the concentration of L-dopa. The nucleus of astrocytes is far away from the vessel. In somatic capillary there are fenestra, not in the NS. There is also a neuron in the brain capillary on the top of the endothelium: it helps to regulate blood stream pressure because the capillary can expand o restrict following the secretion of neurotransmitter from the neuron.

Tight junctions are present only in this barrier, the molecules are claudin and occludin, that are in contact with the cytoskeleton. We find also junctional adhesion molecules.

The cerebral vasculature can change volume according to the need of O_2. In absence of oxygen, neurons can survive 3-6 minutes. The vasculature has an autoregulation, but also can be regulated by the neuronal activity.

A molecule that has to pass the barrier, cannot go through the plasma membrane or between the cells, but it must go on a vesicular transport (receptor mediated endocytosis or pinocytosis). The integrity of the barrier relies on the presence of tight junciont, adherens junction, pericytes and atrocytes.

2.3.1 Tight junctions

Made by claudin and occludin and junctional adhesion molecules (JAM). The occludin are regulatory proteins because they can alter the paracellular (intercellular, or between the cells) permeability. Occludin and claudin assemble into heteropolymers and form ... The loss of occludine is present in breakdown of BBB.

JAM are integral membrane proteins, they are also involved in the monocyte transmigration over the BBB and in the regulation of paracellular permeability. They may have a homotypic binding (interaction with homologous

molecule) and a heterotypic binding with leukocyte, like in multiple sclerosis.

There are also accessory proteins, like ZO-1, ZO-2, ZO-3, cingulin, that link the occludins to the cytoskeleton in order to make a tight junction very solid: they are globular proteins. Also MAGUKS, guanylate kinases, make the same thing, but can be regulates and they can attach or detach.

Caderins are adhesion molecules present in every tissue.

2.3.2 Pericytes

Cells that provide structural support and are important for the phagocytic activity: they are the first calls able to destroy some damaging molecules.

There are regions that are not included in the BBB:

- Circumventricular organs: they secrete hormones in the blood stream, like neurohypophysis, pineal gland, lamina terminalis

With facilitated transport can be carried amminoacids, glucose (a lot!), glycine. Some fat soluble molecules can cross the BBB and cell membranes, like some drugs or treatments. A lot of factors can alterate the BBB and provoke disease or alterate the mood.

Chapter 3

Lesson 3: Neuroglia and myelin

3.1 Oligodendrocites

Oligodendrocites are few branch glia in the CNS. They have been discovered with the technique of Golgi and Ramon y CAjal. The myelin contituets the white matter: they are target of some disease like multiple sclerosys (may occur in young people, is an immune attack against oligo).

Olidodendrocites form myelin around more than one axon. One of the typical feature is that these enrollement of the axons are formend only on discrete portion an there is an interaction of two portion of myelin that is the Ranvier's node. Myelin is formed by a series of levels which has elettrondense and elettronclear appearence at the microscope.

3.2 Schwann cells

Schwann cell is the second type of myel forming glial cells, typical of the PNS. The appearence of the myelin coating the axons is the same of oligo. The main difference between oligo and schwann is that one schwann cell myelinates one axon, an oligo myelinates more than 1 axon.

A pseudounipolar neuron has 2 axons myelinated, but one acts like a dendrite, because the direction of the conduction is from the perifery to the soma. In the CNS, the myelin is exclusively in the axons, in PNS there is also on a axon working as a dendrite (in pseudounipolar neuron). In a motor neuron, soma in in the CNS and axon in the PNS to innervate muscle.

Not all the axons are myelinated in PNS: they are involved in pain detection, and that's the reason why we sometimes feel pain after a certain time. That's because the signal conduction in these fibers is much slower.

3.2.1 Nerves

Once the myelinated sensory fibers and the myelinated motor fibers are out of the CNS, they are pached into *periferal nerves*, and there is a mix of sensory and motor fibers, and they are surrounded by a matrix composing a connective tissue, composed of fybroblasts, fibers like fibronectin, laminin, together with other substances that help keep n position the myelinated fibers, protecting them. There are also elastic fibers to have flexible nerves, to stretch them. In black we see the *periniuron* (inside there is the *endonireon*, that contains lots of water to help the passage of substances), more fibrous. The outside contains some fat cells and is the *epiniuron*, the connective tissue that covers the entire nerve. The nerves can be colorated with ematoxilin or...

The capillaries blood vessels are imbended into the outer fibrous coat, the epineuron.

The periniuron is composed by flat epithelium, lots of connective fibers. The endoniurion we have a structure containing the Renault bodies, that are internal sites for immune system to protect the nervo from any viral or bacterial attacks: the virus can enter into the cytoplasm of these cells, crossing the myelin, and reach the cell bodies into the spinal cord, for exemple after an accident that leads to a break of the nerve. Large capillaries are in the epineuron, bud small capillaries are also inside the endoniurion to bring glucose and oxygen: in the endoniurion there are fybroblasts that produce connective tissues, there are some resident immune cells like macrophages, capillaries and the unmyelinated axons as well as myelinated fibers.

Unmyelinated fibers are the invaginations in the glial cell: the diameter of the myelinated axons is larger that the unmyelinated. The glial cells so protect the axons and has a trophic function and exchanging signals.

3.3 Myelin

The myeli act as insulator for the *vertebrate* nerve cells, formed by wrapping of plasmalemma around the axons. 80% lipids and 20% protein. All myelination is completed at 25 years old, and lady have a faster myelinization. We have a pagkaging of this plasma membrane one on top of the other, and the cytoplasm is eliminated by a physical squeezing.

What influence the speed of a nerve signal is the diameter of the fibers and the presence of the myelin: larger fibers have a bigger surface for signals and:

- Small, umyelinated fibers: 0.5-2.0 m/sec

- Small, myelinated fibers: 3-15 m/sec
- Large, myelinated fibers: up to 120 m/sec (almost istantaneous

Slow signals supply the stomach and dilate pupil.

In myelinated axons we have a saltatory conduction between the Ranvier's nodes. In order to achieve these, in the nodes there are some specializations: the channels that open and allow ions to pass across the membrane are Na channels, at very high density.

The composition of the myelin: the major dense line (very dark at the microscope) is the cytoplasm; the withe part is the plasma membrane and the red is the extracellular part. . The wrapping of the plasma membrane are crossed by some transmembrane proteins: there are *cell-cell adhesion*, so these proteins are cell adhesion molecules that compact the myelin to have a very dense packaging and squeeze out the cytoplasm. There is the myelin basic protein, MBP, that helps the compactation and the squeeze of cytoplasm.

There are some tumoral cells of glia: neurofibroma and schwannoma in PNS, atrocitoma (benign pilocytic astrocytic and the most common is malignant, the glioblastoma), oligodendrogliomas and ependymomas (is the more easily removed by surgical interventi) in CNS. Some diseases are due to mutations that do not permit a good compact of the myelin, like because there is a mutation in MBP. MPD is also attacked in some forms of multiple sclerosys.

The diameter of the myeline is different during the age process. There are 2 big cluster of myelinated diameters, one around 5 μm and one around 12 μm

3.3.1 Mielination

In myelination the cells need a continuous contact with the axons, so the wrapping in conducted to the contrary of what expected: the first wrapping will be the more external one, while the second will be underneath so that the cell maintain the contact with the axons.

The contibution of the external part that is in connection with the cell body in limited: only the free edge can actively produce the myelination. The cell recognized that the first round is completed, because there are some signals in the contact point, and continues growing. There are spaces between the axon and the leading edge, because the leading edge still has cytoplasm, because it is needed to produce the cytoskeletal compound needed to the growth. The leading edge is growing and needs energy: everything is in the cytoplasm. Behind the proteins (MPB inside and cell-cell adhesion outside)

are compacting the wrapping and the cytoplasm is squeezing. How can the cell maintain the contact with the leading edge if everything is closing behind? There are small channels, a narrow corridor in which the membranes are not compacted (and it is the white part).. Laterally, we have places in which the myelin is not present, because the SC has an end-point: it is important for the formation of Nodes of Ranvier. The end point of the myelin needs to be closed to avoid the passage of everything, but there is also an enlargement with passage of cytoplasm: this part is important to make contact and closing, so we find adhesion molecules, TJs. It is also important for the communication, signalling: there is a balancy between closing the gap, avoiding substances to enter, insulating myelin and keep this area as an important site for communication.

During myelination, how can the cell decide when is the moment to compact and close the gap? It's not just about proximity: the proteins of adhesion are produced from RNA granules and, in order to make sure that myelination occurs only when needed, the ribosomes that are packed into the RNA granules receive a signal, coming from the *contact between the axons and myelin*, that opens the granules and the translation of the adhesion molecule stars, for compacting of the myelin.

The position of the myelin on the axon causes a movement of the surface molecules of the axon: channels move laterally, they are eliminated from the places that will host the myelin, move laterally and go into the Nodes of ranvier, also because initially we have several adhesion molecules initially distributed on the surface of the Schwann cell (in blue): these are proteins which are called *neurofascin 155*, associated with the cytoskeleton. Neurofacin takes contact with the contactin, another adhesion molecule on the axon, and then these molecules are squeezed laterally and they move at the top of the myelin wrapping during the compaction, and they remain in the part in which we have some enlargements. Here they provide signals for the cytoskeleton and the Na channels are moved laterally, because the adhesion molecules are linked to the cytoskeleton. The two neighboring Schwann cells position the channels between them, in the *node*. We have the compaction of myelin (P0, MBP) that are squeezing out the cytoplasm. There are part of the cytoplasm which are compacted specifically by Neurofascin 155 that binds to *contactin* and to *Caspr*: this region is the *paranode*, because it is close to the node. The node is the region in which the Na channels bound with each other by a specialized cytoskeletal protein, *ankirin*. This is displaced and brings the Na channels: we have a squeezing of cytoskeleton by ankirin. We have other molecules, Na channels, in the middle, physically separated by K channels in the paranodal region.

The balancy is made by using one simple trick: having adhesion molecule

neurofascin 155 that are initially present all along the space, they take contact with a receptor on the surface of the axon which is attached to the cytoskeleton in the axon. There is a lateral movements of these molecules of neurofascin: in this way they will be concentrated at the paranodal place and they push the Na channels at the nodes of ranvier. Na channels are anchored to ankirin. Ankirin is bring into position from the paranode region: now gap and tight junctions close the terminal part (between the different wrappings of the glial cells).

The terminals that are forming during myelination are called *early paranode*, bring to this position ankirin and Na channels, while in the marginal region we have only K channels. Then everything is closed just like in epithelial cells: we have gap juctions and tight junctions that close the termina part, between the different wrappings of the glial cells, not between axon and glial cell.

Ankyrin binds to specrtin and, since there are molecules connected to the channels, we have the formation of the complex. In case of K channels, placed at a different place (not paranode and node, but under the myelin), we have PDZ protein, a cytoskeletal protein. We have cell-cell adhesion molecules specialized in different part of the node of ranvier that contact the glial cell and the axon, part of the contact is i he paranode and inside the node. The molecules makes a bridge to take the channels in the right place and they are helped by adhesion molecules.

Chapter 4

Lesson 4: Astrocytes functions

(15-10-2015)

Historically recognized:

- Sequestration of K+ during neural activity. K+ are concentrated outside and, if there is too much K+, the astrocytes will remove it
- Removal of neurotransmitter and recycling of them (GABA and glutamate) to make precursors
- BBB regulation
- Involved in neural stem cells generation (regulation of neurogenesis)
- Regulation of synaptogenesis (establishment of new synapses
- Modulation of synaptic activity

Presence of astrocytes closely related with synapse, in particular to the synaptic cleft: over 90% of synapses have astrocytes. Take a brain embryo from rodents, dissociate cells using trypsime and plate them in a Petri dish in appropriate medium: if we take at day 12 of gestation, there are no glial cells that are formed $\longrightarrow$90-95% of the cells in the plate will be only neurons. Synaptotagmin is present in the presinaptic, PSD95 in the post synaptic, these are 2 proteins: if we see them in the same place, that means there is a synapse, because they are co-localized. In a culture using the same neurons, but on the top also a culture of glial cells, we can see both proteins, while in the culture of only neuron we cannot $\longrightarrow$the presence of glial cell plays a role in synapses formation.

The number of the glial cells grows growing the complexity of the animals. Albert Einstein has much more glial cells per neuron than a normal person.

Experiment 1 Astrocytes use Ca to "talk", meaning that the excitability of astrocytes is caused by a variation in intracellular Ca concentration: taking a retina and put this in culture. The retina can be filled with a fluorescent dye which response to Ca increase: every time the fluorescence increase, there is a Ca increase. Touching the cells provoke a flash of fluorescence: this explains why when we get a hit in the eye, we see flashing of light. When we touch the cells with a glass pipette e see a circular wave that goes from one cell to another. There are specific connections between astrocytes that are GAP junctions, that form membrane pores: the Ca ions can freely pass from one cell to another $\longrightarrow$wave (short-range signals). These waves stops after certain distance; there are also long-range signals that go through membrane receptor, because glial cells and neurons can release some specific *gliotransmitters*[1]. In particular, ATP[2] has a receptor on the top of glial cells, connected to PLC, that activates the IP3, that activate the RE $\longrightarrow$release of Ca from the RE. These two systems work in parallel, they are both activated.

Experiment 2 Another experiment: it is possible to use mouse models that were grafted with astrocytes coming from human, so they have much more contacts. The chimeric learned very well to respond to stimulus.

leggi l'articolo

Experiment 3 Another experiment: astrocytes loaded with IP3 caging: with a flesh of light we can uncage the compound $\longrightarrow$increase in the astrocytic content of Ca. At the same time, they put an electrode in the neighboring neurons and recover a spontaneous potential. The average frequency of this spontaneous activity in increasing: this activity is caused by a release of neurotransmitters vesicles. The signal that make this release is: by rising the intracellular Ca in astrocyte, there is a possibility to release Glu, because it is captured by astrocytes and release again, that will activated the presynaptic metabotropic receptor, ad D-Serine, that can activate a part of the Glu receptor, the Gly site (this site can bind D-Ser and Gly): this binding let the receptor opens better. Gly is released by neurons and D-Ser form astrocytes: in this way the astrocytes can modulate the release of Glu by the neuron.

It is possible to measure the electrical activity of the neuron: they found out that astrocytes are activated with the neuron.

Let consider a synapsis which release ACh. This synapse is wrapped by glial cells. Making the experiment 1 and comparing the synaptic activity,

[1] ATP, Glu, D-Ser, which regulate neuronal excitability and synaptic transmission
[2] given by neurons and glia

they found out that when astrocytes were presents, there wasn't a better transmission! O.O There was a decrease in synaptic activity and the ACh was captured by a molecule produced by glial cells, capturing and eliminating ACh. But how the glial cells sense and respond to the ACh? They have a receptor for ACh that is active and responds to an increase of ACh: the response is the production of ACh binding protein that is release in the synaptic cleft. This protein has been identified by Bungarotoxin purification scheme. This protein, given alone without the glial cells, produces the same synaptic depression.

All this plasticity is accompained with morphological changes. The change in the volume over time[3] is more in the processes that in the spines of astrocytes. So, astrocyte can modulate the concentration of the neurotransmitter just increasing their volume into the synaptic cleft. This aspect of the volume is important also for the function of the glia: if glia is very closer to the cleft, there is a higher probability to catch the neurotransmitter.

How can we relate the changing shape and all the molecules described?

The actin cytoskeleton is modulate by Ca: ATP and Glu receptors on the surface of the astrocyte and a pool of actin single molecule that can be added to a growing actin filament. To push the membrane we need something: this is the cytoskeleton. We need to build the cytoskeleton with actin filaments, that are parallel and grow into a direction, and this is controlled by Ca and phosphate groups. Profilin is a proteins that helps in building the actin filaments. Overexpressing this protein we can change the shape of the astrocytes.

Important: the anesthesia takes down the Ca frequency. There is also a difference between young and adult brain in the function of astrocytes: young astrocytes are more reactive to synaptic activity.

[3]Changing the concentration of the neurotransmitter ins the synaptic cleft

Chapter 5

Lesson 5: Dendritic spines

Pyramidal muktipolar neruon: apical dendrites and several smaller basal dendrites. They have some small protrusion of the surface and show the highest density of number of spines (number diviso length).

Most of the studies are done with neurons filled with fluorescence proteins. Spines are very different but there are specific features: SER, spine apparatus (cellular cisterne), polyribosomes[1]. Spines are twitching: in addition to the classification of the shape, we have to deal with the motility of these spines. In particular, in learning and memory spines contributes substantially in changing their shape. Treating spines with isofluorane is going to immobilize the spines.

The shape is important because a loss of the shape is linked to certain pathologies: if there is a neuronal developmental disorder, like mental retardation, the shape of spines is involved and a decrease in number of spines.

Spine density decrease normally during normal aging and a change in shape during hormonal change. Glu and growth factors are important to modulate the nubmer and shape of spines. There are fillopodia, the first structures that take contacts with the axon, in order to establish synaptic contact. There is a polimerization of actin filaments and the fillopodia is produced: then it establish the contact, that can be temporary or not, establishing a synapse.

Once created, spines will not rest like that forever, but they will move ant they require a continuous signaling to be maintained. Spines that are not required will be selectively[2] eliminated: this process is called *pruning* and occurs during the development a phase in which there are a lot of spines. At 11 years old we have the maximum number of spines, then there is a

[1] They can be also in the periphery of the neuron

[2] What is not stimulated is lost

decline during the teen-age time. Fillopodia come out rapidly and are rapidly retracted.

In the early stage of development (10 days in vitro) we can se several fillopodia, while in the mature stage there are more established synapses.

5.1 Dendritic spines types

By looking at the Golgi staining, there is a classical classification that distinguish 3 types:

- Stubby
- Thin
- Mushroom

The shape is mutant in time. There is a *mathematical rule*: some spines (type 1) have a diameter of the head is the same of the length. Type 2 there were longer than large, ad had a thin neck; type 3 have a very long neck. Type one are stubby spines; type 2 are mushroom spines; type 3 are thin spines.

How we distinguish a filopodium from a thin spines? by the motility, that is more rapid than a spine, and the length: a spine is smaller that 3 μm $\longrightarrow$there is also a maximal length of the spines.

The mushroom spines can also have a cup shape. Sometimes we can se a spinule covered by clatrine.

In the thin spines, the connection with the dendrite is very narrow, while the stubby shape have a complete connection with the dendrite. In red excitatory synapses, in blu the inhibitory: note that the red synapses are placed on spines and the blue one on hte parental dendrite.

In the pedunculated spines: large head and small neck. . In the simple spine: no constriction between the spine and the dendrite, so the exchange of material is easy. This means that there is not a restriction in exchange with synapses! The morphological shape and its function are related. We can have also thin spines that have a small neck: there are lots of possible variations!

In a dendrite we can find en enlargement, the *varicosity*, very common in invertebrates. The synaptic crest is very extendend in 3D, even if it looks like a mushroom. The aim of complexes spines is to have contact with different axons to make an integration of different signals. In the case of thorny excrescence, it is one of the most important structure for the memory: they are so big because they are the most active synapses in the brain and

is important to integrate different memories and recall sequential memory. During the evolution, the hippocsmpus was the site of the spatial memory, but now we know that also the time memory is built it this site. These synapses are so strong that cells die and have to be renovate.

Several of these complex spines are present in the cerebellum: they also make spines biforcated to maximize the number of contact.

5.1.1 Internal structure

Basically, the most highly concentrated molecules are related to the cytoskeleton and are responding to Ca: there are a lot of actin filaments and in the central part of the spine we find filaments that are called *stable actin*: they don't undergo an intensive polymerization od depolymerization because they have a cap on their endings (yellow and bleu points). To keep a stable actin filaments, we have also to distribute the actin filaments in a certain direction $\longrightarrow$we need to bind them with transversal proteins, the α-actinin. We have seen that spines are twitching: the most mobile part is related to the head, not to the neck. In the periphery of the spines there are the *dynamic actin* filaments: these are twisted or attached in the center thanks to the actin polymerization proteins and on one side they are attached to the surface of the cell membrane, via *spectrin*.

Here we have to achieve 2 important features: anchor and dynamic possibility to elongate or restrict, There are proteins that bind the dynamic actin to the cytoskeleton in order to have more stable spines.

There is another molecule, the *myosine*: actine and myosine contribute to the contractile of the cells. Here they have another important feature, the transport of vesicles and proteins and small organelles from the parental dendrite.

When the spine is stable, the peripheral filaments are bound to the central core; when there is a need to elongate, the filaments can change length.

5.1.2 Regulation of dendritic spines motility

Cytochalasin D block the spines movement by blocking the actin polymerization. We can block polymerization by blocking Ca: there is no changing in the shape. But Ca is coming from intracellular stores or from outside? Both. At the end, we can block step by step the cascade and we can demonstrate that for having this phenomenon we need all the cascade.

Cellular model for the memory

we can have long-term potentiation (LTP) of synapses, but in some other cases memory is made of long-term depression (LTD) of the synaptic activity. One of the molecular an cellular mechanism to achieve this in to change the shape of spine: for LTP a bis head shape and so much actin filaments while for LTD is the contrary.

The consequences of having different shapes is linked to LTP and LTD, but why?

There is a link with Ca: once the neurotransmitters arrive on the surface of the post-synaptic membrane and open the channels, there is a huge amount of Ca entering in the cell. It can go into the soma and make a signal there, or stay in the cytoplasm and activate signals there. Large spines have also a big smooth ER, that is the intracellular storage for Ca. So, there is Ca entering from the channels and releasing from the intracellular stores. thereis also a relationship between the dimention on the spine and the amount of CA: if it is small and isolated from the dendrite, we have a huge increase of CA, while when the spine is large, the concentration will be lower but also rapidly spread laterally in the dendritic shaft.

The rising in intraspines Ca can be through the channels and from the intracellular store: if we have 2 synapses close, we have a huge amount of Ca that enters in the stubby spine and the Ca stays inside the mushroom spine, so 1 synapse is very active and the other one is less active. If the Ca from the stubby one can enter in the mushroom one, the Ca can exit from the spine apparatus and this will make possible for this poorly active synapse (the synapse with the mushroom spine) to be stimulated equally well that the other one (the synapses with the stubby spine) (coordination). So we have a syncronized activity of these synapses: if there isn't a syncronized activity, we don't have strong synapses or integration at the level f the soma.

Chapter 6

Lesson 6: Synapses

What is the main function of a neuronal cell? To send messages. That's why we need a network! The shape of dendritic arborization is functional to the type of connection that we need. We have an *input layer* that collect the initial information, like the sensorial neurons located in the periferal ganglia; then a *hidden layer*, which is the interneurons: then a *output layer* that are the spinal motorneurors. Multiple interneuron segnals converge in a single output signal. This is the *signals integration*.

Cajal and Golgi observed baskets of a very long and ramified axon and basket cells: at the end these cells have a boutons, like a claw(clava) that are covering the cell bodies of the neurons in the cerebellum.

Sherrington understood that the impulse could be also an inhibitory signal, but he was wrong on the point that the transmission involve only electrical principles: Elliot was able to increase the heartbeat by incubating frogs with adrenaline (he was not believed). Few years later, Otto Lewi made a more convincing experiment (Elliot didn't start from the old models): a vagus nerve connected with a heart immersed in a physiological solution. He could stimulate electrically and see contraction: but, if there is a chemical substance which is released ad the synaptic level, this substrance sould go into the physiological solution! In order to determine if this substance is sufficient to increase the beat, I could put the solution containing this compound with the heart not innvervated: he sow contraction and he was believed.

The presynaptic ending contains mitochondria and other organelles; the postsynaptic has neuroreceptor on the membrane; the space in between is called synaptic cleft, 20 nm, is also surrounded by astrocytes. The adult human brain contains between 10^{14} to $5 \cdot 10^{14}$ synapses.

Synapses are funcitonal connection between neurons and between neurons and other cells. Def: a presynaptic terminal that is able to synthesize a neurotransmitter, that is contained in vesicles.

6.1 Criteria for Chemical transmission

The most of synapses in the CNS and PNS are chemical synapses. There are some recurrent features of these synapses:

- The neurotransmitter is synthesized in presynaptic terminals
- Neurotransmitter is stored in secretory vesicles
- The release is regulated
- Presence of receptors in the postsynaptic membrane
- The action of neurotransmitter is controlled by termination steps (like astrocytes that release ACh binding protein).

6.1.1 Classification of synapses

The excitatory synapses are mainly located on spines while inhibitory on the dendritic shafts, soma and axons.The neurotransmitters for excitatory synapses are glutamate, ATP and ACh, the ones for inhibitory synapses are GABA and Gly. Those synapses contains channels, that create a pore in the plasma membrane: when the channel is open, ions can cross the membrane according to their potential. There are other type of synapses that have no channels: they are using a particular type of receptors, the *metabitropic receptors*. In addition to ion channels, we can have receptor channels, line NMDA type for Glu: one part binds Glu and the rest of the subunit forms a ion channel. In metabotropic receptor, they do not form an ion pore.

Electrical synapses can work only if the membranes are very very close: like in GAP junctions. It works as a syncitium, even if structurally it is not because the cytoplasm is not shared. GAP junctions are not always open.

cosa le fa aprire e chiudere???????

The unidirectional synapse is a a-symmetry synapes: it is between an axon and a dendrite at the top of a spine and the membrane is thickening (*postsynaptic density*)in just one side.

Symmetric synapses are presents between axon and cell bodies usually.

The collaterals branching of the axon can have a synapse on each branch end. We can also have a single axons with *boutons en passage*: everitime a axon touches a dendrite in a spine, it makes a synapse, so there are multiple synapses.

Typically, a-symmetric synapses are excitatory and symmetric synapses are inhibitory. The axon-somatic synapses is usually inhibitory.

lo chiede all'esame!!!

So, synapses classification can be based on:

- cytoarchitecture
- method of signal conduction and
- conductance of the postsynaptic element

6.1.2 The synaptic bouton

By freeze-hatching we can detach membranes and separate the lipid bilayer to look at the protein that are inserted: we can see what arrives at the surface of the pre-synaptic membrane. There is an array of trans-membrane proteins aligned: looking on other synapses we can see vesicles fused on the membrane, that occur laterally on the array of proteins. After the release of the content of these vesicles, the membrane is recovering and taking back with endocytosis part of the membrane, by coated-pits vesicles that has clatrin.

The vesicles are aligned along these arrays of transmembrane proteins, and they fuse laterally.

Neurotransmitter

They can be aminoacids, monoamine (dopamine, noradrenaline, adrenaline, serotonine, melatonin), then ACh, adenosine, histamine, ATP etc. There are also neuroactive peptides: CGRP, SP, PY.

Monoamines are most regulatory neurotransmitters: they are often together in the same synapses.

The secretion of neurotransmitters is very very fast: all the vesicles are kept inside until the arrival of the action potential and the increase of Ca in the presynaptic terminal.

Ca controles:

- Exocytosis
- Mobilization of synaptic vesicles, that are linked to the cytoskeleton

The consequence is the fact that, since these vesicles contain a defined amount of neurotransmitter, the synaptic transmission is quantal: a fixed amount of neurotransmitter cause a release oaf a certain amount of vesicles. This is due to the anatomical specialization of the synapses and the properties of ion channels.

Chapter 7

Lesson 7: Organelles and secretion

Synaptic transmission is a specialized type of exocytosis. We need to have a vesicle targeting and the release has to be event-specific and fast release of chemicals. The molecular biology reveals conserved components:

ER $\longrightarrow$AG $\longrightarrow$vesicles to the bouton. Vesicles sometimes have to do a very long journey. The proteins inside the secretory vesicles are mainly *enzymes* to produce the neurotransmitter, which has a precursor in the bouton: sometimes this process occur in the cytoplasm, sometimes in the synaptic vesicles. Vesicles proteins are involved in:

- Trafficking
- Exocytosys
- Synthesis of neurotransmitter

The cell must sort out the different vesicles produced by AG in a very specific manner. Neurons do have a ER and a AG in the cell soma, but they also have some peculiar characteristics: GA is huge and numerous, because neurons are highly metabolic active cells. Most proteins last 1 to 2 days, but some proteins that can last moths and proteins that can last few minutes, so that explains why there is a lot of ER and GA.

GA has many functions: modifies N-linked oligosaccharides and sorts proteins so that when they exit the trans Golgi network, they are delivered to the correct destination.

Vesicles with a coat made by COPI are in the pathway from the GA to the ER; COPII from the ER to GA. In the vesicular transport models, vesicles brings back the enzymes to be recycled; in the cisternal maturation model there are some vesicles that move, basically backwards, to recycle enzymes.

Clathrin, COPI and COPII are very different: clathrin forms the triskelion structure that imposes a change on the surface of the plasma membrane[1]. One of the key issues is that in order to bind to the membrane, clathrin needs receptors and adaptors: the ligands (the proteins that have to be secreated) binds to the recpetors and signals that the proteins are ready for being secreted $\longrightarrow$the receptors recruites adaptine, that is attached outside the receptor $\longrightarrow$signal to clathrin to bind $\longrightarrow$formation of the secretory vesicles $\longrightarrow$clathrine goes out after the formation of the vesicle. the detachment of the vesicles is made by dynamin, the squeeze the neck of the vesicles.

All these secretory vesicles are transported by microtubules: they are going from the ER to GA, and then motor proteins that can bind the vesicles.

A change in the pH from the Er to GA permits the detachment of the proteins from the receptors on the plasma membrane (that binds the ligand that has to be secreted). All coated vesicles contain GTP-binding proteins, very important for the assembly of the vesicle. These proteins are associated to different vesicles and different coated proteins.

see slides

The adaptor proteins provides the information on where the vesicle have to go, so the target. AP is a complex of different subunits. How a coated vesicle is created?

We need a receptor that binds to a ligand, that can be the secreted protein or the enzyme of the maturating cisterns. Trough the maturation, the cisterns reaches a different pH to activate different enzymes and increase (or decrease) the affinity for the receptor. The vesicles form only when the ligand is there: this prevent the formation of empty vesicles.

In the regulatory secretion, we need a signal to start the secretion, while in the costitutive secretion aussi tot que the vesicle is created, it is fused with the membrane to secrete.

A secreted proteins has a signal peptide in the N-term that let the protein to go in the ER: this is sufficient to have the signal peptide in order to go to the secretory pathway; when a protein has to go on the regulated pathway, there is a signal sequence into the protein that causes a folding to bind a specific receptor. This give 2 information: the protein is a secretory regulated one and it's properly folded.

In the brain, the protein that stimulate the growth of neurons is a secretory protein, addressed both to the costitutive and regulated pathway, but for BDNF the 90% has a regulatory pathway, the 10% the costitutive one. In contrast, NGF (nerve grow factor), very similar in sequence and structures and receptor and pathway activated to BDNF, is secreted at 90% with the costitutive pathway and 10% with the regulated one. What makes the

[1]Its shape is given by the cytoskeleton

difference between these 2 similar factors?

There are 4 aa in the core proteins that when the protein is correctly folded form a signal for a receptor, the *carboxy-peptidase E*: it recognizes the aa of BDNF. If we exchange the aa that differ from BDFN and NGF, the researcher saw that the pathways were exchanged. (in particular, the made 2 mutations. BDNF...).

So, how neurotransmitter are secreted?

Basically, the principle is the same: vesicle goes by a donor compartment to a target compartment (formation, transport and fusion). The neurotransmitter has to be stored in vesicles because:

- They can undergo degradation, so they have to be protected
- Allows for regulation
- Provide a storage system
- Can be docked at active zones
- Differ for classical transmitter vs neuropeptides

Synaptic vesicle components are made in the soma, in particular in the Golgi, and they are transported to their sites by the cytoskeleton etc. Once the vesicle is fused to the membrane, we have a endocytosis that forms and endocytic vesicle: two vesicle can fuse to forma endosome, that can go to the catabolitic route (late endosome) or form new vesicles. The late endosome, on one side produces new vesicles that come back to the Golgi, on the other side they become a lysosome. We also have the recycling of the neurotransmitter outside.

In neurons, we have he classical coated pits pathway or cisternae models, then the kiss and run model to recycling: the vesicles arrives to the membrane, that te vesicles (regulated pathway) are activated by the phenomenon of priming, then when Ca arrives they fuse (not completely(with the membrane. The neurotransmitter is secreted and the membrane closes back $\longrightarrow$recycling of the vesicle without the clathrin pathway. If the vesicle fuses completely, we need energy to create the curvature to recycle it: this is given by clathrin (this is not the kiss and run model).: in this case we have a fusion with the endosome, an additional way to regulate because, in the case of kiss-and-run, most of the vesicle are filled by neurotransmitters and if we don't want to have transmission, the route of the endosome eliminates everything.

Chapter 8

Lesson 8: Cytoskeleton and axonal transport

Synaptic vesicles and every vesicles that is transported at the axon terminus make a long journey along the cytoskeleton. The function of the cytoskeleton are:

- Dynamic scaffold
- Internal framework to divide the different compartment of the cell
- Network of highways
- Provide the force to generate apparatus-cell movement
- mRNA anchoring
- Cell division

We have microtubules, microfilaments and intermediate filaments. MT and MF are made by a single protein; IF are made of several proteins. Nurofilaments, ore IF, are very heterogeneous.

These 3 types of filaments are differently distributed in the cell: the actin filaments are at the periphery of the cell, constituting the cortex, spines, microvilli etc. Actin can be elastic, so gives an internal coating and the shape, being on the periphery. IF are inside the cytoplasm, attached into particular structures of the membrane, forming *junctions*, anchoring 2 cells. MT irradiate from the centrioles.

MT have a cavity inside, 13 colons of tubulin molecules (α and β tubulin): they are resistant to compression and keeps the cell shape, in some cases are involved in cell motility, because they are shifting one on top of the other,

making very large movements. They are involved in chromosome movements, organelle movement.

MF are involved in the mantainance or change of the cell shape, in dynamic contraction.

IF are a serious of fibrous proteins, rigid and super coiled: these proteins are of the keratin family. They have no polarity! Glial cells can be identified by staining these proteins of the IF, because they express a unique type of IF (type III). The type 4 proteins like NF triplet (heavy, medium and light) are important because they form tangles in many disease like Parkingson, Alzheimer.

MF have a polarization because they can be elongated by actin monomers only at one side. These monomers has to be bound to ATP to bind the filament. The de-phosphorylation of ATP into ADP make the dissociation of these monomers. The most difficult step is the *nucleation*, then we have a *dynamic stability* because the length of the filament won't change.

In MT, the polarity is given by the fact that we have a dimer. If it has at the tip only monomers bind to GDP (because of the loss of GTP cap), there is a catastrophic depolymerization. In addition to MT, there are accessory proteins, like MAP2 for the dendrites and tau for the axon: the distance by microtubules can be regulated by phosphorylation of these proteins. In excess of phosphorylation tau, there is formation of tangles in neurodegenerative diseases.

Studying that, the cell culture were so important: it provided the majority of the information.

In highly polarized cells like neurons, the presence of the cytoskeleton is very important: we need to have more space between the cytoskeleton filaments, to let the organelles flow along the axon, so the MT do not to be bound via covalent bonds. The spacing is very important and there are a lot of connections that create a very irregular an wide space of NF triplet.

These were the static properties of the cytoskeleton: now we consider the *cell motility*.

8.1 Cell motility

Growth and retraction of cellular processes: the cell is emitting some filopodia to explore the environment and, when it finds a clue, there is a signal that goes in the cell and the advancement of the cell. We can have contractile bundles of AF because of the opposite organization of AF; in the central part of the cell, we have a gel-like network, very similar to the cortex, because there a re AF going in all directions, forming a mash; if we need

to push a filopodium, we have the convergency of the AC with the + end polymerizing in one direction, at the tip of the exploring filament. When the filopodium finds an environment not suitable, the machinery of the actin stops the growth and the depolymerization occurs.

In filopodia we see parallel fibers. We can use electro-microscopy, but the immunofluorescence staining is more useful. Growth cones have very different shapes, the filopodia can be very long and highly motiles. The tubulin staining is in the axonal shafts , and this part is stick, while the part containing actin is flat, there are boundles and filopodia.

In the filopodium we have polarize F-actin boundles, with the + end at the tip. Then, a flat area in which we have the actin cytoskeleton forming a network, and some *exploratory microtubules* that follows the actin filament and, in case of establishment of this filopodium, they reinforce the structure to keep growing. In the axonal shaft we have a stable domain of MT; in the part of the actin domain we have some unstable MT. There are also some structures, the *actin arc* that contribute to the central core of the growth cone. There are also some accessory binding proteins that contribute to the stabilization.

8.1.1 Molecular details

An extracellular stimulus activates GTPase ans PIP2: this activates a protein complex, the WASP, that binds to the GTP and is able to interact with Arp2/3 complex $\longrightarrow$this is able to help enucleating the first actin filaments, that is the rate limiting step that requires energy (starting from the plasma membrane). Some ancillary proteins can make biforcation in the filaments (barbed ends). The *cofilin*, an actin-depolymerizing factor promotes the dissociation of filaments, so give an highly dynamic form of depolymerization and polymerization, by bringing the ATP-bind actin to the initial site.

Role of microtubules

MT have a distribution along the axon that is polarized (+ end to the terminal tip), due to the way in which they are assembled, and gives information about the transport. In dendrites, this is not always true: in the initial part we have a mixed polarity, while in the more distant part it is like in axons.

Axonal transport is a very difficult task because in some cell axon can be more than 95% of the neuronal volume. There are many components of the synapse that need to be transported: this was seen thanks to a ligature experiments, that showed an enlargement before the ligature, that disappeared at the removal of the ligature. There is a group of proteins that is moving

along the axon with a fast axonal transport; a second group with an intermediate rate transport and a third group with a slow transport. Even the cytoskeletal proteins are transported because of their turn-over.

Are the MT transported along the axons already assembled or in monomers? This was solved by using tubulin made fluorescent by fusion with GFP: we load the neurone with that and see a fluorescent axon, than we flash with laser 1 point and bleach the fluorescence in a point to create a gap $\longrightarrow$we see a stretch of MT that are moving in this gap! They are moving very fast and in both directions! Figoooooo.

The problem of the retrograde transport was demonstrated in this way: ligation of the nerve + fluorescence. In the initial situation, we have molecules which are labeled, in red anterograde moving and in blue retrograde moving: after the ligation we have on one side an accumulation of red proteins and on the other side an accumulation of the blue proteins.

Another experiment can be made by labelling the motor proteins: the ones that are involved in the retrograde transport (dyneins) are on both sides of the ligature:that is because they are produced in the soma, so they are first transported on the terminal and than back to the soma.

Chapter 9

Lesson 9: Cellular transport and molecular motor

There are two types of microtubule proteins: kinesins and dyneins. Another family f proteins uses actin for trafficking, this is the myosin superfamily. They have separated motor proteins, because the transport from the center via microtubuels end is in the terminal, so the coming back is helped by actin filaments.

Also cilia and flagella use one of such motor proteins, in particular the dyneins.

Myosin II is a muscle motor proteins: it has a coil-coiled of 2 α helices and 2 globular heads linked by a neck to the coil-coiled. The globular structures are useful to interaction; in the part between the heads and the filament, there is a sort of light chains that allow the changing in conformation. When they are assembled together with actin and look like a millepiedi.

Even the kinesins have a similar structure. The motor domain changes in the class, so we can classify the kinesins ad N-kinesins (N-term), C-kinesins (C-term) and M kinases (middle). These kinesins proteins works as a monomer or a homodimer.

C-kinesins and dyneins are minus-end directed; N-kinesins (ressemble the myosin) are plus-end directed; M-kinesins are involved in the depolymerization of the microtubules at the + end.

Depending of the binding of the heads to the α or β tubulin, they go in different directions; in N-kinesins, the head is directed in the - end, the "tails" in the + end, so the kinesins can move only in the - end. These proteins are capable to transport huge vesicles and organelles so it is probable that these molecules act in groups to transport them.

General features about motor proteins:

- They generally mone unidirectionally
- They move stepwise: this is given by the structure and the fact that they occur as a dimer
- Series of conformational changes: due to chemical cycle and mechanical cycle that requires energy. ATP binds to the motor, than hydrolysis, that release of ADP and Pi and then binding of a new ATP.

The hydrolysis causes the attach and the release of ADP causes the movement and the detachment.

9.1 In the neuron

MT are generated in the centrioles, they elongate in all directions and the + end are in the periphery, where we have the growth of MT: in axons is the same, and while in the cell body MT are directly linked to the centrioles, here we can have some discontinuities. Kinesins motors goes from the cell soma to the cell periphery, while dyneins go from the periphery to the soma.

In transport, there are families and other proteins that allows to discriminate the cargo:

- Synaptic vesicles: they have to go only in axons
- MT: they have to go everywhere in the cell

There are kinesins that transport from the trans-golgi network to dendrites, like KIF7 and KIF5, they transport *dense core*, vesicles with proteins[1] like membrane receptors. The most important receptors in dendrites are the receptor for neurotransmitter: having them at the proper place, in the right amount ecc is very important. In the axons, we can also transport mitochondria, but the transport could take hours or days: there are some specific transporters that transport the cytoskeleton, to contribute to the formation of axons collateral.

9.1.1 Kinesins

They are involved in ATP hydrolysis and binding to the MT (in the globuler head). There are light chains that bind the vesicles. They share a molecular domain, then there is the coil-coiled domain and additional domains that "sense" the environment, to target the right cargo to the right place.

[1] synaptic vesicles have a clear core

THe structure of the molecule has been seen with electronmicroscopy. The classification was a mixture between genetical analyses and morphological analyses.

There are different kinesines that can transport the same organelle: KIF3 and KIF17 are founf in dendrites and transport different cargos because they have different adaptors, like GRIP. An additional group of proteins involved in transport is KLC: they are involved in the axons and they aren't present in the dendrites.

GRIP1 is related to the ampa type of the Glu receptor; KIF5 can use different adaptors, like milton and miro for mitochondria. Considering the synaptic vesicles, there are some cargos that contains only them and other cargos that contains only the protein for the machinery of the docking site.

The principle of inhibiting the usage of the material during the journey is to avoid secretion and loss of the components.

The different Glu receptors use different vesicles.

The signals that these motors can send are several: there is one class of cargos regulated by Calmodulin-kinases. When Ca high, the cargo in detached; that is useful to deliver something in a region rich in Ca, like a synapses! By this mechanism we can deliver the GluR in post-synaptic membrane, like in spines. Another mechanism is sensitive to GTP, in particular to Rab proteins, that are involved in reacting to protein kinases that active the death domain. In this place we can have energy by GTP: a synaptic vesicle precursor contain the enzyme to produce the neurotransmitter.

Another mechanism has the miron milton proteins, that attach a small kinesins and remains attached to the mitochondria.

9.1.2 Cytoplasmic dyneins

There a re multiple subunits: they have 2 feet that can move, but the foot is not a simple globular domain like in kinesins, because there are multiple subunits, so they can be much more regulated that kinesines. This protein complex can accept multiple cargos.

We have intermediate chains, that are dimers: 2 genes, it can be Pi. Ligth intermediate chains also can be Pi, are a dimer and controlled by 2 genes. The heavy chains are also dimers.

In time laps analysis, there are spots moving: they are the cargos. We can plate and bound the motors to a glass surface, and then put the MT, giving ATP: plating kinesines, the MT structure moves in a direction in which the barbed end in in the tail; plating the dyneins, the stretch is moving with the barbed end in front

If we make a ligature, we see that kinases accumulate before the ligation; dyneins are in both sides! That's logical, because both these motors have to be produced in the soma, so also dynein is transported to the plus end $\longrightarrow$they are transported in aggregate not to be functional until they reach the + end of the MT. When label in red kinases and in green dyneins, in the anterograde transport are co-localized, but when they move retrogradely they are not. So, the revised model is the following: the trick is to have the kinesine molecule transporting a non-functional dynein structure on the top. In case of dyneins, the steps can be long or short: this depend on the ATP loaded.

Kinesines can be transported back like dyneins are transported to the + end, but it is not clear what occur from the detachment of the cagro from the kinesin and the retrograde transport of kinesin.

In dyneins, the entire structure is moving: the head is composed of ATPases and when ATP is at low level, we have very long step (25 nm); with intermediate load, 15 nm and with a high concentration ATP is 8 nm, probably some quick steps.

Chapter 10

Lesson 10: Presynaptic secretion

Synaptic transmission is a specialized form of normal exocytosis, but the molecule sinvolved are specialized for the synapse. The basic mechanism is the *SNARE hypothesis*: synaptic vesicles have a specific protein that directs them to a receptor on the plasma membrane, with the aid of SNAPs proteins. The vesicles contains v-SNARE and the target the T-SNARE: the V-SNARE is the synaptobrevis and the T-SNARE is represented by a complex of syntaxin and SNAP-25. Vesicles exiting from the Golgi apparatus can be directed to different sites: moving the vesiclec from golgi to specific target is regulated by mutual recognitionbetween the vesicle and the target with ligand-receptor mechanism. The V-SNARE is locatend in the synaptic vesicles membrane and interacts with the T-SNARE: they make a *coiled-coil interaction*. These proteins and these mechanism are high-conserved from yeast to humans: we can find similar mechanisms in the fusion of endosomes, but the proteins are different (VAMP 8, Syntaxin 7 and 8, Vti 1b).

The reason of the coiled-coil structure is that hey work together to bring the 2 plasma membrane (cell and vesicles) to create an hydrophobic environment: the 2 lipid bilayers are initially separated by water, while when the V and T SNARE interact, the water is excluded and the hydrophobic environment favours the fusion of the two membranes. The initial phase is the *emifusion*, then the complete fusion makes the 2 cytoplasms in contact.

The specificity of T-SNARE and V-SNARE is not the only information that allows the specific interaction: if we have secretion and a mutation and all dopamine vesicles are secreted not the the axon ending but to the soma, the mechanism is disrupted. There is a principle of *redundancy* that allows specificity: this is given by *Rab proteins*. THe issue is the selective vesicular traffiting: to reach this selectivity there are SNARE proteins and also Rab proteins. SNARE govern vesicular docking and fusions, that are 2 different steps of the same mechanism. The SNAREs are more involved

in the fusion phase while the Rab proteins are more involved in the docking phase. This mechanism requires energy, given by GTP that is attached to Rab via monomeric GTPases: the presence of GTP allows the first step of docking. At a certain point, the vesicle is released from the cell.

So, in a vesicle we have V-SNARE and Rab+GTP: the first step is docking, and this first interaction is given by Rab-GTP that binds with the Rab receptor. The binding of SNAREs comes after. If the Rab receptor don't recognizes the Rab-GTP, the GTP is hydrolized into GDP and the vesicle is detached. In both cases, when vesicle is wrog or after release and fusion of the plasma membrane, there is the release by hydrolisis of the GTP. Then we have the coiled-coil. Then the problem is: when the vesicle fuses, the V-SNARE and T-SNARE ends u in the same membrane, close to each other: we need to disentangle this interaction, so there is an unwanding, because in the coiled-coil interaction the α-elix are coiling. This requires energy: since this structure doesn't have a mechanism to unwind, we need a molecule that does it. In building the complex, the unidirectionality of the mechanism assure a faithful and stability of the plasma membrane. These molecules can be sent to the lysosome or re-used: in case of recycling, the NSF protein (NRM-sensitive factor, blocked by N-ethylmaleimide) unwind the complex. NSF is atteched to SNAP: after the fusion of the vesicle to the plasma membrane, the NSF complex arrives and the hydrolisys of ATP into ADP+Pi, coupled with this complex, allows the disassembly of SNARE complexes.

If there is a wrong endocytosis to recover the molecule, there are adaptors proteins that bind NSF and unwind the SNAREs.THis situation of SNAREs attached to each other and maybe re-used in a vesicule is dangerous! NSF comples gives a rotation to the SNAREs proteins to disentangle them. ATP, which is in red, provides the energy to turn the NSF complex and the system is released after the hydrolisis of ATP into ADP.

10.1 The role of Ca

AP_{pre} is the presynaptic potential: we have a AP, so a depolaryzation, that a Ca wave and the release of the vesicles (after the Ca spike): measuring the EPSC, we can see the action potential in the post-synaptic temrinal. These things do not happen at the same time, but there is a timing: AP pre, wave of CA, EPSC, AP post. The interesting thing is that these steps do not occur at the same time, so in takes time to do something and between the AP and the release of the vesicle we have docking, fusion ecc that takes time. What is Ca doing?

There are several Ca channels close to the sites where the synaptic vesicles

are docked, bind by the Rab but not fused yet: the fusion occurs a moment after the Ca entering into the cell, so there is a mechanism in order to allow the coiled-coil interaction and the fusion. Up to the arrival Ca everything is frozen at the docking: Ca channels are exactely close to the docking sites, so when these channels open, the highest concentration of Ca is close to the synaptic vesicles. Sometimes this is not sufficient in order to release the vesicles: we need that several receptors are opened at the same time or repeatedly. A single AP in some cases do not provide enough Ca, but if we have a train of AP we can reach the level of Ca that allows the vesicular release: the mechanism that keeps frozen the vesicle has a sensor that is sensitive to a specific level of Ca and below that there won't be any fusion.

The vesicle contains Rab3-GTP for the binding, then trasmitter transporters, a huge molecules that provides the channels trough which the trasmitter is released, a proton pump, synaptin I, synaptobrevin and synaptophysin, and a protein which can bind Ca, the *synaptogamin*. Synaptotagmin has a Ca binding site: synaptotagmin is not present in other vesicles, only in the nervous system. V-Glut is the transporter for Glu, Munc 18 is important also. The most abundand component is *synaptobrevin*, a V-SNARE that protrudes from the surface of the vesicle.

10.1.1 Synaptotagmin

This is a transmembrane protein, has a large cytoplasmic domain (outside of the vesicle!!!): in that part, there is a *situ domain* that is involved in Ca and P binding. These situ domain are 2: the injection of this peptide can block release after the vesicle is docked. Synaptotagmin forms tetramers with each binding of CA: the binding of the fist molecule of Ca favours the formation of tetramers cooperatively. Synaptotagmin can sense different concentration of Ca, expecially from 10 to 100 μM for the release. In the absence of CA, synaptotagmin may serve as a brake for release. Ca removes the brake.

What happens when there is a firts docking and the first interaction between SNAP, V-SNARE and T-SNARE? The first interaction is between Rab3 and RIM (Rab interacting moecule), which is not known yet. Now he vesicle is docked: there is a first step which requires the molecule Munc13. Mutations of Munc13 is involved in autism. There is a first interaction between V and T SNAREs that is very looze: this step is *reclutanty primed*. We need a rigid structure, so synaptotagmin cooperates with the SNARE complex, helping for the coiled-coil: this step is the *fully primed*.

Betheen the looze interaction and the tight interaction there are some steps which are not known yet.

Syntaxin has an additional domain that do not partecpate to the interac-

tion in the first step, that is called *nucleation*[1]: there is an initial part that is in contact and form a rode structure, while orther parts form a looze interaction: this is called *zippering*. The winding promotes the zippering. Then we have the fusion of the vesicle to the plasma membrane, NSF unwind the SNAREs complex and the vesicle bud back.

During the zippering, there are other proteins that contributes to make this complex rigid and stable: one of these proteins is *complexin*, that came at the priming stage II. This does not allow the reversibility of the zippering (the process of nucleation is reversible), because complexin reduces the reversibility creating a more stable complex; Ca is the final signal that induces release and at his arrival, the process becomes totally irreversible.

Once Ca arrives, we have release: we know that synaptotagmin can bind 4 Ca, so there is a certain flexibility in the mechanism: at 100 nM we have a spontaneous release, even in the Ca concentration is low. This spontaneous release is important to keep the synapse alive, like during development. Then Ca rises to $100\mu M$: we have a non-coordinated release. When Ca is $10\mu M$ we have a *sinchronous release* of vesicles.

The fully primed system is the coiled-coil and now is well-formed: we need synaptotagim for the last step of release. When Ca concentration is low, sometimes some release can occur: then 1 CA binds the synatotagmin and we'll have an asyncrhonous release; then 4 moleculebind synaptotagmin and we'll have a synchronous release. We can regulate this further: if we change the isoform of synaptotagmin, there will be able to bind Ca at different concentration. In some cells, synaptotagmin is a dimer, so the range of Ca sensor is bigger.

Munc13 is binding to this structures only when complexin is there: it is one of the main element that gives to this structure more stability by giving an additional twisting.

10.2 Where the synaptic vesicle fusion occurs?

There are active zones that contains RIM, because the vesicle needs to be bound to that molecule, considering that RIM is just in front of the postsynaptic membrane. In these zones, we have docking a fusion and rneurotrasmitter release. There is also the *cytomatrix*, a matrix that looks like the cytoskeleton and is the place in which the vesicles are near the plasma membrane. The *electron dense projection...* Even when the vesicles are not yet interacing with the target membrane, there have transporting ribbon (the cytomatrix) that brings them to the right place. By making 3D reconstruction

[1]The dockingis already done, now we are at the beginning of fusion

we can see that there are filaments that keeps the vesicles togheter, composed by:

- Actin, tubulin, myosin, spectrin etc
- Scaffolding proteins
- Active-zone specific proteins like RIM, Munc13 and Basson, Piccolo, Aczonin and CAST.

Piccolo and Basson came out from the active zone like a spine (?). They connect the cytoskeleton and the plasmamembrane like a bridge: they collect the vesicles and put them in contact with the central ribbon, the point that will be on the way to the right place for the vesicles.

Profilin interacts wth the cytoskeleton (actin) and with basson an piccolo, capturing the vesicles, transporting near Munc13 and Rab3: Rab3 allows the vesicles to move in this passage and the vesicles can interact with different RIM present on the membrane.

Chapter 11

Lesson 11: The postsynaptic density

The postsynaptic density is a specialization of the cytoskeleton at the synaptic junction and faces the active zone of the presynaptic terminal. PSDs have a variety of shapes. It's a dense area that occupies the 10% of the area of the postsynaptic membrane: it contains most of the Glu receptor of the AMPA and NMDA type. One of the most important protein is PSD95. AMPA and NMDA are 2 chemicals that activate 2 different classes of Glu receptor: both receptors respond to Glu, but they have different affinity for Glu, different structures, NMDA is a channel for Ca while AMPA do not, they have different time of reactivity to Glu. THey're both constitute from 4 subunits that change during development. These receptors are activated by AMPA and NMDA respectively. There are other receptors like the dopamine receptors, that can be more in the periphery.

In the PSD there are also *adhesion molecules* that allows to bind to other transmitter receptors located in the presynaptic terminal but also to the matrix. There are also signalling proteins and cytoskeleton: in this way, the efficiency, accurancy and speed of the synapse is increased, because the signal is situated near the presynaptic terminal.

11.1 Geography of PSDs

With the *crioelectronmicroscopy* technique, we use frozen sections of electronmicroscopy and we can do the 3D reconstruction. If we use antibodies against specific molecules, we find vertical filaments inserted into the membrane, that are PSD95 molecules. PSD95 is attached to the plasma mem-

brane in the vertical direction[1]. In blue we have the AMPA receptors and they are associated with PSD95 in red (the green part is the extracellular domain of the recetor). From the bottom, there is a core dinstribution of NMDA receptors in the center while the AMPA receptors surround them: a central region of PSD2 contains 16-25 NMDA receptors and it is surrounded by 30-100 AMPA receptors. This is a *rimbic structure*: the trapping of NMDA in the center is due to the combination of special lipids and the cytoskeleton and the scaffolding proteins, in particular the central part of PSDs is rich in cholesterol (like lipid drafts). Cholesterol is important for memory ant to keep the synapse alive.

We have an additional Glu receptor, the *metabotropic*. These receptors (metabotropic, NMDA and AMPA) are linked by GKAP and by scaffolding proteins that keeps the receptors together, and there are also multiple domains of these proteins. This form a toolbox and work in the same area in the same position. There are 2 ways to have multiple functions in the same place:

- A Multiprotein domain: several proteins with the same function that create subunits of a complex
- If we have many function in multimeric proteins, we can change subunits to change the functions

Here we have multiple subunits: each has its own regulation, but, we want to have them together: for 1 NMDA receptor we want to have 3 AMPA receptor. How to do this, if they are not interacting? We put the scaffolding proteins.

Even the scaffolding proteins need to be regulated: we have PSD95 that brings together NMDA receptors and CaM Kinase II, which is a calmodulin kinase so it's sensitive to Ca: Ca flow trough the NMDA receptor and arrive right to the CaM Kinase. This gives a rapid response, because we'll have a high concentration of Ca at the NMDA receptor, with no dispertion of the signal. PSD95 also has additional connections with a scaffolding protein that brings together 2 different complex (AMPA-receptor complex and NMDA-receptor complex): these proteins are GKAP and Shank.

THe fisrt molecules that we meet are the adhesion molecules, that are heterodimers and are found between the pre and the post synaptic temrinals: heprin, N-caderine, β-neurexin, ephrin receptors and neurolidin (?). THey are importnat to synaptogenesis, to keep the synapse in place and to keep the distance between the pre and the postsynaptic terminals.

[1] In previous studies they hypotisized that it was in a parallel orientation

N-CAM and N-CAderins are in the postsynaptic terminal. On the surface we have NMDA receptors surrounded by AMPA receptors. We can also find K channels and Ca channels. There are additional receptors that do not respond to neurotransmitter, but to nitric oxyde, hormons and growth factors. There are dopamine receptors, neuropeptide receptors: there will be a series of receptors on the surface that sense what is secreted by glial cells and by presynaptic terminal (tripartite synapse).

All these receptors are kept in position by the presence of scaffolding proteins that connect these receptors to signalling molecules: they are very close to the plasma membrane. We finde also nNOS (neuron nitric oxyde synthase) and scaffolding proteins and signalling proteins: the scaffolding proteins have 2 functions:

- Clustering the receptors
- Adapting all the signalling molecules close to the receptors
- Connect all these structures with actin cytoskeleton

Shank is a multiple protein that binds IP3 and the smooth endoplasmic reticulum and connect the NMDA with metamotropic Glu receptors cluster and with actin cytoskeleton. A different type of scaffolding protein connect adhesion molecules to the receptors: GRIP connects the ephrin to the AMPA receptors.

The cortical actin cytoskeleton, the so called *cortex*, contributes to the thickening of the cytoskeleton near the plasmamembrane. Actin contributes also to the all spine (because excitatory synapses are located in spines). Along the F-actin cytoskeleton we can have movement of the molecules thaks to the presence of myosin V, that can also transport vesicles containing the AMPA receptors in the plasma membrane.

11.1.1 PSD95

Lies at the core of the PSDs and has the role of binding the intracellular part of transmitter receptor to the scaffolding proteins. It is known to be a very labile protein, very unstable, that last few hours before it is degraded. This instability is something that is general for our body: most of the parts of our body are constantly rechanged. This happens also at the molecular level in synapses.

Plasmid with a modified version of PSD95 that has a sequence encoding fo a particular type of GFP: this is the *photo activable GFP*, in normal condition is black but upon a flash of light the protein is activadet and become green.

This is the GFP-PSD95. A second plasmid was introduced in the neuron with another fluorescen protein, the m-Cherry: they open a window in the scalp of a mice and put a glass into it, so they can monitor these neurons. A pioneer of these studies is Wen Biao Gan of NYU: he is an expert to observe ehat happens to dendritic spines during time.

read the paper

In adult, spines are highly stable, while expecially 14 days postbirth, the spine sinteracts with the envinorment in order to select the connections that are usable. Multicolor DiOlistic labelling. o They had 2-photon laser placed in the head of mice and the could activate the GFP while the red fluorescent protein was always activated. Spines appear and disappear on daily basis; dendritic spines and PSD are quite stable around the second week. At time 0 there is a photoactivation of GFP-PSD95: 2 spots appear in correspondency to PSDs. After 90 min, the disappearance of the labelling in one synapse and the change of position in another synapse, that was in the neck of the spine. After 1 day the activation was not detectable anymore. It is possible to measure the decay of GFP-PSD95 in individual synapses: there are some synapses which lost the signal in the first 20 min, while in other it takes longer, so we can identify 2 population, one that loose the signal in short time and other that have a longer time decay. Maybe this phenomenon (unstable compared to stable synapses) is due to different developmental stage.

listen the record

In adulthood the spines are more stable and keeps the PSD95 in the scaffold by molecular binding or it's a physical trapping because spines have a smaller neck. SO, they comapred the dynamic of actin and GFP-PSD95 and GFP alone: the diffusion of GFP alone is modulated by the spine structure and PSD-95 is still a question mark. GFP-actin depend on the cycling of actin in dendritic spines.

GFP has a very short retention time because it doesn't bind to anything; actins has an intermediate retention time and PSD95 has the highest retention time.

The retention time is given by interaction with the PSDs: after a certain time the labelling appears in the neighbor synapses that were not labelled at the beginning. The time of disappearance from 1 spines depend in the dimention of the spine: when the PSD is larger, the retention time tends be higher. Is the retention time modulated by synaptic activity? Yes, because in sensory deprivation (cut sensory wiskers), the time of retention was longer (these experiments are done by Mcdiamond is SISSA) for PSD95.

read the paper!!!

11.2 Synaptogenesis

These experiments highlights the fact that synaptogenesis is a dynamic process: it starts during embriogenesis and continues during teen-age time. The brain volume increases during development, and so the number of synapses per neuron. In order to make synapses, the machinery is connected to cell adhesion molecules to spines and contains specialized molecules. There is a need of the pre and post synaptic terminal to exchange information to build the synapse in THAT place. These first contacts are stabilized by adhesion molecules that give the signals for the formation of the clusters: the first cluster is given by scaffolding proteins connected with the receptors, then the formation of non-active synapses containing only scaffolding protein, then receptors are included in PSD formic active synapses.

Before day 12 in vitro, we can see the components of presynaptic density and PSD, but this site is not active, because there are no active receptors in the PSD. In the Pre synaptic terminal, synaptic adhesion molecules give the signal to build the synapse in this place, then the scaffold in guided by the presence of adhesion molecules, the PSD scaffold proteins and the receptors attached into the membrane. The receptors AMPA and NMDA are located in vesicles because they come from the Golgi (they are transmembrane proteins!) so they need to have an exocytosis, but if we have the basis of the forming PSD with the scaffolding protein, is difficult to insert a vesicle! The machinery is given by SNARE proteins: this machinery is located in a different place. The experiment is related to PSD95 and they target the endocitic zones with *clathrin*: the endocytosis and the exocytosis occur outside the PSD95 area, lateral to that area (in red). The mechanism is that vesicles containing the receptors are fused laterally and then the receptors are taken in the PSD zone floating on the plasma membrane. It's a continuous fusion of receptor on one side and elimination to the other side. So, even the receptors, and all the constituents of the PSD are continuosly recycled.

Chapter 12

Lesson 12: Dendritic local synthesis I

(18.11.2015)

Translational control in dendrites for the next three lessons: neurons require translation of a huge number of genes and this occurs in respond to stimuli from the environment, requires activation and deactivation of translation in specific domains and time window, so it is regulated in space, time and quantity. All these informations are important to avoid aberrant products.

Translation is divided into 2 phases:

- Initiation
- Elongation

12.1 Initiation

Initiation is the phase during which the mRNA contacts the ribosomes and there is a large number of initiation factors that are required to assemble the ribosomes and to start translation. Elongation is the phase in which there is a continuous assembly of amminoacid to form the proteins. Eukaryotic ribosomes have 2 subunits: 40 S and 60 S. 40 S binds to the 5' end of the mRNA and starts a movement along the mRNA, that is the *rybosomial scan* until it reaches the initiation site. This site is the AUG codon: in addition to it, we have additional 10 nucleotides that provides the signals for starting translation. This signal has been discovered by Kozak: she discovered these 10 nucleotides around AUG that indicates to the ribosome that this is the site to start translation. This 10 nucleotides can change, so that we can have *high efficient Kozak sequence* or *low efficient Kozak sequence*: this will give

an information for higher grades of translation or lower grades of translation. When the smaller subunits arrives at the start codon, the large subunit will attach and then form the complete ribosome.

The first step is that the small ribosomal subunit binds to the 5' cap, the starts migrate until it reaches the initiation site, this is called *ribosomal scan*. If the 5' is very long, it will take longer time for the smaller subunit to scan it, if they are very short the ribosomes will arrive soon. If this is long, many subunits will arrive and it will form a *ribosomal queue*. The small subunit is not sufficient alone start the initiation phase of translation: there are additional proteins called *initiation factors*, required for all stages of the initiation, including the binding of the firs tRNA and the binding to the RNA, also for the movement along the mRNA.

The *pre-initiation complex* is formed before the ribosome is attached to the mRNA. The first tRNA contains the Met, which is inserted in correspondence to AUG, then the eIF2, 1a, 1 and 3: they are initiation factors that partecipate to the pre-initiation complex. This complex need to encounter the mRNA and the mRNA needs to be prepared to receive it. There is a complex on the RNA which binds to the cap, that has methylated nucleotides. On the top we have another proteins, the eIF4 A, B, E, G.

Every time we have a complex $\longrightarrow$regulation. If we have so many proteins in the complex and the RNA it means that this process needs to be regulated, because we don't want an automatic translation of every RNA present in the cytoplasm.

eIF2 binds the initiation Met tRNA and forms a ternary complex that associates with the ribosome. Each protein recognizes different component, so a complex is facilitated in binding a structure. These processes need energy and the conversion of eIF2, that is a GTPase, and eIF2B is the exchange factor that exchange GTP with GDP.

Let's go on the RNA side: the 5'cap is made of guanine methylated in position 7 and is a signal for binding the entire complex, the *eIF4 family complex* made by a scaffold protein, the eIF4G, one another protein that is initally bind to eIF4G and is responsible for the binding to the RNA, while other 2 components, eIF4A and eIF4B are involved in folding of the RNA by helicases, that open the unwanted 3D structures. This complex is the G, A, B, E: it is collecting eIF4 and it is known as *eIF4F complex*. This complex (G, A and E subunits initially) binds to the 5' cap: the A subunits sense the presence of the mRNA and recruits by consumption ATP an additional helicase that is the B subunits. We can introduce regulatory mechanism and the main one is Pi: eIF4E, which binds the cap,can be Pi. eIF4E can be bound by specific proteins, *4E binding proteins*. If eIF4E is trapped by a binding protein, this will prevent the possibility to bind the cap. How can

we regulate this?

Through the Pi of the 4E binding protein. We have the scaffold protein 4G, which is the helicase, that can bind the 4E protein; if 4E is seqestrated by the binding proteins there won't be the formation of the *eIF4F complex*. What regulates this? A simple Pi. When the 4E binding protein is Pi, it cannot bind 4E, so this makes the protein inactive so that the 4E is free to bind and partecipate to the eIF4F complex. 4E binding proteins has different Pi sites and the signalling cascade is now known: there are hormons, neurofractors etc that activates some kinases. There is one effector that is called *mTOR*, the mammalian target of rapamycin which is an antibiotic that can block protein translation at this level. mTOR can Pi the 4E binding protein: when it is Pi, it releases 4E that can partecipate to the initiation complex and start the translation. If the kinase is blocked, it cannot Pi the 4E binding protein, which remains attached. We also need several Pi to to shut off 4E binding protein: this is an additional way to regulate the process. The different Pi sites can be Pi also by different kinases, so we can have additional complicancy.

Pi of the eIF4E binding protein is plural: another step that regulates the initiation step is the binding of the binding of the polyadenilate binding protein (PABP) to eIF4G, the scaffolding protein. There is also another step which is the Pi of eIF4E itself: this allows the detachment from the initiation complex. So, the steps are:

- Pi of eIF4E binding proteins;
- Binding of polyadenilate bindinf proteinsto eIF4G;
- Pi of eIF4E itself.

Now we are in the situation in which the 4G scaffolding protein is present and 4E is already attached, then we want to detach it: there is one kinase, Mnk, that is activated by a signalling cascade that involves MEK and ERk and also by stress, and is able to interact with eIF4G; in addition, it Pi specifically 4E. When 4E is Pi by this kinase, it detaches from scaffolding 4G and is recycled. So, after the pre-initiation complex is formed, it is possible to Pi 4E to detach and recycle it.

Considering that we are at the 5', why are we also talking about the polyA, which is on the other side of the mRNA? Because in many case, the RNAs which udergoes translation are circlerized forming the polysome. This situation is due to the interaction between the cap, in particular eIF4G that is able to bind the poliA binding protein: in order to be able to bind to poliA, poliA must exist! One of the step which is fundamental during translation is

also the *elongation of the poliA*, otherwise the PABP is not able to bind. So, at the beginning there is a eIF4E binding protein that captures the 4E, so there is no pre.initiation complex; then Pi of the binding protein that releases 4E, which arrives on the 4G $\longrightarrow$formation of the pre-initiation complex and there must be a signal to say that the 5' prime is ready and it's time to elongate the RNA. Is there any trick in order to make the communication simple? The signal is given by the fact that by folding structures at the 5' is possible to continue inducing the formation of rybosomes. The signal is given by the 4E binding protein itself, because also the 4E binding proteins are multi-domain proteins: on one side they bind to the 4E, on the other side they bind to proteins at the 5'. There are 2 tyoes of 4E binding proteins:

- Those which are free to float in the cytoplasm and that prevent any interaction of the 4E with any other RNAs;
- Those which can bind to the 4E when the 4E is already attached to the methylated cap.

In this way, they exclude from the pre-initiation complex the 4G: this is an entire class of different molecules and when they are Pi they release the 4E so that 4G can be attached. On the other side, there is a signal that allows the attachment of the poly-A polimerase, that elongates the poliA and allows the binding of the poliA binding protein.

Let's consider one specific case: there is one case which has been found to give one of the major mechanism occuring in neurons. In dividing cells in S-phase, there is a blockage of the translation: eIF4E is bound to the cap, then there is one protein that has the role of 4E BP that prevents the bind of 4G: this protein that blocks the 4E is called *maskin*,[1] is a multi-domain protein that is also binding another protein, the *cytoplasmic polyadenilation element binding protein* (CPEBP). The CPE is the signal for poliA polimerase to start polyadenilation: it is a small sequence in the RNA which precedes the starting sequence for poliA polimerase binding. The binding of CPE binding protein block the binding of the polimerase in that site. So, we have a double blockade: the 3' is blocked and also the 5'. These configurations are called *translationally silenced RNAs*.

What happens when maskin and CPEBP are Pi? We have the attachment of CPSF (cytoplasmi poliadenilation soluble factor), which binds to the short sequence of 6 nucleotides and this is the signal that allows the poli-A polymerase factor to do its job. When it starts to elongate the poliA, then it's possible for the poliA binding protein to be attached. On the other side, the

[1] It masks the 4E preventing the bind of 4G.

4E which is now free to bind to the 4g starts to form the initiation complex with the small subinit of the rybosomes and 4G can interact with the poli-A binding protein. The 2 mechanism coexist and start at the same time, so there is a coordination between the stabilization of the cap and of the poli-A. Stabilization because the presence of the rpotected cap and the elongated poli-A is the signal that this RNA is necessary and it's not degraded, so they *protect RNA from degradation.*

Maskin is able to block translation of the RNA making it silet for translation, by binding at the same time to the 4E and preventing attachment; on the other side, it binds to CPEBP preventing the fomration of the poli-A. How do we switch from the 2 situations? In the 3' UTR we have the CPE sequence and a kinase, the *Aurora* kinase, that can be disregulated in cancer and is able to Pi CPEBP: there is a conformational change and maskin[2] displaces from binding 4E and 4E is now free to interact with 4G and to the 40S pre-initiation complex.

There are 2 classes of 4E binding proteins: there is a soluble type (4E-BP1) that is Pi by Erc/m-Tor mechanism, so in this we have a translationally silent RNA which contains the complex that prevents translation, but 4E is already bind to the cap; in m-TOR regulation, 4E is completely sequestered by 4E-BP1 so that is no more able to bind to the cap. This situation however may have a normal poli-A, because the poli-A polymerase is not prevented to bind. This is a general translation inhibitor. So, Maskin binds only the RNAs that contains the CPEBP: the RNA that don't have in the 3'UTR the CPE sequence cannot undergo this mechanism (specific inhibition of translation) and when 4E is no more available, the general transcription is blocked.

If I don't have the CPE sequence, can I still have a specific regulation of translation? I can change the sequence, the binding partners and there is an entire family of 4e-BP: in yellow we see the 4E and then different BP.

- 4G gives the active translation condition;
- 4E-BP prevents the binding to the cap and there will not be translation;
- Maskin block the 4E through the CPEBP;
- The last mechanism is the one in which maskin is replaced by *cup* and instead of CPEBP there is *Bruno*;
- CYFP1 that binds to the RNa through a non-coding adaptor RNA (BC1).

[2]Maskin is binding at the same time CPEBP, which binds the CPE sequence, and 4E, which bind the cap.

We may have CPB1 in one RNA, Bruno in another one, FMRP in another one, so we have these RNA responding to different signals. I can put under the CPEBP element all the RNAs important for excitatory synapse, while I put under the Bruno all the RNAs which are important for an inhibitory synapse or for the pre-synaptic terminal, so I can have translation at the same time of the all pool of mRNA that makes a certain protein: we get translation of an mRNA belonging to a certain cellular program. There are many mRNA belonging to PSD that are regulated with the same mechanism and undergo translation together. There are also other mechanisms: these are the one related to the regulation of the 3'UTR region, but other mechanism involve the 5' UTR: the *top sequence*, typically very rapid in translation and regulate all the translation factors we have seen above. TF have a top sequence that tells the cell to translate first the TFs.

There are more compliancies: two RNAs that have the eIF4E and CPEB connected through Maskin, which is also connected with TOG: TOG binds to other RNA binding proteins. When CPEB is Pi, Maskin releases the 5' and the eIF4E, but is not yet activating the poliA site: there is an additional step which is the movement of the all complex using the anchoring of the hnRNP that put the terminal part of the 3' in conjunction with the CPEB, although the CPEB is in the middle of the sequence. Once the 5' is released, there is the possibility to interact with the 3' to reconstruct the complex and this will allow the poliA.

what is the role of MBP in this case?

12.1.1 Assembly of the pre-initiation complex

There is another factor, eIF2 which carries the Met aa. There is GTP: the GDP-GTP exchange on eIF2 is the mechanism to control translation. eIF2 is inhibited by the Pi and Pi of eIF2 is one of the mechanism that the cell has to block general translation.[3]

The 2 complexes start to interact: the interaction is such that we have the connection between the pre-initiation complex and the 4G. The helicases are close to the basis of the ribosomes: the pre-initiation complex travels along the 5' and arrives at the start codon and recognizes the interaction between the eIF2-GTP complex and tRNA complex. GTP is important for each aa that is incorporated for elongation. GTP in this case is important for the complex with the large subunit.

Then there is the usage of the fist GTP to attach the fist Met and then there are the involvement of the eF, the *elongation factors* which use GTP to elongate and incorporate aa.

[3] Another is to have a soluble 4EBP which prevents binding to the cap.

4E can be highly regulated: once this blockage has been released, there is a scanning of the RNA until the ribosome reaches the start codon. eIF2 is Pi and this is a general control of translation.

Chapter 13

Lesson 13: Dendritic protein synthesis II

(19.11.2015)

Neurons have to be very rapid to respond to stimuli, but they are also highly polarized cells: in some cases, inputs due to anatomical distribution can be very separated to each other, *segregation of synaptic inputs*. Neurons must discriminate what happens at different synapses: when to potentiate one synapse or when to decrease the efficacy of another cell. In axons, there is need to transport proteins at very long distances: in some cases is sufficient to transport them along microtubules but in other case it might be necessary to degrade the proteins in axons. Is there a synthetic machinery for proteins in dendrites? Yes.

The first study came from Stuart, who was using electron microscopy and discovered that there were some pictures who showed the presence of some ribosomes close to synapses (and it was considered an artifact). Stuart repeated the experiments and find the presence of *rosettes* at the basis of the spines. This was also repeated later and brought a 3D reconstruction of a spine: inside, there is a huge number of ribosomes. If we want to have proteins synthesis, we need also tRNA, elongation factors, RNA. tRNA and all the translational partners are present comes some years later with radioactive in-situ hybridization in cultured neurons.

Another issue is related to transmembrane proteins: there is a big debate because it has been impossible to show the presence to rough endoplasmic reticulum, but we can find smooth endoplasmic reticulum in the dendrites.

For glycosilation of proteins in the golgi, there is no evidence of on-going glycosilation or the presence of specific markers of the golgi apparatus. A marker for golgi apparatus is GM130: using Ab against the trans-golgi network, we can see that the large majority of the golgi cisternae is present close

to the soma, but there are some small cisternae that can go far away and move very nicely. GM130 is present in the soma, proximal dendrites and increase upon stimulation of the excitatory synapses; the central cisternae remain close to the soma. We see a huge increase of trans-golgi network in stimulated dendrite. It seems that the GA in dendrites is kind of disassembled. if we consider the hypothesis of golgi-maturation, we don't need to have cisternae one on top of the other. In the spines we may have only the more mature trans-golgi network cisternae moving: the less mature are in the proximal part but then move to mature in the distl part of the dendrites, because the small cisternae are able to travel.

Neurons do not only produces proteins in cell soma, but also in dendrites and, in some cases, in axons. It is hard to find rough endoplasmic reticulum. Trans golgi network can move fast in the dendrites. Using confocal microscopy, we can reconstruct these structures.

Golgi cisterne are impilate in the first part of the dendrite until the branching point. When there is high synaptic activity in one part of the dendritic arborization, the machinery for translation can be moved there (floating trans golgi network). We need the RNA: thanks to Oswald Stewart it was discovered that also the mRNA can be targeted in dendrites. He stimulated in the entorinal cortex some fibers that makes synapse in the hyppocampus, in particular in the dentate girus: this medial perforant pathway arrives in the center the lateral perforant pathway arrives in the outer molecular layer. *Arc* protein is a cytoskeletal protein that is produces only when neurons are stimulated , is an immediate early genes IEG, which mRNA are rapidly degradate. It take 30-40 min to translate them. We can label that and follow the mRNA into the brain. Stimulating the medial perforant pathway, we can see that not the entire lenght of dendrite is label: the labelling stops in the medial cortex, not in the outer molecular layer. Analyzing the intensity of these area, we see a pic in the medial layer, so after the strong stimulus the mRNA is produced in the nucleus and move through the synapse zone.

We can see the time course: after 2 hours full accumulation in the activated lamina.

What happens if the stimulus is not specific to a given synapse but for the entire arborization? The mRNA should fill the entire arborization. The mRNA is the same, but the stimulus is different.

13.1 RNA localization

There are high conserved mechanism, in particular they have been studied in Drosophyla. We want to have, by moving RNA in a specific place, that translation occurs only in that place: it is important to have a protein in that part and no in other parts where it would be dangerous. For example, the localization of maternal egg in Drosophila egg or in Xenopus ovocites. The edge of growth cones is filled of rybosomes and when the right signal arrives, there is translation. If you consider that there are very complex neurons, dendrites can represent the 90% of the entire cell! We may want that some proteins that are able to compact the myelin are not expressed at this stage, but at laters stages, only in other part of the cells where the myelin formation has already occured. Tere are granules transported along the microtubules that are only translated in myelin.

Erin Schuman from the Max Plank Institut, by sequencing and mass spectrometry, discovered that the number of mRNA in dendrites is very huge!

It is possible to follow individual granules: the red one is simply oscillating around one point (stationary granules), the blue one is moving only in the primary dendrites while the green one enters in the lateral dendrites. The 25% are actively moving while the rest is stationary granules, some other are making giant moves, because they move and stop and then move again.

13.2 Intracellular signals that regulate translation

Ribosome and RNAs are located close to the PSD: when we have a nice spine and the intracellular signaling cascade is activated, we need the machinery to be placed at the rite time and place. Many RNAs are produces in situation in which they cannot be translated, because it's the synapse that gives the signal. ERK and eIF4E-BP target the eIF4 that target the methylated G-cap. We can put these pathways in relation with the receptor on the surface, that is Trk receptor, that is a sensor for grow factors, like NGF and BDNF. It is possible to modulate the cap-dependent initiation phase. PiP3 regulates a signal mediated by Ca: it activate Ca calmodulin and, one activated, it regulates the signaling of eIF2, an elongation factor, and also the initiation with a factor that binds to the first tRNA containing the Met amminoacid, forming a complex with the ribosome. By activating just one receptor on the surface, the Trk receptor, we can modulate eIF2 and also the 4E binding protein: we can regulate the initiation (eIF4E) and the elongation (eIF2).

Another signalling pathway is related to the 5 top RNAs: all the RNAs encoding for translation factors and rybosomal proteins are called *top RNAs*. Other signalling pathways are able to regulate signals involving the 3': in similar mechanism activated by Glu receptors.

There is a series of other mechanism that involve the presence of micro-RNAs.

The first limiting step in protein translation is the formation of the cap complex, that requires that eIF4E is bound to mRNA: the problem is the presence of the 4E binding protein, that is bound to the 4E and only when is phosphorilated leaves the 4E to make it free to bind the mRNA. m-TOR is responsable for the phosphorylation. When Mink1 arrives 4E leaves the mRNA because fosforilated. This signaling cascade is linked to the membrane thanks to TrkB receptor.

There is another possibility to prevent translation while the eIF4E is bound: there are some 4E binding proteins like *bruno*, fmrp that connects the 5' and 3', blocking the translation. These binding proteins respond to different cascades:

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- Respond to NMDA receptors and the cascade phosphorylate the Aurora protein
- Metabotropic receptors for Glu control the binding proteins mediated to FMRP protein

13.2.1 Signalling cascades that influences translation of mRNAs

mGluR and homer: homer is a scaffolding protein that connect the receptor with the PI3K, that is attached to the PIP2: this complex transfort PIP2 into PIP3 and this activates the cascade of m-TOR. m-TOR phosphorilate 4E-BP2 and eIF4E can interact with eIF4G activating the translation. ERK activates the Mink1 pathway that block the translation. How can the system know which pathway activate by the same receptor? If the PI3K is no more available, the other one pathway will be activated. While the receptor can activate the signalling cascade, the cascade may not be there or ready to start!

This signalling cascade can move. The PIP3 cascade is ore stable so it is the favored one. The PI3K is located in the membrane and converts PIP2 into PIP3. PIP3 is linked to PDK1/2 and Akt, that inactivates the complex TSC1 and TSC2, the tuberous sclerosis complex: a mutation in these 2 genes causes multiple sclerosis. These 2 genes activates m-TOR that,

binding Raptor, can activate translation after the formation of the complex with raptor. S6K is activated and is able to phosphorylate the S6 protein of the ribosomes. mTOR is phosphorylating 4E BP2 in the previous pathway switching off translation, while here activates it activating translation. So, the interaction of m-TOR with the tuberous sclerosis complex determines the activation of transcription.

There are regulations in 5' and 3' because some elements influence the transltion, like hairpin, IRES structure and some sequence that have a trans-acting regulatory factors; we may also have some ORF reduced translation of the main ORF, that are called *uORF* $\longrightarrow$they can have a weak kozak sequence or a really strong one; then some sequences at the 3' that can bind some regulatory factors. uORF means upstream ORF, they slow down the scanning of the ribosomes: if the ribosome find a sequence of uORF, if the sequence is weak this will slow down the ribosome, while if it is strong, there will be a first translation. In order to get to the real ORF, we could have this kind of waste of material.

There are 3 mechanism of regulation:

- Steric blockage: IREs mechanism
- Sequestration of eIF4E
- Cap-indipendent inhibition interference with ribosome scanning

IREs mechanism

IRE binding protein: IRE is present in a protein that is ferritin. Mutations in this gene are able to causes the hyperferritenemia-catarac syndrome. Ferritin is involved in regulation of iron, which is important for respiration (hemoglobin). In the cytoplasm of the cells there is an iron pool which is bound to a protein, the *iron regulatory protein*, that is closed when bound to iron and in such state activates the translation of ferritin gene. When iron is low, this iron regulatory protein opens and it is able to bind to the IRE-stem loop of the ferritin: this blocks the transcription. The stem loop structure, when it is not bound to the regulatory protein, can be unwind by helicases and the ribosomes can scan the sequence; when the protein is bound, helicases cannot unwind that sequence and the ribosomes cannot scan it.

The block of RNA can be achieved also in another way: the fragile-X syndrome protein binds a structure, the g-quartet and FMRP binds this structure, blocking the translation.

IRES means internal ribosomial entry site: viruses enter into the cells and inhibit global translation, but since they have IRES they make a shift of the translation from a cap dependento mode to a IRES depdendent mode. Some initiation factors are reclutated by ITAF, IRES trans acting factor that bypasses the cap-dependent translation, THis can be found in many neuronasl RNA, expecially those that are transported to synapses: these RNA can be translated even when the global translation is repressed. Bcl2 is the gene for apopthosis: we are in cellular stress and we have a repression of global translation, but Bcl2 survives because of these IRES sequence.

registrazione

There are RNA that contains TOP sequence (terminal oligo-pyridine). m-TOR activates S6 kinase and phosphorylate S6 that can attach the sequence in respecting the cap-initiation complex. In this case, S6 phosphorylated ribosomes don't need cap-initiation complex, but binds directly the initiation sequence.

During evolutio $\longrightarrow$progressive elongation of 3' translating region, while 5' is the same: in the 3' there are many sequence that interacts with specific RNA binding proteins, not global one. The main regulatory global mechanisms are related to mRNA stability (elav binding sites causes instability of mRNA so it can be degraded), the rest are specific mechanisms.

Chapter 14

Lesson 14: The ubiquitin proteosome pathway

Protein degradation has 2 roles: prevents that misfolded proteins are involved in several processes and these proteins have functions that are not required for the cell; the second is to limit the availability of the protein in order to reduce its activity.

The turnover of protein is not costant: the half-life vary from minutes to years. There are short lived proteins, life TF, regulatory proteins, and long lived proteins, like those in the crystallin of the eyes. THe time of half life is the time required to degradate 50% of the proteins. Protein degradation may depend on tissue distribution. This is a regulated process, for example Acetil-CoA carboxylase half life depends on the nutritional state.

Not all intracellular proteins are degradates, because of lysosomal process. The proteosomes are composed by large multiprotein complexes and are degrades ubiquinated proteins. In lysosomes. we have an un-selective degradation, or selective for proteins that have a special aa sequence, triggered by cell starvation.

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Heat shock proteins can help refolding the proteins and, when the proteins are uncorrectly folded, they are degradated. the degradation occurs in:

- Lysonomic extracellular protein degradation of proteins contained in vesicles, occur in the lysosome
- Cytosolic proteins degradation: occurs in the protosome

Lysosomes contain many degradative enzymes.

The proteasome is a structure made by several subunits: the poli-ubiquitin chain of the protein is recognized by the proteasome. The need of ubiquitin is given by the fact than more that 80% of the proteins are not correctly

folded and chaperonine are not enough. Proteins undergo the removal of oligosaccharides, catalysed by N-glycanase. The deglicosination is followed by the ubiquitination: there is a very precise mechanism to extrude these proteins from the RE to the cytoplasm:

- Through vesicular transport
- From the RE to the cytoplasm

Ubiquitin is a small protein of 67 aa, is very stable because of its compact globular structure: there are 3 α-helices and 4 β-sheets. The aa sequence is highly conserved. The proteosome consist of a central cilinder with a cavity inside and is formed by multiple subunit: the cilinder is formed by 4 multimeric proteins. The cavity is were the proteins are degraded. THe initial and the terminal part: in the initial part the protein is inserted because it recognized the ubiquitin, while in the terminal part there is the exit of little peptides. The central cylinder is composed by 2 α and 2 β subunits: this central part is a huge aggregate of proteolytic proteins.

14.1 The ubiquitin pathway

Achieve the attachment of several ubiquitin molecules to a protein. Degradation is not the only function. Ubiquitination is catalyzed by 3 families of enzymes:

- E1: activating enzymes
- E2: conjugating enzymes
- E3: ligating enzymes

Ubiquitin is activated by enzyme E1, then it is transferred to several types of E2 and finally we have the ligation with E3: the protein that will be degraded receive the ubiquitin with an *isopeptide bond*. There is the phenomenon of poli-ubiquitination with E4 that elongates the ubiquitin chain.

E1 It can form the thio-ester bond and associates with E2 enzyme. Is can be modulated by adenilation. E1 activates the C-ter of ubiquitin and force and acyl-adenilate intermediate, then it transfers the ubquitin to the E2 enzymes and after the release of E2 the cycle can start again.

E2 Bring the ubiquitin from E1 to the substrate: it is the active pocket. In bleu we see the ligase which contains 2 hydrogen bonds that make weak interaction of the corbonyl group of E2, so these double bond to O of the carbonyl group become one and now this molecule is able to interact with the N-term of the substrate.

E3 Ligase, can recognize the final target, there are 3 ways:

- Specific pocket in E3
- Phosphorylation of the target
- Ancillary proteins that plays as an adaptor

There are 2 categories of E3 ligases: RING and HECT.

E4 It is involved in catalyzing the poli-ubiquitination.

There is also a process called *deubiquitination* that require some proteases, the UBP (ubiquitin processing enzymes) and UBH. The difference is that UBP eliminated Ub from polu-ubiq proteins, while UBH regenerates monomeric Ub.

Poli-ubiquitination is important for degradation, but also the mono-ubiquitination, because it is responsible for transcription, histone function, endocytosis and membrane trafficking. Ubiquitinated proteins can occur in just one sequence of the protein or in many. In the main signal for degradation, we have a stretch of linear poli-ubiquitin. Different lisines can be recognized by different enzymes: K11 and K29 are recognised by E2, while K63 is important for the ligase TRAF, involved in many tumor and apoptotic processes. These lisines can be targeted in many part of the cell after the ubiquitination. Ubiquitin is attached to many lisines: the presence of these lisines in the N-term of the proteins gives the possibility to the degradation or to go in different parts of the cell.

14.1.1 Degradation pathway

The degradation is achieved in the 26S proteasome. The core of the proteasomes if formed by octameres of α-subunits and β-subunits. In the central part, the β-subunits are proteins that have a catalytic activity; there are also hydrophobic aa, in particular the β-5 subunit has a trypsin activity, β-1 is a caspase-like subunit that recognize acid aa; β-2 recognsines basic and positive charged aa, is a trypsin like subunit.

14.1.2 Deubiquitination

Ubiquitin can also be recycled: there are some specific enzymes that remove ubiquitin. There is a possibility to remove and edit ubiquitin. Ubiquitination do not occur only in the proteasome, but it can also take place before that proteins go to the proteasome.

14.2 Pathological implication of defective ubiquitin-proteasome pathway

E3 enzyme is involved in Parkinson disease and also in Angelman sindrome, that affects children in movements and mental retardation. Ataxin-1, when is mutated, cannot unwind when arrives to the proteasome, so it blocks the proteasome and cannot be degraded.

In neurons, the proteasome is found in the pre and post synaptic terminals: it is involved in the internalization of the receptor in vesicles (when the ligand is present), for degradation or for multi-vesicular bodies which are transported in retrograde trafficking at the soma.

The proteasome degradate multi-ubiquitin proteins: Ub is involved in synaptic growth, synapse developmend, vesicle dynamic and neurotransmitter release in the pre-synaptic termina, while in the post-synaptic it is involved in spine mantainance, downstream signalling. There are E3 ligases for Ub.

When there is an overexpression of E3, this accelerates the degradation. The degradation process can be also affected by a mutation in ne enzyme or in the protein itself, so this will stop the degradation.

Oncoproteins, all substrates of U-P pathways, tumor suppressor genes like p53 or p27 can be involved in these diseases. There is also the formation of inclusion bodies, some insoluble aggregates that cannot be eliminated by the immune system and can be toxic.

In Angelman syndrome, there is a defective synthesis of gene coding for E3 ligase. UBE3A promotes the degradation of ARC, a protein accumulated in spines that is important for the formation of new synapses: it is an immediate gene, so undergoes very rapid production and degradation cycles, therefore there is a need to a continuous degradation of ARC, because an accumulation of ARC cannot remodel the synapse.

In cystic fibrosis the gene encoding for CFTR (Cl channels) is mutated and there is a strong activation of protein degradation that completely eliminates the receptor from the surface.

In Parkinson disease, in one group of patients there are mutations in

parkin that causes the up-regulation of many genes: a group of proteins in the PSD are disregulated. These proteins include PSD95, homer, glu receptors, GKAP, shank etc, involved in the formation of PSD. Most of the enzymes are located everywhere besides the E3 enzyme. Treatments need to be given for short time to avoid toxicity.

Chapter 15

Lesson 15: Mechanisms of control of mRNA

All the translational machinery is present in dendrites. RNAs, when transcribed, are forming the primary RNA transctript that in the nucleus undergo the splicing, the cap and the poliA: these steps are essential to protect the RNA from degradation. The mRNA can be transported, translated or degraded.

ZBP1 and the proteins are trnasported in the growth cone. How this can be achieved?

- Local protein synthesis: when there s a change in the plasticity if synapses, this need proteins. mRNA can be localized in proximity of the synapsis and the translation occurs only in active synapse
- Synapse-specific targeting: mRNA is in the nucleus and proteins are synthesized there and have to be transported in the synapses that are active.
- Synapse-specific capture or marking: synthesis in the nucleus, then diffusion and proteins are captured by the synapses

We think that the first model and the second are both valid.

15.1 RNA localization

Sorting of mRNA to subcellular domains is an evolutionary conserved mechanism. All the RNA that are located in wrong position are degraded and there is an active directional transport on cytoskeletal elements. We can also

have a random cytoplasmatic diffusion and trapping. In neurons the major one is the active directional transport on cytoskeletal elements

In Xenopus oocites there is the *mitochondral cloud*, a system that is coupled to METRO that contains material for the animal pole of the oocite. Another mechanism for the localization of β-actin is still active directional transport on cytoskeletal elements.

Different parts of the mRNA contain information for its own regulation: many of these information are for the translational control at the 3'. To exploit these functions, the majority of these informations require the binding of specific proteins which recognize either some linear sequences (zip) either 3D RNA structures: we call them *Cis-elements*. In some cases, these binding factors can have as co-factors some non-coding RNA. The interaction between RNA and proteins occurs in the nucleus and it is important to export the mRNA out of the nucleus: these proteins start to arrive during the translation. One family is the hnRNP (heterogeneous nuclear ribo-nuclear proteins). These studies have been done in yeast and there are 2 principles conserved:

- mRNA are translational repressed during transport
- Repression is relieved at specific cellular location and specific signal

One mechanism of translational repression and transporting has been studied is ASH1 yeast. In the initial step, Khd1 is bound to the mRNA and this occupy the site of 40S subunit: only when it is removed, the translation can occur. Then we have Puff6 that is close to the poliA site: it prevents the assembly of the full ribosome by blocking the interaction with 60S subunit. The two proteins are present at the same time: the trasport is made by the attachmento of She2 to a motor protein along an actin filament. The Pi of Kdh1 and Puff6 release the mRNA and the translation can occur.

15.1.1 How mRNA are transported in neurons?

We can see RNA granules in oligoendrocytes: they contains the mRNA for myelin basic protein. This protein must be active only when needed. These granules are attached to microtubules in oligodendrocytes, tha tin culture have dendrites like neurons.

These granules are also found in neurons: we can see them like a pearl necklace. The RNA are completely covered. The diameter of these granules may vary: some granules are very big (1 micron) and other are very small (0.1 micron) $\longrightarrow$maybe there is a correlation between the size of the mRNA and the size of the granule. In dendrites initially we have mRNA with mixed

polarity, while in the axon mRNAs have all the same polarity. In dendrites, also kinases can go in both direction: once they arrive close to the synaptic spine, the microtubule don't enter in the spine $\longrightarrow$change by MT-based trafficking to actin-based trafficking.

Some kinesins are specialized to transport RNA granules, in particular Kif5 and Kif2. There were several attempts to purify these granules with 2 approaches:

- Classical biochemistry approach based on salis (chromatography): not so good because we can have mixed granules, like ribosomes, RNA transporting granules, stress bodies
- Immunoaffinity precipitation: RNA transporting granules are transported selectively by one kinesin, so they used an antibody for that protein, run mass spectrometry and find a list of protein which contain hnRNP, staufen and FMR1 (involved in trafficking and blocking translation), then EF factors, ribosomal proteins

These granules are a short program: they contain the information to make a synapse, like the receptors, the cytoskeleton, a PSD for the excitatory synapse. They contain multiple RNAs for the same program. different granules contain different RNA binding proteins.

15.2 Fragile X syndrome

Overproduction of certain proteins that causes a higher number of immature spines: there is no the blocking of mRNA translation because FMRP is lacking. FMRP is a RNA binding protein containing 2 central domains KH, which are involved in RNA binding. FMRP have several targets that can interact with a poliG sequence or a structured RNA which takes the form of G quadruplets. Some RNA targets are GluR mRNA etc. Deletion of mutations in FMRP decrease transport of mRNAs into dendrites. It appears to block/decrease rates of dendritic mRNA translation and it binds to multiple target. One of the principal in this course is that *shape is involved in function* and *we can't consider a process isolated from the others*: RNA continuously shuttle from one granule to another, depending on the situation.

RNA binding proteins are involved in stability, trafficking, translational control and localization in synapse. This circular effect might be the called *sushi belt model*: mRNA can also go back and circulate within the dendrites and in some situations they can stops in activated synapse or in degradation

centers (depending on the signal). The can jump from te MT to the actin cytoskeleton or a small stretch of MT can enter into the spine if the spine is big enough.

Another example of dynamic MT is in the growth cone: the MT were stabilizing the fillopodia, bringing RNA for stabilization.

In the axonal compartment, there are not ribosomes but there is RNA. There is a local synthesis of proteins in the axon and 1000 different mRNA. In dendrites, 3000 mRNA encoding for gene for plasticity and housekeeping genes.

15.3 Neurotrophins

BDNF, NGF, NT4, NT3 are growth factors (discovered by Rita Levi Montalcini). BDNF is expressed in all neurons and under certain conditions, like after epileptogenetic .., the mRNA move in another part of the dendrite. RNA granules od BDNF can be actively moving or not.

Branching point is the region in which AG is accumulated: there is a stack of vesicles there. Upon depolarization, BDNF mRNA is translated expecially at the branching points, because they contain the machinery for translation. This are the *hotspot for local protein translation*. Position, dimension and localization have a function in the cell.

15.3.1 BDNF

By studying BDNF we can study many processes in the cells. 11 different 5'UTR region, one CDS and 2 different 3'UTR from the same proteins by different splicing. Cloned the 5' with the CDS , or the CDS alone or the 3' alone; then transfected all the different RNAs into the neurons and got a surprise: some of the 5' brought the RNA in the distal part of the dendrite, other only in the soma, but the CDS alone was completely far away from the dendrite! Right in the middle of the CDS there is a signal for binding a protein, *translin*. Then the RNA binding protein site (for translin) is the site where is human is a polymorphism: we have 2 nucleotides that produces Val-Met polymorphism. Transfected the human wt (val) and human with Met: the met one cannot go in the dendrites. Human beings having this polymorphism have deficits in memory and they are more prone to have diseases like Alzheimer's disease and post-traumatic stress disorder.

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A modelling of translin: there is a specific pocket in each of the monomeres that is bound to a G and there is a tandem repeat containing 2 G: G_{196} (site of human polymorphism) and G_{178}. The space is too narrow, so how it

is possible? In that area, there is a perfect Watson and Crick pairing for the RNA and this is highly conserved throughout evolution (in human 1 aa is mutated). Mutating the other gene, it's the same thing: suppression of trafficking.

RNAs differ from each other because of the alternative splicing in the 5'UTR and the presence of 2 different 3'UTR: alternative splicing and alternative polyadenilation signal. We have the coding of a part which is inside a single exons. This generates a series of RNA and it was unknown why this protein required so many transcripts in order to make just one protein. The work of the lab was to try to find different functions for this variance, in particular we studied the subcellular localization. We started doing analysis of different exons: some of these transcripts remain in the soma while others are actively transported in the dendrites. This transport is induced by activity of neurons: there is a basal transport in dendrites, but when neurons are depolarized we have an *inducible transport*. Everytime we have an inducible mechanism it means that there is something that can activate this function. hypothesis: the motors could be in the 5', because some of these transcripts remain in the soma, others go in dendrites, so the regulatory sequence could be in the 5'. We cloned several of this domain, togheter with the coding sequence in frame with GFP, we transfected this in neurons, then looked at the localization of these RNA under basal condition as well as i nneurons depolarized for 3 hours. As control: coding sequence alone, to be avoide f any responsivness. Wrong hypothesis: the coding sequence alone was able to go in distal dendrites under basal conditions!

It means that it contains some signals recognized by the transporting machinery. The CDS (coding sequence) contains a signal that is recognized specifically by the transporting machinary, comosed by RNA binding proteins and kinesin motors. Scanning the region, we found a sequence highly conserved from Drosophyla to humans: this is the recognition site for *traslin*. Measure the distance in dendrites under basal conditions of these neurons: they showed that RNA reaches 80% of the dendrites. With iRNA, almost completely abolished the targeting. In addition, the recognition site is exactly overlapping with a very important human polymorphism linked to memory deficits and vulnerability to neurodegenerative diseases and depression. We got the human coding sequence of BDN and trasfected the construct in the wt and in the mutated for this polymorphism, consisting in a single nucleotide change from G to T. She found that this trafficking is constitutive, not regulated.

Transfecting the human $\longrightarrow$similar to rat sequence. Trasfection of mutate allele $\longrightarrow$50%. This means that either the trafficking was not completely blocked or there is another mechanism which is not translin-dependent, but

is not completely effective. 2 monomers of translins: each has a pocket that is suited to surround a G. There are 2 G, one mutated in humans and one not mutated, that can fit perfectly. When the molecular modelist did the modelling was taking only 4 nt at time: they fit perfectly, but there is a long stretch in between. With bio-informatic analysis, we could demonstrate that there was a stem loop in which we had a perfect Watson and Crick pairing.

The all sequence of BDNF is composed by 5', coding and 3' and responds to electrical activity: where are the signals that respond to this activity? The 5' do not contain any signal to go in dendrites. There are 2 which cointain the signals that keep the RNA in nucleus. We have a motor always active in the coding region and selectors that allow to stay in the nucleus or go in dendrites. The final hypothesis is that if we have just a suppressor or permissive sequene and a moto in the CDS, the only signal to move will be in 3'.

Cloned 3'UTR under GFP $\longrightarrow$a lot of small stretches highly conserved in vertebrates. The end part is identical to fish to humans! We identified the presence of several sequences bound by families of RNA binding proteins:

- Elav: involved in stabilization of RNA
- Fragile X proteins: involved in translation
- CPEB: CEPB1 interacts with Maskin

CPE is in the end and in the middle: there are stretches of many conserved binding sites in the middle and in the end. Then we did a co-localization analysis looking at proteins and mRNA for BDNF and we see that they are physically interacting.

To demostrate the involvment of RBP is to have an experimental setup and a redout: hypocampal neurons in culture, transfect the costruct, stimulate for 3 hours with high Kand the that RNA gets to the 80% of the distance. We also have an inhibitor of the proteins, iRNA: if we K-O these proteins, we see if we still have trafficking or not or we make a mutation in the RNA sequence of the protein. The construct is still GFP with 3'UTR. We can block trafficking by inhibiting CPEB1-2 but not with CPEB3-4. Mutating the CPE binsing site, we still abolish the trafficking: this sequence is important for trafficking due to electrical activity.

Then we mutated the 2 Elav binding sites or we did a silencing of the different Elav: by K-Down elav-1 we don't have any effect but we can suppress the inducible target by eliminating elav 2 and 4. We mutated one site or the other and see thet both sequence were required to target to the dendrite. The short is in the initial part and contains 3 signals:the 2 elav and CPE.

The long 3' also contain these, but apparently it is not active because it has in the middle and in the end high conserved sequences that are hotspots for binding, so it is crowded by many RNA binding proteins. We extracted the three families of protein and run an assay in which we transfected neurons with the long construct, depolarize and add BDNF. While the short requires CPEB1 and 2, in this case (long) we need only CPEB1. When we silenced Elav binding protein we had a surprise: by silencing Elav1, the basal level was equal to stimulated one, and when we stimulated we have an over-shooting of the basal level of the trafficking, also by knocking-down elav3 and elav4. Eliminating some RBP, the RNA goes further, so these RBP are keeping the RNA, are negative regulators, but they are not involved in the response to electrical activity, because eliminating them the RNA is still able to respond to electrical activity. It is a complex mechanism: the same happens silencing FMRP protein (fragile X).

Took the CD, put ahead and this was able to suppress. If I took only the 3'UTR short, when I stimulate the RNA goes more in dendrites while when I eliminate the RBP it doesn't go. For the long, this mechanism doesn't exist: in some cases it might be folded, so other sequences in the iddle and at the end will take place. The initial part of 3'UTR is not important anymore: when we eliminate the CPEB1 the RNA doesn't go in dendrites $\longrightarrow$the signal at the end gives responsivness to electrical activity.

Let's see if the Elav binding proteins are important and work at the same way: silencing Elav sequences we saw that the trafficking in dendrites was enhanced. So, the Elav BP and fragile x proteins could work as a brake. If these peaces in the middle are isolated and put in the neurons, we expect to have a suppression of the trafficking. The 3'UTR long has a basal level which is more dstant in dendrites and respond to electrical activity; the central part has a basal level whis is much closer to the soma and does not respond to electrical activity. Is it not respondins because the sequence was wrong. Put the suppressor was in competition to another signal $\longrightarrow$the basal level goes closer to the commander, then slightly respond to BDNF: when we stimulate BDNF there is a specific signalling cascade that removes the brake. If the terminal sequences contains CEPB and Elav binding site $\longrightarrow$green signal, the RNA can go in dendrites and respond to activity.

if we have only the part that contains fragile x protein and elav $\longrightarrow$red signal, cannot go in dendrites and no response to electrical activity.

By combining the coding and the central part, using BDNF we have a green light: regulation of the localization of the RNA is not as was described in the past, but we have a combination of opposite signals that tell to the RNA what to do in different time in response to different signals.

In vivo we gave seasures to the animal and we see an increase of trafficking,

the *electrical activity induced*. Here depolarizing by high K didn't make change: this is a *constitutive active signal*.

Chapter 16

Lesson 16: Mechanisms of control of mRNA processing and degradation in neural cells: P-bodies and stress granules

Eukariotic mRNA subcellular localization: the transporting granules are not the only possibility for mRNA. They can also be found in other subcellular aggregation, P-bodies, stress granules and transporting granules and polysomes. Polysomes are the rosettes. Stress granules are constituted by RNA which are stopped at the translation initition and don't go in the elongation phase (only the small subunit is there). P-bodies do not have ribosomes and we have RNAs stored, waiting for a decision by the cell.

16.1 P-bodies

Aggregates of RNAs and proteins that contains translationally repressed RNAs. Involved in translation repression, mRNA degradation and silencing. There are core proteins, in particular decapping machinery and de-adenylase complex. The protections, cap and polyA, must be removed. We have additional proteins involved in repression and can bind micro-RNAs, in particular the RISC complex. We may also find specialized machinery, the *non-sense mediated decay*: it's a mechanism of rapid blockade of translation and degradation. P-bodies are a non-membranous aggregate or a non-membranous organelle. These molecular machinery are heavly exploted in virus infections and can dirottare the RNAs.

Deadenylase chop the polyA and the decapping enzyme reduce the me-

thilatex 7 G.

sbobina at 7 min of 27/11b

A state of active translation of mRNA in which we can see that the mRNAs do contain already 4E and 4G with 48S, the 40S subunit with the initiation complex. The cell may decide not to go on ith translation and there is a substitution of translation initiation complex with the capping complex. THis complex is attaching to the cap in order to remove it to degrade the mRNA. The major mechanism for degradation is starting from the cap, even if deadenylation is present. After the formation of the complex, there are 2 pathways:

- Formation of Stress granules: we need P-bodies to do that, because if we block their formation we don't have stress granules neither
- Translation

The stress granules are reversible: the switching to a translation state to a repressive-translation state is made by the removal of the cap-complex. It is possible to go back by adding aagin the cap complex. Stress granules and P-bodies come closer to each other: stress granules are stable while P-bodies tend to move along the MT and they reach the position where stress granules are located (immobile). There is then a contact and exchange of material, in particular RNA, so that the mRNAs that are transferred into the stress granule acquire the cap complex.

There is a point of *triage*: there is a monomeric structure that goes into aggregates, so it is similar to the configuration of P-bodies (that are not monomeric), or go into exosome that degrades mRNA. There is a debate on where degradation occurs: maybe activation of the degradation pathway occurs in competition with the formation of P-bodies. In the stress granule, the protein that recognize the RNA and accompain if throgh the migration to the stress granule and the P-bodies is TTP (tris-tetra proline).

Let's consider a P-body, where we can identify the cap and the *decapping complex*. The P-body attach to MT and moves along them thanks to a protein that binds to motor protein. THere is a possiblity to have an exchange with stress granule. There is also RISC complex in the P-body that contains argonaut-2[1] and brings the mRNA into the P-body: it exploits the presence of complementary RNA called *microRNAs* that match the sequence that will induce the degradation of RNA. If they do't perfectly match, they induce the stop of the translatio; when they match, they induce degradation in the *exosome*. Exosomes contain the microRNA of argonaut and some constituents of the P-body responsible for degradation.

[1] A major component of the RISC complex.

THe P-bodies contain some proteins that are part of the 2 types RNA transporting granules: one contains ribosomes, the other one doesn't. There is the presence of SYNCRP, mRNP. When they enter in the transporting mode, they reglease the GW complex and through the presence of staufen they enter into the transporting state. RNA transporting granules can also be transformed in stress granules, although there there is the capping system and TIA and G3 binding protein, that are highly specific. It seems that in the end the mRNA is working as a swiss knife, having all the possible tools to do many different things but we don't use them all together. The mRNAs in the different granules can contain various proteins that have different important functions and features:

- They may not be in contact with RNA
- The presene of different proteins will activate a complex instead of another one

CBP is important for prevent translation but also for transporting the RNAs. It is possible that these P-bodies represent a part of the sushi belt: there could be some granules that are docking to a dendrite. What makes different the RNAs that are actively transported to the ones that rest into the dendrites?

Riassumendo P-bodies are non-membranous organelles that contain mRNA which will be eliminated or stored in an inactive state. Degradation of mRNA in P-bodies occurs in 5' $\longrightarrow$3' direction, while degradation in 3' $\longrightarrow$5' occurs in the exosome, a complex of esonucleases.

Chapter 17

Lesson 17: Neurotrophic factors I

The neurotrophic factor hypothesis has been made on the chick by Victor Hamburger in 1934: when he was removing the limb bud, he could see that in the ventral horn of the spinal cord there were much less motorneurons. There must be a relationship between the target and the neurons, so he made this hypothesis: the targets of innervating neurons provide signals that recruit undifferentiated cells to develop into sensory or motor neurons. He was wrong.

Several years later, Rita Levi Montalcini proposed that the target derived signals maintain survival of differentiating neurons. This is the *neurotrophic hypothesis*. Giuseppe Levi has been the first to introduce molecular neurobiology in Italy. Salvador Luria (the one of the LB medium to culture bacterias) and Dulbecco where companion of Rita and they were in the lab of Giuseppe Levi.

The first change in the model was to use an aleograph, a murine sarcoma transplanted into chick embryos: it is possible to move it into different parts. If you move it far away from the spinal cord, we see sprouting anyway. If we put it in extra-embryonic parts (allantois etc) that are in contact only with the blood vessels, you don't see prouting $\longrightarrow$this is not a soluble molecule. We have a simple source of the material, the neurosarcoma. It can grow very easily: the problem is that we end up with a series of fractions containing different proteins, so how can we test them easily? A lab in Rio de Janeiro developed the colture of dorsal root ganglia.

NGF is found in mandibular nerves, while BDNF is found in all neurons of the brain, maybe it is the most abundant neurotrophic factor. NT-3 and NT-4/5 were obtain by PCR cloning in mammals ad frog. Then other families of trophic factors were discovered.

Growth factors include substances, not necessarely proteins, that stimulate cells to divide (hyperplasia) or increase in size (hypertrophy).

Trophic factors include substances that have effect on cell differentiation, cell survival, expression of specific phenotypes, cellular morphological plasticity as well as hypertrophy.

A single molecule can have both growth and trophic function, also in different periods of the development.

17.1 Neurotrophic factors

Soluble endogenous proteins regulating survival, growth, morphological and synaptic plasticity os synthesis of proteins for differentiated fuctions of neurons or glial cells. Cytokines are produced by astrocytes and microglia: they are important to regulate the synaptic plasticity and morphology and brain immune system connections.

They are produced by all cells of the nervous system, including neurons, glial cells, ependimal cells, blood vessels endothelial cells etc. 20-60 micron is the diameter of the cell soma and the distance between cell soma and capillary is 15 microns, because of the exchange of oxygen and glucose. There are a lot of possible situations for the release of the signal:

- Transport from tje target to the cell soma: retrograde transport
- Anterograde transport
- Secretion and intracellular communication

Autocrine: 2 neurons close to each other and in contact.

Paracrine: 2 neurons close to each other but not in contact.

Juxtacrine: receptor on the neuron that is secreting.

Holocrine: the membrane is disrupted and the neuron is dying $\longrightarrow$secretion (like mammary gland).

Ependymal cells: surrounds ventircles and the central canal of spinal cord.

Telocrine: where there is no BBB like in hyppocampus.

glial cells contributes to this secretion.

The trans-cellular release means that glial cells, in particular astrocytes can catch the factor, put them in vesicle and send that vesicle in the synapse or in other synapses to spread the signal.

17.1.1 Functions

They have a trophic action as cells survival factors, trophic action by stimulating growth of cellular process. They have a role in the cellular pheno-

type to mature into ACh secreting neurons. They have a mixed trophic and growth functions, because cells are becoming bigger, and they have an action on synaptic plasticity. We have a population f immature neurons which are growing the axon: the axon find the target in other neurons or in peripheral tissues and when it finds the correct target it receives fro mthe target the NF and send a message to the cel lbody: we have find the target! If it doesn't find the target $\longrightarrow$apopthosis.

The possible targets are neurons that innervate the periphery, in particular the Merkel disk:[1] this terminal require BDNF for the survival; the sensors for pain respond to NFG; the muscle spindle responsible for contraction is sensing NT3. NGF is also found in salivary glands: some animals, while fighting, they lick theirselves and NGF helps to heal the wounds.

(3.12.2015)

Like many hormones, neurotrophins are synthesized as *pro-neurotrophins*: pro-insulin is produced in a large protein that is then cleavated. In contrast ti hormones in which the pro-form is not active, pro-form of neurotrophin has a receptor and produces a biological effect, P75, a low affinity receptor. The major biological effet is *apopthosis*; in contrast, upon proteolitic cleavage, the mature neurotrophin binds a class of high affinity receptors:

- TrkA
- TrkB
- TrkC

Trk means tropomiosin-like kinase (trak), because it contains a domain which has kinase activity. The biological effects are cells survival, Growth and differentiation. NGF can bind only TrkA, BDNF and NT4 bind TrkB while NT3 is promiscuos. At high concentration of these neurotrophins, they can bind also P75, but it is a non-physiological state. When neurotrophin binds to Trk receptor. the kinase domain is activated resulting in autophosporilation. Trk doesn't autophosporilate itself, but need to Pi a neighbour Trk $\longrightarrow$dimer. Each neurotrophin is a *dimer*: each monomer interacts with one receptor. AutoPi results is *activtion of kinase domain*: there are Tyr domains that are Pi leading to the activation of different cascades. Even the pro-neurotrophins can act as dimers, but in case of P75 receptor it is more complicated: in some cases we have a dimer of P75 or an heterodimer of P75 with different receptors.

It seems that a specific receptor for NT4 (TrkD) was lost in evolution or didn't exist at all.

[1] A sensore that senses pressure on the skin

Dimerization of receptors is not unusual: GDNF has 2 co-repceptors. We can find heterodimers for several neurotrophic factors, cytokines: GDNF is interesting because it has been proposed as a possible cure for Parkinson's disease. These are the models of interactions: in the classical situation we have a dimer which in interacting with a dimer of Trk, so the pro-neurotrophin (dimer) is interacting with P75; there is also a situation in which P75 an Trk interact forming a tetrameric complex in which the interaction of P75 increases the affinity of Trk receptor for the mature neurotrophin.

There are also co-receptors that increase the affinity of the receptor for the ligand, in particular P75. P75 can interact with Trk receptor in order to increase the affinity, or make a cluster interactong with amyloid precursor protein and with the prion protein, or with the Nogo receptor, important because it is expressed on the surface of oligodendrocites and prevents the regeneration of the axons following injury of the spinal chord.

Neurotrophic factors can be transported in many way (retrograde, anterograde, paracrine etc). In the *retrograde trafficking*, during development neurons grow their axons and the growth cones reach their target: when the target is reached, they form synapses that are stabilized by target-derived neurotrophic factors. The cone secretes the NF that inform the neurons that the contact has been estabiliched with the right target $\longrightarrow$signal for cell survival, differentiation and growth. The major issue is how to transport the signal from the synapse to the cell soma, expecially when there is a very long axon.

A vesicle through endocytosis that includes the receptor has the cytoplasmic part of the receptor still in contact with the cytoplasm and the ligand is in the vesicle: if we have the right pH the ligand is still present and the receptor is activated. The vesicle is transported by *microtubules-based retrograde transport* thanks to dyneins. At the soma, since the receptor is still activating the signalling cascade, it cans send the message to the nucleus in order to produce proteins to survive and reinforce the synapses. It is possible also to secrete a neurotrophic factor on the synapse. The vesicles that are sended to the terminal contains only the ligands.

Using dorsal root ganglia, Campenot used a chamber that has a center comparted separated by telfon walls sealsed with parafilm (water cant cross): neurons grow throug these compartments and in the end the cell soma will remain trapped in the central compartment. It is now possible to put labelled neurotrophins in the axon and see if they reach the soma. It is possible to stimulate only one side with the neurotrophin and see the difference: there is sprouting only in the compartment with the NT (NGF).

In order to have this growth of axons, NGF needs to be present on the growth cones, not on the cell soma, but after 15 minutes some labelling

was enteng in the axons, so even the cell soma was labelled with NGF. To understand the molecular mechanism: phospho Trk receptors can be targeted with Ab and when they are fluorescent it means that the receptor is activated. There is a co-localization in the section of an axon of dynein and PiTrk, so activated receptors can be transported retrogradely through dyneins.

Is it only with dynein? What about kinesin? The co-localization is not present, only a few, because in the back of the vesicle there might be some kinesines.

Once the receptors are binded by the ligand, we can have endocytosis but in some case these vesicles might end up in macro-endosomes. We may also have early endocytic vesicles and recycled. Some others are incorporated into a larger vesicle but they don't fuse (*multi-vesicular body*). These bodies cannot provide the signal and they ends up into late-endosomes.

Mutlivesicular body can be targeted to lysosme degradation; in other cases we can have a *signalling endosome* (in yellow) or the recycling endosome.

The three main singalling cascades from Trk are Rac, GIPC, PLC-γ, PI3K (mainly involved in cell survival) and MEK (mainly involved in differentiation). The Trk receptors are activated because they can cross-phosphorylate each other to activate a signalling cascade. Which signalling cascade? It is possible to activate all the 5 cascades, but if one component of a cascade is missing in that moment, that cascade cannot be activated. Like the receptors can be dinstributed in the surface of the cell, the proteins of the cascades can be located in different parts of the cell. There are some other signalling cascades that can be localized: in the soma, the ERK cascade is mediated by ERK5, a particular form activated by MEK (MEK can activate ERK5 or ERK1-2, the first one in the soma and the second one in the periphery).

Generally, Trk receptors are Pi on the membrane and also during the retrograde transport. The members of the signalling cascade are found in the cone and in the soma, not during the transport along the axon. The building blocks in the soma are different frome those in the growth cone (see what we were saying before).

A typical effect of MEK in the periphery during development is the growth of the cone, while in the soma is cell survival, so different position = different function. The activation of ERK5 is something specific for the receptors coming from the periphery: the cells can activate ERK5 only when the signal coming from the periphery is present.

Trk activation $\longrightarrow$PI3K cascade $\longrightarrow$axon growth + endocytosis of the receptor and the ligand $\longrightarrow$signalling from the endosome $\longrightarrow$activatio of ER1/2 $\longrightarrow$axon growth.

It seems that NGF and BDNF are those with the main capability to

form signalling endosome, while the others members of the family of NT are involved in membrane signalling. In the vesicle, there is a binding between the cytoplasmic part of the receptor and the dynein. THere is also the Raf cascade, Rab5 (one of the main proteins on the surface of the vesicle for Rab family). This structure is tellong us that some parts of the sigalling cascades are present, but not all of them, like PI3K. One of the signalling cascade which are activated by phoshorylation is involved in the recognition of the vesicle by the dynein structure; GIPC is involved in the binding with actin filament (for the initial step) and MT (for the forwards steps) and it is an adaptor proteins which contains PBZ domains, tipically used for protein-protein interaction. We have also Tctex-1 which is part of the dynein motor complex. Trk receptors have recognition sites for the cytoskeleton. These systems can compete and the vesicle must choose.

How these vesicle switch from one direction to the other? It seems that there are proteins that help switching between forwards and backwards transport is *huntingtin*. We have both motors attached to the vesicle (dyneins and kynesins): Dyactin complex goes with actin and HAP-1 woth MT. When HAP-1 is Pi, kinesine motor is attached; when dephosphorylate, dynein is attached. This is the case of signalling endosomes.

(11.12.2015)

Chapter 18

Lesson 18: Neurotrophic factors II

Nt works through different receptors: mature binds to a family of receptors, Trk. There are 3 receptors, TrkA specific for NGF, TrkB for BDNF and TrkC with NT-3 and 4. P75 binds immature NF: it promotes apoptosis and is involved in the initial development of the axon, response to injury and some aspect of develompent. Trk receptors are involved in opposite functions: survival, synaptic plasticity, growth, differentiation. Another family of co-receptor is the *Sortilin* family: 4 members, all involved in the singalling of pro-neurotrophins. There is also another type of receptor for neurotrophins, *Nogo* receptor, a complex made by 2 proteins, Nogo and LINGO-1 (transmemnrane): it is the reaso why axon cannot regenerate after spinal chord injury. All these 3 class of receptors interact with P75: it increase the affinity of mature neurotrophin for TrkA; it facilitates high affinity binding between sortilin and pro-neurotrophin; in case of Nogo-receptor, p75 facilitates the interaction with some myelin-associated proteins also. It is important for suppression of T-killer cells.

There is a family of sortinlin receptors: 4 main types very similar to each other that are Sortilin, SorCS1, SorCS2 and SorC3 and other 2 receptors very different that are VPS10P and SorLA (bigger and much more complicated structure). They are all involed in signalling between neruons and glial cells: even the simple binding between a soluble molecule and one receptor is challenged by the fact that individual receptors can interact with different partners. This is no more true for neurotransmitter, becaue receptors can change in subunits. In case of growth factors we have 1 receptor but also co-receptors that can hel pthe binding.

18.1 Trk signalling

Let's consider TrkA: in the cytoplasmic part there is a kinase domain that can make a cross-Pi to the other partner. There are several residues that can be Pi: Tyr (iuxtramembrane) and 3 more distant Tyr. the one which is more proximal to the membrne is the most important and can activate 2 different cascades: one mediated by PiP3 (cell survival) and one mediated b Ras cascade (cell survival and differentiation). These 2 cascades are found in all Trk cascades. The initial proteins of the cascade recognize a Pi residue: Shc, that binds Grb2. Grb2 can bind different partners, like SOS or Gab1 and depending on this it activated different cascades. What decides which signalling cascade is activated? 2 ways:

- Availability of the component
- Specific Pi that allows interaction with only 1 component

All of these signalling cascade will arrive into the nucleus or in the endosome.

Let's focus onf PLC-γ, a singalling cascade that modulates the intracelular Ca creating IP3 and DAG: these 2 signalling molecules are important for mobilitation of intracellular Ca stores. PI3 kinase arrives on AKT1 kinase, that has an important funciton in supressing pro-apoptotic genes like p53 and Forkhead and on the other side it acivates proteins involved in cell survival and cell protection. NfkB is a transcriptio factor that activates survival genes and its Pi is able to enter in mitochondria and promote their survival. The different signalling cascades of Trk have many points of contacs

chiarisci questa ultima parte

18.2 p75 signalling cascade

A p75 signalling cascade is not in contact with Trk cascades: this is a very race case! There is the *death domain* that is able to activate a signalling cascade involved in cell death and this is the TRAF cascade. There is also the RhoGDPase that is activated by this domain $\longrightarrow$cytoskeletal movements. These are proteins called *small G proteins* that can regulate actin filaments and this signalling cascade regulates the growth cone mobility preventing it. p75 interacts with Nogo $\longrightarrow$that's why they have a mutual help in prevent growth of axons after spinal cord injury. It is possible in the end to activate NF-kβ, a protein involved in transcription of other apoptotic genes that can be found also in Trk cascade at the level of Ikβ.

Most of these receptors have the possibility to activate TRAFm which activates JNK and Jun. Another group of receptors mobilitate Caspases and FADD. p75 can also be called NGFR (NGF receptor).

18.3 Sortilin

Sortilin is a co-receptor because it can bind the mature part of the nerotrophin and offer the pro-domain of the neurotrophin to p75 by immobilizing the mature part to prevent its interaction with Trk.It offers the pro-domain to p75. p75 was called *low affinity receptor*: the presence of the coreceptor orient the pro-neurotrophin in a way in which it can fit better with p75. Sortilin per se doesn't have any singalling properties! It only promotes the binding to p75.

Genes that contain the jun binding site in their promoter are genes of the *caspase family* involved in apopthosis. Since we know that p75 is bimding to the pro-domain of neurotrophin, the mature part remain available for binding, so we can have a situation in which p75 does the same job of sortiling, helps the pro-neurotrophin to bind to Trk with the mature domain. There are 4 receptors: the fact that neurotrophin work as a dimer force the receptors to work also as dimers. We have the formatio nof tetrameric receptor: 2 p75 and 2 Trk. In this way, the binding is favoured.

Sortilin is present in vesicles that transport the receptors, but also in the vesicles that transport the neurotrophin: sortiling is a signal to sorting ligands and receptors in the golgi apparatus, so it is one of the receptors that work also intracellularly. It recognizes the central part of the neurotrophic factor.

Chapter 19

Lesson 19: Neurotrophines—signalling mechaisms

19.1 RTK receptor family

They all contain kinase domain in intracellular portion. Insulin-like receptor detects ILF1: it is used to stimulate the growth in dwarfism and consider a possible therapy for diseases which involve brain atrophy.

They all share the presence of Ig domains.

19.1.1 Structure of Trk receptors

They contain Ig domains (D4-D5), typical domains which are used for protein-protein interactions because they are very stable, have cys-cys interactions and are involved in ligand binding, so the ligand is binded very deep in the groove of dimerization of the receptor. D1, 2 and 3 are the cys-rich domains. This structure is shared by all Trk receptors. The NT binding occur very deep into the groove: the leucine-rich domain is external.

The structure of NGF has a core part, the part of the mature NGF, while the pro-NGF is surrounding this area. The core is made by 2 monomers with a flat surface used for their interaction, thes 2 huge arms which have a much more looze structure, is long and corresponds to the pro-NT. The pro parts are highly motiles and can muve up or down while the core part is immotile. There are 3 cys-cys interactions (n black) and when they are inside each monomer, they cross each other: *cystein knots*, located at the core of each monomer and bind different peripheral parts of the molecule.

So, cys are found in the monomers which represent the core of NGF.

19.1.2 Activation of Trk

The 2 catalitic domain of Trk are: PTB and SH2. PTB domains: Phospho tyrosine (p-Y) binding domain. The part that is bind to p-Y contains basic aa and a second domain recognizes hydrophobic aa and can bind few aa, helping adapting this structure to be very close to Pi sites.

SH2: sarc homology 2 domain. The 2 sites ensures that the PTB sites are non-specific and, in order to have specificity to that particular p-Y, we need a sequence of 6 aa that is provide by the protein. So, there are also here 2 different sites: one for p-Y and one for the sequence of 6 aa (p-Y + 3 usually hydrophobic). If PTB is linked to a series of 6 specific domains in the SH2 domain, this can become a specific bind. The structure of SH2 domain allows to recognise the 5 different Y on which Sh2 has to bind (Y 490, Y 670, Y 674, Y 675 and Y 785).

Only less than 1% of proteins will have a p-Y, so this is a very special signalling pathway. The majority of Pi occurs in Ser of Thr residues.

These proteins can have mutliple interactions with SH domains: SH2 is involved in protein-protein interaction and SH3 that is responsible for plasma-membrane interaction. It is important to be anchored to phosphsolipids to cluster the receptors together in lipid rafts.

19.2 Ras pathway

Ras is a membrane boud GTPase. We have multiple SH2-SH3 domains followed by GRB2 or SOS. The very same cascade can be activated by different receptors:

- For EGF, GRB2 and SH interacts with Pi tyrosine kinase domain of the receptor EGFR, activating SOS, a proline-rich domain, that activates Ras;

- For NGF, before SOS and GRB2 there must be SHC that binds to a Pi residue of Trk receptor in the tyr-kinase domain, because GRB2 cannot recognize the Pi residue on Trk. To let GRB2 recognize a Pi domain, SHC must be Pi, so GRB2 recognizes the Pi residue on SHC. In this case, we can say that the tyr domain of Trk receptor is on Pi-SHC.

19.2.1 Activation of Ras

Grab2 recognise a Pi residue, so FRS2[1] is acivated. In order to amplify the signal, we can have an adaptor Pi on multiple sites, so it's recognized by multiple Grb2. . The second component of the cascade is PIP3 that is transformed in Pip2 by Pi-3K. It can further amplificate the signal: it can bind additional adaptor proteins like Shp2 and additional Grab2. SOS can be activated by a lot of Grab2 $\longrightarrow$amplification: Sos acts on Ras and ERK modulating them.

riascolta l'ultima mezz'ora

After the activation of Ras that consists in exchange between GDP and GTP, active Ras recruits, binds and activates Raf.[2] GTP hydrolysis leads to dissociation of Ras from RAF, which is then Pi many times and can activate MEK. MEK is further Pi by ATP and activated MAP kinase (MAPK/ERK) by Pi in 2 sites. The dimeric form of active MAP kinase translocate in the nucleus and activates many transcription factors. This occurs only if we have a post-translational modification of Ras (break in the bond between Cys and Ala).

19.2.2 Regulation of gene expression by ERK

MAPK/ERK (they are the same thing!) dimer can translocate in the nucleus, where Pi TCF (ternary complex factor, Elk-1), which can bind the SRE sequence (serum response element) of the c-fos gene, activating transcription. Another pathway that leads to the activation of transcription involves the activation of $p90^{RSK}$ out of the nucleus by Pi: this translocate into the nucleus, bind SRF (serum response factor) and Pi it, - Pi-SRF can also bind SRe sequence helping TCR. So, activation of Trk receptors induces rapid and transient cellular responses in addition to slow and long lasting effects.

there is a part missing!

Timing and number of passages is a big deal: we can have signalling cascades activated for hours because of the huge number of steps and helpers. Signalling cascades became more complicated during evolution because fo the possibility to have multiple regulatio points, possible substitutes or integrative pathway. Once they are Pi, this signalling cascades for cluster form complexes in hwich scaffolding proteins can help.

[1] FGF receptor substrate 2.

[2] Inactive Raf is a complex with the protein 14-3-3, which reognises 2 Pi sites.

Chapter 20

Lesson 20: Hyppocampus

Very peculiar structure. It allows to make nice models at matematical level. Series of folded areas: we can open up it and have all these cells in a line. Once we unfold this structure, we have a cylindrical structure, very close to the thalamus and it is the cortical structure. A coronal section and a Nissel staining, in the rat, we see the structure on the vetrino that we have done in lab. In human brain, the hyppocampus has been folded and invaginated inside the cortex. Comparing human and rat hyppocampus, the human one is 1000 times larger that the rat hyppocampus (like the all rat brain).

20.1 Anatomy

In the anatomy, we recognize different parts:

- Enthorinal cortex: in contact with the hyppocampus through a region, the *subiculum*
- Hyppocampus proper: main layer of cell bodies
- Dentate girus: another layer of cell bodies, separated from the rest of the hyppocampus by a boundary, the hypocampal fissure
- CA1, CA2, CA3 and CA4 regions: the largest areas are CA1 and CA3. These areas are called *hyppocampal proper*

In CA regions we can see stripes: in this area we have *apical dendrites*. The area under CA region contains a lot of blod vessels. Under this area there is dentate girus: these neurons are different from the CA neurons, they look like a chandelier, they are called *granule cells*. The different layers are:

- Stratum oriens: external layer, outside the hyppocampus

- Stratum pyramidale: contains the cell bodies and makes a boundary between inside and outside
- Stratum radiatum
- Stratum lacunoso molecularis: we have a lot of holes due to the blood vessels and the molecular layer is the name of area in which there are a lot of myelinated axons

N.B. Sl (stratum lucidum) is found only in CA3, there is a mistake in the picture

From the enthoninal cortex we have myelinated axons, the *perforant pathway* that passes from the fissure and runs parallel to the dentate girus and arrives to the dendrites of the granular ccells. The feature of the hyppocampus is to have nice separated areas that contain different cells: the granule cells have the axons inside and the dendrites are in the DG. The cell bodies are aligned in one layer. The axons form another pathway which is called *mossy fibers*: contain a lot of varicoristy, like muschio. These mossy fibers are the second segment of this 3 synaptic circuit and make synapses not only on dendrites, but in an area very close to the soma, the *stratum lucidum*: there are very big spines and have mutliple synapses on the spines. The third segment is emerging on the opposite site respect to apical dendrites from CA3, cross the pyramidal layer and the axons arrive on the apical dendrites of Ca: these fibers are the *Schaffer's colaltera*, that have a branch making synase with the dendrites of CA1 and second branch of axons that can exit the hypocampus in the septal way.

registrazione 33 minuti

Enthorinal cortex have 3 sites of entry: perforant pathway, subiculum and lacunoso moleculari. Different part of the dendrites may have different funtion.the 2 series of axons are coming from different directions and the neurons have to integrate these information.

registrazione

20.2 Basic circuits

EC have different layers: from layer 2 there is perforant path to the dendtate girus. Dentate girus sends mossy fibers to CA3: form multiple synapses. For each neurons stimulated n DG, we have 3-20 neuron stimulated in CA3 $\longrightarrow$huge *amplification* of the signal. Then CA3 sends Schaffer collaterals to CA1, but there are additional collaterals tht exits the hyppocampus from the septum, but some can form synapses to the CA3 itself. These are the *recurrent colalterals*. So, CA3 receives synapses from EC, DG and CA3 itself:

these are hot synapses, they require a lot of oxygen in the brain. There are renewable cells because in dentate girus we have stem cells.

Perforant pathways divide in the lower part (medial perforant pathway) and an external part, the lateral perforant pathway. We have also 2 more pathways from the EC: one goes ot to the hyppicampal cortes, LEC and MEC: lateral and medial EC.

41 minuti

A pathway from EC makes synapsesi with subiculum and CA1 layer. Some fibers do not stop and continue to the more peripheral part of CA3 neurons.

registrazione

So far we have consider only pyramidal neurons, excitatory, but there are also inhibitory neurons, like stellate cells (like in the cortex). Granul neurons are completely different from pyramidal neurons.

CA1 We have the cell body in the stratum pyramidale, the basal dendrites in the stratum oriens, the main apical dendrite on statum radiatum and large and thin rmaification in the stratum lacunoso-molecolare. This different geometry accomplish different functions. Stratum oriens contains all the fibers coming from the EC, in the stratum radiatum we have the fibers form the CA3 (excitatory) and in the stratum lacunosum-moleculare they are coming from the EC. In stratum pyramidale collects information from internal interneurons. The neurons will have to make a balance between the different inputs.

Dentate girus The medial perforant pathway formes synapsis in the inner molecular layer, then the medial PP formes synapses in medial and outer molecular neurons. Intrinsic neurons form synapses on granul cell layer and in the area with axons we have other interneurons and the mossy fibers collateral. We have the polimorphic neurons, the granul cell layer and the mlecular layer. ArcRNA in the medial molecular layer: after 30 min from stimulation, RNAs are accumulated in this medial layer that contains axon terminals of the stimulated fibers, the medial PP. Other type of synapses are located in GCL and also in the sub-GCL and polymorphic layer that says the speed, intensity etc. to modulate the signal.

CA3 The majority of mossy fibers arriving from DG make contacts with the proximal segment of the apical dendrite, the *thorny excrescences*, that contain spines. They can concentrate Ca spines. Mossy fibers form 6-10 contacts, then jump to a second neuron and make another 6-10 contacts and maybe a 3rd neuron. Each granul cells from DG can contacts many pyramidal cells and each pyramidal cell can receive fibers from 72 different granul

cells.

In DG in rat, there are 1.2 million granule cells that contacts 330K pyramidal cells in the CA3. We have a 4:1 number and each granule cells form from 10 to 40 synapses with one CA3 cell.

EC have only 200K cells, so there is a real mplification in DG that converge on CA3 neurons. We can take these neurons and plate them in a petri dish: this is genetically determined, so mutants will have a different growth and number of spines and length of dendrites.

Scholl analysis: by electromicroscopy of synapses and classify them, we could analyze the % of symmetrical vs non symmetrical, so excitatory vs inhibitory. Looking at the proximal part, 98% of synapse is inhibiory, while the majority of excitatory synapses is in the stratum radiatum. In the more distal part, about 17% of synapses are inhibitory.So, the % of inhibitory and excitatory synapses change above the length of the neuron.

Looking at pyramidal neurons in CA1, we have 2 populations:

1. Not ramified in the periphery, only near the soma and a few only in the middle

2. Ramified also in the periphery but not in the basal part

These 2 population encode respectively for spatial information and non-spatial information. The 2 different circuits, one contains information that are called *eaarly bursting* and *late bursting*.

The MEC and LEC: MEC is sending informations on DG, some collaterals in lcunoso-molecularis. Also LEC. They are separated and MEC is more involved in map-like spatial processing that the LEC. This circuits help us to find palces, roads etc. LEC is involved in recognition of objects etc. When we have to remember a place where we have already been, we use LEC while for a new place we use MEC.

20.3 Cellular diversity

read article lesson20 neuronal diversity

...

20.4 Functions of hyppocampus

Formation of new episodic memories? Incorporate new experiences in the brain. It is important for cognitive maps and to associate different memories

into a single cognitive experience. We need hyppocampus also to combine information to make a story.

registrazione

there is a strong interaction between stress hormones (from ipothalamus) and hyppocampus. How can different parts of the brain affect hyppocampus in addition to basal circuit? There is locus coeruleus that sends fibers that provide noradrenaline, involved in awardness. This is a facilitatory neurotransmitter that is helping the functioning on DG stimulating the making of new cells. Serotonin is present in subiculum, DG and output areas. Dopamine controls the output while noradrenaline control the modulaiton of the signal. Serotonin controls mood, dopamine control reward and Ach controls novelty.

Chapter 21

Lesson 21: Synaptic plasticity

Research in Aplysia, Drosophila and Mouse. These animal have simple learning behaviors and few neurons, so circuits are very simple. We talk about *reflexes* more than network: they respont to a stimuluswith always the same circuits. Aplisia ad Drosophila were chosone because the simple mechanism and Drosophila also for its genetics (also the mouse). For the mouse, some of the anatomy is similar to human and also some behaviors. By mutagenesis we can have link between genetics and behavior.

riascolta tutta la lezione

21.1 Long term potentiation

LTP: synaptic potentiation that can last for long time (days, weeks, years). An example is that we know that fire burns and is dangerous. It can be induced by tethanic stimulations,. Hyppocampus has been studied, in particular the Schaffers collaterals from CA3 that make synapses with CA1. During stimulations, the post-synaptic neuron needs to be active. A slice of this tissue can be put in a Petri dish and this lives for 8-12 hours.

There are several type of stimula, like the theta-bursts or the high frequency stimulus train: 10 Hz, for a minute. Early LTP is given by one theta burst, which is less than doubling of EPScurrent wich lasts for hours and then goes back to the basal level. If we give at least 4 stimulations, which is 1 stimulus per minute, then a pause of 1 minute, we have the establishment of late LTP, so we have a 4-fold EPScurrent. So, after 30-40 minutes we have a decrease of the EPSP, but the level is higher than before. It is importatant that the frequency is at least 10 Hz.

It is also possible to stimulate mossy fibers and then we may record CA3 and measure LTP, and also in CA1. THe one in CA1 works better.

What makes the difference between early LTP and late LTP?

We have a release of synaptic vesicles: the terminals do contain a limited numver of vesicles ready for docking. By depolarizing we induce the release of neurotransmitter. Why do we need a separation in timme? To allow the recycling of vesicle and to have new vesicles in the pool. The neurotransmitter is Glu: if blocked, we won't have LTP. Glu is important for LTP because if we block Glu receptors (inparticular NMDA receptor) this phenomenon disappears. Ca bind Ca-Calmoduline which activates the Ca-calmodulin kinase: on one side it Pi the NMDA receptor, in particular the 2 B subunits $\longrightarrow$open the channel, so everytime more Glu arrive, more Ca will enter through the NMDA receptor. On the other side it release NO that passes through the pre-synaptic terminal and reinforce the release. This is part of early LTP (first 30 minutes).

The later phases involve RNA transcription and protein translation, so in cell body and parental dendrites, while early LTP occurs in the post-synaptic terminals. How the transcription in nucleus nvolve the termina? Cam kinase activate AC that produced cAMP, which activates the cAMP kinase (PKA). PKA can activate the transcription of genes by Pi CREB-1 (cAMP responsive element binding protein). Timing is different, and also space. The late LTP has a retrograde traficking and require hours, so transcription could occur also 6 hours later. Sleep is important to the conversion of early signal into the late signal.

21.2 Long term depression

If we use stimula 1-5 Hz, after an initial rise in EPSP slope, we'll have a depression that lasts for days. We have a *train* of stimula at low frequency. There is a low level of Ca entry: calcineurin is a protein that can bind to Ca in low concentration. Ca-calmodulin have a low affinity to Ca, so we need a higher concentration of Ca. In case of high frequency stimulation (LTP), there is the requirement of Ca-calmodulin kinase (we also have calcineurin like in LTD).

If the regulatory subunit block the catalytic part, the kinase cannot Pi. The cAMP opens the 2 part of the kinase, so it can Pi the other proteins. Also the Pi itself keep the kinase active for longtime. Synthesis of new proteins is essential for conversion between early and late LTP. Inhibitors provide deficits in learnins and memory.

Low concentration of Ca activate calcineurons, while high concentration of Ca activates both calcineurin and CAM kinase. Calcineurin induced inhibition of the phosphatase.

The umber of AMPA and NMDa receptors on the surface makes a big

difference. The number is determined by the rate of insertion of the vesicles that contains receptors laterally by exocytosis and the rate of internalization of the receptors by endocytosis. Stabilization of receptors in the PSD is given by the amount of PSD95 protein and other scaffolding proteins. If the scaffolding proteins are not sufficient or not there, there ismuch more lateral movements of these receptors and they can end-up in the region where the endocytosis is active. Proteins like GRIP and PSD95 are important for stabilization of the synaptic content of NMDA and AMPA receptors.

The circle with calcineurin is the recycling of receptors AMPA that occurs during LTD: this is a type of receptors that contain only a short tail of the AMPA receptor, and they are low-conductance less sensitive AMPA receptors. This receptor can bring only few Ca inside. When CAM is activated, not only short-tail subunit are expressed, so this receptor is more sensitive: this allow a higher flux of Ca inside the cell.

Since calcineurin activate phosphatase and Calmodulin activates CAMK, these 2 systems are anthagonists. Also LTD and LTP are antagonists.

mGluR doesn't bind PSD95, but *Homer*, that bins IP3 receptor that modulate intracellular Ca store: by modulating the amount of mGluR we can modulate the activity of the intracellular Ca stores.

During synaptic activity, neurotrophic factors are released: BDNF can activate Trk receptors. Trk activate the cascade that promotes protein synthesis. LTP tipically activates NMDA and BDNF that can activate in a sinergic way the same signalling cascade, so the same kinase. A partially different cascade is activated in LTD by mGluR.

(16.12.2015)

How the changing in synaptic activity lead the changing in shape? In order to keep the shape: accompain the changes in plasma membrane to changes in cytoskeleton. So, proliferation and ramification of actin cytoskeleton must be controlled: the turning point is the small GTPases among which we have the Ras signalling cascade. Ras is downstream the BDNF-Trk cascade: through Erc it can Pi another protein linked to the actin cytoskeleton to stabilize it. Myosin 2 is important for trafficking along actin cytosk: link between NMDAR and Rap GTPase. Growth is activated in LTP: all the signalling cascade activate Rac, the small GTPase that controls the enlargment of cytosk. In LTD, we have Rho GTPase that activates different signalling cascades for actin deconstruction.

EpfB receptors controls Rac and ADF/cofilin, involved in actin regulation.

We have an increased number of adhesion molecules: between axon, dendrites and extracell matrix and pre and post synaptic terminal. Immediately after LTP induction: extracell matrix dissassembly. Later: enlargment of

spines, new extracell matrix produciton and dendrites and spines goes closer.

Chapter 22

Lesson 22: Quantitative analysis of cell images

The purpose is to discriminate cells based on their appearance, because their morphology can give us an idea of underlying phenomena.

We have 2 cells: in an image we see pixels which have a different gray intensity. By increasing the number of pixels, we'll have a different grade of gray. Each pixel will have an assay grey level: the computer assign a number to each pixel and create a matrix. Once we have the matrix, we can make quantifications to measure the intensity in an area of the mean intensity; we can measure the area and don't count pixels that are below a certain value $\longrightarrow$we create a *mask* that eliminate the background $\longrightarrow$now we can recognize all the areas that are separated to each other. The computer can count and se how many objects are separated.

Process: first picture (staining has already been done), ask the computer to separate objects, track[1] cells for parameters that we set (with the hypothesis), classificate the cells and make an evaluation.

22.1 Cell count

Red cells: biomarker of reproducing cells. In bleu we see the nuclei. We can count the number of cells with belu nuclei and the number of cells having red spots, so we can count the % of cells that are reproducing

Another example: efficacy of tubercolosis treatment. Determin the infection power of these bacteria in human cells. So, put bacteria in human cells and take photos: in green we have the labelling for bacteria and in bleu the

[1]Follow the cells and give them a number. We can do a life-cell analysis taking pictures every day and the computerrecognizes the cells by their position.

nuclei. We can count cells that have the bacteria: but how many bacteria can infect a single cell? How to get this?

The first step: identify the cell nucleus by the mask and ask the computer to identify boundaries between cells. The second step: find in this area the number of green spots. We see a multiplicity of infections.

22.2 Classification of cells based on actin distribution

Actin is the green spots. Aks the computer to distinguish between round cells and elongated cells, so the criteria are: shape and presence of actin (uniform of polarized?).

22.2.1 Uniform and polarized actin

Set a second threshold, related to actin intensity. Per each cell, we say to the computer to color the region with actin (color the pixels). We can count the AreaFT50: area of the filled 50% threshold mask of the actin image. There are 2 ways to analyze the brightness: in the gray par, we can set this as the background so this is the threshold. The second way is semi-automatic: place a square i nthe center of the cell and take 100 cells and look for the threshold.

For neurons, we can measure the length of the dendrites: we have to avoid measuring filopodia, that are >5microns long, so we can exclude them. We can also measure the average length of dendrites or the entire dendritic length.

22.2.2 Scholl analysis

Make circles around the soma and measure each time the dendrite crosses these circles. We can make a plot and count the number of crossing at a given distance. Different neurons could have completely different profiles. If we add the total dendritic lenght, we have 2 informations that are complementary. The branches that emerge from the soma are primary dendrites, then secondary then tertially etc and we don't go above the 5th order, fllowing the ramifications. In order to establish how ramified is a neuron, we can do this analysis.

For spines, there are 3 main types depending on lenght of the neck and diameter. To decide if it is a spine or not, we have to find the neck and then ... (vedi slides).

We can also follow the development of a neuron *in vitro*: neurons in vitro from rat hypopcampus follow a stereotipate progression. Dendrites are very small and one of them become very unstable, start to move and to elongate and it became the axon, then we have dendrite outgrowth and mamturation. From day8 to 11, there is one of the dendrite that becomes unstable, until it stabilizes and become the apical dendrite.

22.2.3 Synaptogenesis

We can establish how many excitatory and inhibitory synapses do we have. We have synapsin-1 ad PSD95 co-localized: in the soma, we have only 80% of co-localization, because close to the soma we have more inhibitory synapses (PSD95 is in excitatory synapses). Measuring the synaptic activity during different days, we found that the activity is present after day in vitro (DIV) 12 in rat, while in mouse at DIV6.

Why we look at mouse? Because we can use transgenic mice as models for pathologies: the Rett syndrome is a neurodevelopmental disorders that involves in females and males with XXY pthenotype, because it affects the X chromosome. This support the *growth arrest hypothesis*. Neurons are smaller because they cannot grow, but they form a normal number of synapses that they cannot maintain.

Chapter 23

Target identification in drug development

Elena Di Daniel - UCB (Bruxelles).

FDA in US and EMA in europe.

Cimzia: humanized Ab against TNF.

Vimpat: for epilepsy.

Keppra: for epilepsy.

2 main research centers: Immusology (slough) and Neuroscience (Braine).

23.1 Drug discovery

Determine the needs of the patient. What is the cause of the disease? Consider also the biology of the target.

It is a very rational process: hard work!

A druggable target is a functional molecule (protein, peptide) that can be modulated and this modulation is connected to therapeutic effects. We have to be able to identify an assay: the target doesn't have to be distributed in all the body.

Target classes can be distinguished by the type of drug that hits them:

- Small molecule: less specific. Examples are receptors, enzymes, transporter, protein-protein interactions
- Biologics: Ab, really target. Examples are extracellular proteins, cell surface receptors

For CNS, the target has to be expressed in the brain and in disease-relevant regions. The target has to be genetically associated with the disease and modulated in disease conditions.

From a new target to a new drug: 10-20 years. The probability of success is low: 10%. The identification of target is the most important and critical phase.

23.1.1 Target identification approaches

- Ab validating targets: like Biogen aducanumab for beta-amyloids in Alzheimer's disease. Ab are specific and the cost for production is high. The long-term dosing effects are unknown. They don't get through the BBB.
- Protective Abs: they protect from the disease.
- Mutations: sclerostin. Sclerosteosis is characterized by high bone mass: it is caused by a LOF mutation of a gene which encodes for protein sclerostin, that inhibits bone formation.
- Human insights beyond genetics: epigenetics, environmental factors
- Transgenic mice
- Phenotypic/translational screens: Keppra (levetiracetam). It binds to SV2A.

23.1.2 Target validation

We try to demonstrate that the target is involved in the disease and modulate the target will have a therapeutic effect. We use KO mice, antisense knock-down/ RKNA interference and stem cells.

For mutant mice, we can generate different types of KO: complete loss of gene function (conventional KO) or tissue specific (conditional KO).

For nucleic-acid based gene silencing/editing: RNA interference (cleavage of a long dsRNA into small siRNA) and CRISPR.

For iPSC cells (stem cells): cells derived from skin or blood re-programmed to embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell. They can use to study compound-mechanisms of action, to model diseases, develop personalized medicine.

23.1.3 Assay development

Use purified protein and measure activity +/-.

The cellular assay is used when the molecular target is known but not isolatable from cells.

Lipinski rule for chemical properties for CNS molecules: low MW, good solubility, fewer than 10 H bond acceptors and few that 5 H bond donators.

Toxicity: cell-based cytotoxicity, hepatotoxicity, cardiotoxicity (beat rate) and genotoxicity.

All that we have said is about small molecules.

What about biologics? They are selective but more complex. Ab come out as next generation of therapeutics.

23.1.4 Target neurodegenerative disease

Parkinson's disease: increase DOPA levels to correct the disease. Ritigotine is a dopamine agonist approved for treating PD. Alpha-synuclein aggregate and form insoluble fibrils in PD. There are duplication of the gene and some point mutations.

An immunotherapy to block these aggregates is made with Prothena. Another approach culd be stimulate the microglial phagocytic activity, or inhibition od alpha-synuclei mediated microglial inflammation, or to inhibit aggregatio or exocytosis of alpha-synuclein.

Chapter 24

Cell based drug screening

Fabio Bianco, chief of NeuroZone in Milan. info@neuro-zone.com

Blockbusters drug: selling over 1 million. It is "protected" for 25 years to recover the costs of production, then it becomes available to everyone (brevetto, patents). A new drug has to show significant new benefits from the previous one.

Cell-based assays are spreading all over the world. The drug quest: Discovery, lead optimization, toxicology and clinical development.

Fundings are: governments, private investors.

TTO: techonoly transfer office, responsible for making the connection between the marker or the academia. This is the place to go if you have an idea and they help you find someone that is interested to your idea.

24.1 Cell based assays

There are technologies supporting those cellular systems: cell spot microarrays, high throughput screening, high content screening etc.

We use fibroblasts, iPSC, NSC and neurons and cerebral organoids to translate the findings in animal models to humans. Some companies are using human cells!

Cell lines vs primary cell culture. By using primary podocites, we know that there is an incredible similarity between them and neurons. Translational cell science in NY: they collect the human cells directly and deliver the material on them. This is going to be the future.

2D vs 3D models: 3D system have a matrix and the molecule is like in the extracellular matrix in vivo. 3D are better in mirroring the environment; replicates complex tissue structure, better reflects normal differentiation. We use in 3D systems fibroblasts (40% of the assays), endothelial cells while more

delicate cells are less used because it is difficult to manipulate them once in the matrix. On 3D culture we run very simple assays like cell viability, while more complex assay are done in 2D.

A good assay must be quantitative, cost effective, reproducible and scalable (you must be able to run your assay on many compounds. There are piastre da 96).

Electrophysiological recordings are essential to understand how the drug works. They are coupled with biochemical experiments. 25 pg/mL in assay miniaturization: micromosaic immunoassay. Microfluidics: reduce volumes and get more control systems in which growing cells. One example is Cellix, which is able to reduce a mechanical stress.

Microtissue: to support growth of cells and study complex cells system. We can culture different cells population separately and run multiple experiments in a blind way and see how the cells respond. I get multiple data cells and I can combine them. This platform is used in RD level, in preclinical scenarios, in Phase1-2 of clinical trials. Throughput is still an issue.

24.2 Target identification in Neuropsychiatry

Enrico Domenici.

Brain Cloud: exploring the gene expression and the genetic regulation.

Chapter 25

In vitro pharmacological assays in drug discovery

Mauro Corsi, Aptuit.

In vivo testing and translational approaches are part of the lead optimization process.

25.1 Principles in assay set up

Firstly, we need *assay robustness*: the assay must stay effective when we make some little changes. It must stay stable during time and the signal over time must be maintained. Another important aspect is to be able to set up assays with a limited number of cells. The assay myst be amenable to miniaturization and utomation and low cost/test point.

For the assay validation, an assay must have precision (same result when you repeat the experiment), accuracy, the limit of detection/quantification must be set, it must have specificity, linearity and robustness.

25.1.1 Cell-free assay

Classical targets are kinases: they are the most relevant intracellular target. An assay can involve a kinase inhibitor. We have to define certain parameters: kinase optimal concentration, ATP Km, substrate concentration and pharmacological identification.

Setting ADP: transform it in ADP that generates H_2O_2 and resofurine, that becomes fluorescent $\longrightarrow$measuring ADP formation with a fluorescence read-out.

Then we set the optimal concentration of the kinase: increasing the kinase up to a very high concentration $\longrightarrow$make a linear graph of fluorescence of y axis and kinase concentration on x axis.. The curves have a linear behaviour in the first part: this is the concentratio selected for the assay.

To set the concentration of ATP: simple saturation curve. The Km is the good point to set the concentration.

To set up cell based assay, there are 2 levels of cell system: recombinant cell system and native cell system. For the pharmacological read-out we use radiometric methods and ... Both systems have disadvantages and advantages: the native system comes from animals wherease a recombinant system contains immortalized cells. The number of receptors in native is limited and they not replicate at allo, while in recombinant we have a lot of receptors and they can grow very fast. Native cannot be used for screening, have multi-type of receptors and it's a physiological condition; recombinant can be used for screening, there is a unique type of receptors.

In its lab, they use recombinant systems.

Radiometric binding assay

Determine the affinity od a radio-ligand with a specific binding site. To prepare the cells for radiometric experiment: prepare some membranes from a cell line transfected with $\alpha 1$ receptor subtype. Cosidering he total binding and subtract the a-specific binding, we have the curve of the specific binding.

Second messenger based assay

G-protein coupled receptor: intracellular determination of cAMP levels. Receptor coupled with G-protein $\longrightarrow$dissociation of the G-protein $\longrightarrow$AC stimulated $\longrightarrow$production of cAMP. How can I measure cAMP? One way is to combine cAMP with a detection solution which allow protein kinase A to Pi its substrate using ATP: more cAMP is produces, less ATP is present and more light coming from the oxidation os ATP with luciferase is produced. To see a modulation, we have to increase the basal level of cAMP.

Another assay is the Ca assay that will determine the intracellular accumulation: ligand-gated ion channels let Ca enter. To measure the level of Ca, it is a direct measure of the modulation of these receptors. Once inside the cell, a fluorofore makes a complex with Ca producing fluorescence at 495 nm. We can generate a cell line expressing recombinant NMDA receptors. The subunits change in terms of expression when the age of the animal increase, and so their distribution.

For IP1 we can use the FRET technique.

Chapter 26

Electrophysiology platform for drug validation

Ion channels are in the second position of targerts (after G-protein coupled receptors), in particular Na channels and GABA channels. Lacidipine invented in Verona. To study ion channels: electrophysiology. At least 200 targets for therapeutic classification: the majority are voltage-gated Na and K channels, then cys-loop (like GABA) and glu metabotropic receptors.

Channelopathies: GOF or LOF mutations.

Output systems based on planar patch clamp: QPatch HTX uses plates of 48-wells, so we can perform experiments with 48 cells at the time. It provides pression controlled pathways. Cells are kept in suspension: at least 2 hours to work properly. After resuspension, the cells are applied to each wells $\longrightarrow$giga seal $\longrightarrow$whole cell configuration. In each well there are more than one cell (up to 10 cells) $\longrightarrow$increase of the signal, less variability between wells and increased possibility of success. With IonWorks Quattro we can record up to 384 wells: is a balance between quality and thoughtput and it is possible to reach manually each wells.

Population of cells: we can record a bigger amplitude of the signal and we have a tighter distribution with PPC that with single cell recordings. In ion works we use perforated membrane. Signel channel recording is not useful for screening, that's why we use whole cell configuration or perphorated path. With a single concentration: 4000 compounds per day (with Ion quattro), while manually only 3-4 compounds per day. Now we use ainly Qube and SyncroPatch 384PE. These devices must support giga-ohm seals (Barracuda is not able to perfom it).

The ligand-gated ion channels are still a challenge: we can measure the activation time constant, then the desensitization. There are really fast channels (AMPA/KAIN, $\tau < 1$ms). The time resolution of compound application

of automated system is between 10-100 ms, so studyin the fast channels is difficult because the time to applying the compound is longer than the time of activation of the channel. For this, we use *fast perfusion system* for manual patch: glass capillary is bend, a hole is made through the tube, the solution flows and the agonist goes out from the hole and goes to the cell. In order to have a faster application $\longrightarrow$Dynaflow: the cell is pathced, lifted up and placed in front of the channel for the solution you want to apply; we also have microperfusion.

If the cell is liftd up, the surface of the cell is invested synchronously by all solutions: the shape of current will change. There are few compounds that hit only one receptor subtype: mosly are non-selective.

We use recombinant cell-line: also iPSCs are used, but not for screening, and cardiomyocites. They prefere to use human derived cell lines (immortalized) or neuroblastoma cells (the ones used with Baj) for drug screening. It is important to look at native systems.

26.1 Drug discovery process

If thetanus in presence of AP5: no amplification. AMPA receptor is involved in long-term plasticity phenomena. Field potentials near basolateral amigdala, the signal il sblocked by AMPA receptors blocker.

We can look at the interaction of compounds with the different states of the channel, so look at the mechanism of activation/inactivation of the channel. Prolonging the depolarization phase force the channels to go in a slow inactivation phase: this is important to choose the physiological relevant protocol. Seres of depolarizing steps that mimics the AP: we can characterize the tonic blockers and the use-depending blockers.

We can follow the current amplitude in time upon application of increasin concentration of drug and construc the responsive concentration curve. To increase the potency of the compound, the chemist add some sostituents to the molecule.

26.2 Safety: lead optimization

Avoid side effect: submit the compound of interest to targets invlolved in adverse events. The aim is to anticipate safety evaluation: we need assays predictable of the in vivo effect of the compound, higher throuhgput and possibly low cost.

Chapter 27

mGluR5 Antagonists

Fabrizio Gasparini (Novartis).

PoC: working on a new target, if we have the proof of principle (prove the efficacy in a certain group of population) we can start the development.

mGlu5 receptors are expressed in brain areas invvolved in diseases such as fear/anxiety, depression, fragile X etc. The receptor was cloned in 1992. For the assay: many possibilities. Binding assay, targeting the Glu site, but there will be also some molecules similar to Glu that binds $\longrightarrow$we look at intracellular signalling of the receptor (PLC $\longrightarrow$IP3, that we can measure $\longrightarrow$release of Ca). The optimization process is not only for improve potency, but also for example to make soluble the molecule $\longrightarrow$make patentable the molecule. If the molecule is not patentable, the pharma will not accept it.

MPEP is very different from L-Glu: it is a non-competitive antagonist and interacts in the TM domain of mGlu5 receptor.

The expression of the receptor is high in hyppocampus and striatum while is low in cerebellum.

Chapter 28

Technical drug product development

Fabio Fais. Work in research and development.