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Cytoplasmic polyadenylation element binding protein (CPEB): a prion-like protein as a regulator of local protein synthesis and synaptic plasticity

1.INTRODUCTION

With this paper I would like to describe you what is my research project here at Columbia and how I am trying to address the many questions underlying my project by working everyday in the lab. But before doing this I feel somehow obliged to give you an introduction on the basic concepts of neurobiology. Therefore we will start with a brief definition and description of what is a neuron, how neurons interact to form synapse and neural circuits, how synapse activity can be modified and finally how these changes in synaptic activity underlie high cognitive processes such as learning and memory.
After providing you this, I hope not too boring introduction, I will go deeper into the molecular aspects of these phenomenon and I will illustrate you the main goal of my research, which is to characterize the role of a particular protein called Cytoplasmic Polyadenylation Element Binding protein with respect to the morphological and physiological changes that occur at the synapse after neuronal stimulation.

Memory

In psychology, **memory** is an organism's ability to store, retain, and subsequently recall information. Although traditional studies of memory began in the realms of philosophy, the late nineteenth and early twentieth century put memory within the paradigms of cognitive psychology. In recent decades, it has become one of the principal pillars of a new branch of science called cognitive neuroscience, a marriage between cognitive psychology and neuroscience.

There are several ways to classify memories, based on duration, nature and retrieval of information. From an information processing perspective there are three main stages in the formation and retrieval of memory:

- *Encoding* or *registration* (processing and combining of received information)
- *Storage* (creation of a permanent record of the encoded information)
- *Retrieval* or *recall* (calling back the stored information in response to some cue for use in a process or activity)

Classification

A basic and generally accepted classification of memory is based on the duration of memory retention, and identifies three distinct types of memory: sensory memory, short term memory and long term memory.

Sensory

Sensory memory corresponds approximately to the initial 200 - 500 ms after an item is perceived. The ability to look at an item, and remember what it looked like with just a second of observation, or memorization, is an example of sensory memory. With very short presentations, participants often report that they seem to "see" more than they can actually report. The first experiments exploring this form of sensory memory were conducted by George Sperling using the "partial report paradigm." Subjects were presented with a grid of 12 letters, arranged into three rows of 4. After a brief presentation, subjects were then played either a high, medium or low tone, cuing them which of the rows to report. Based on these partial report experiments, Sperling was able to show that the capacity of sensory memory was approximately 12 items, but that it degraded very quickly (within a few hundred milliseconds). Because this form of memory degrades so quickly, participants would see the display, but be unable to report all of the items (12 in the "whole report" procedure) before they decayed. This type of memory cannot be prolonged via rehearsal.

Short-term

Some of the information in sensory memory is then transferred to short-term memory. Short-term memory allows one to recall something from several seconds to as long as a minute without rehearsal. Its capacity is also very limited: George A. Miller, when working at Bell Laboratories, conducted experiments showing that the store of short term memory was 7±2 items (the title of his famous paper, "The magic number 7±2"). Modern estimates of the capacity of short-term memory are lower, typically on the order of 4-5 items, and we know that memory capacity can be increased through a process called chunking. For example, if presented with the string:

FB IPH DTW AIB M

people are able to remember only a few items. However, if the same information is presented in the

following way:

FBI PHD TWA IBM

people can remember a great deal more letters. This is because they are able to chunk the information into meaningful groups of letters. Beyond finding meaning in the acronyms above, Herbert Simon showed that the ideal size for chunking letters and numbers, meaningful or not, was three. This is evidenced by the tendency to remember phone numbers as several chunks of three numbers with the final four-number groups generally broken down into two groups of two.

Short-term memory is believed to rely mostly on an acoustic code for storing information, and to a lesser extent a visual code. Conrad (1964) found that test subjects had more difficulty recalling collections of words that were acoustically similar (e.g. dog, fog, bog, log).

Long-term

The storage in sensory memory and short-term memory generally have a strictly limited capacity and duration, which means that information is available for a certain period of time, but is not retained indefinitely. By contrast, long-term memory can store much larger quantities of information for potentially unlimited duration (sometimes a whole lifespan). Whilst short-term memory encodes information acoustically, long-term memory encodes it semantically. Baddeley (1966) found that after 20 minutes, test subjects had the greatest difficulty recalling a collection of words that had similar meanings (e.g. big, large, great, huge).

Short-term memory is supported by transient patterns of neuronal communication, dependent on regions of the frontal lobe (especially dorsolateral prefrontal cortex) and the parietal lobe. Long-term memories, on the other hand, are maintained by more stable and permanent changes in neural connections widely spread throughout the brain. The hippocampus is essential to the consolidation of information from short-term to long-term memory, although it does not seem to store information itself. Rather, it may be involved in changing neural connections for a period of three months or more after the initial learning.

One of the main functions of sleep is thought to be to improve consolidation of information, as it can be shown that memory depends on getting sufficient sleep between training and test, and that the hippocampus replays activity from the current day while sleeping. For example, if we are given a random seven-digit number, we may remember it for only a few seconds and then forget, which means it was stored into our short-term memory. On the other hand, we can remember telephone numbers for many years through repetition; those long-lasting memories are said to be stored in our long-term memory.

Physiology

Overall, the mechanisms of memory are not well understood. Brain areas such as the hippocampus, the amygdala, or the mammillary bodies are thought to be involved in specific types of memory. For example, the hippocampus is believed to be involved in spatial learning and declarative learning. Damage to certain areas in patients and animal models and subsequent memory deficits is a primary source of information. However, rather than implicating a specific area, it could be that damage to adjacent areas, or to a pathway traveling through the area is actually responsible for the observed deficit. Further, it is not sufficient to describe memory, and its counterpart, learning, as solely dependent on specific brain regions. Learning and memory are attributed to changes in neuronal synapses, thought to be mediated by long-term potentiation and long-term depression.

The coding unit: the neuron

Neurons (also known as **neurones** and **nerve cells**) are electrically excitable cells in the nervous system that process and transmit information. Neurons are typically composed of a soma, or cell body, a dendritic tree and an axon. The majority of vertebrate neurons receive input on the cell body and dendritic tree, and transmit output via the axon. However, there is great heterogeneity throughout the nervous system and the animal kingdom, in the size, shape and function of neurons.

Neurons communicate via chemical and electrical synapses, in a process known as synaptic transmission. The fundamental process that triggers synaptic transmission is the action potential, a propagating electrical signal that is generated by exploiting the electrically excitable membrane of the neuron.

The neuron's role as the primary functional unit of the nervous system was first recognized in the early 20th century through the work of the Spanish anatomist Santiago Ramón y Cajal (reviewed in Grant, 2007).

Cajal proposed that neurons were discrete cells that communicated with each other via specialized junctions, or spaces, between cells. To observe the structure of individual neurons, Cajal used a silver staining method developed by his rival, Camillo Golgi. The Golgi stain is an extremely useful method for neuroanatomical investigations because, for reasons unknown, it stains a very small percentage of cells in a tissue, so one is able to see the complete microstructure of individual neurons without much overlap from other cells in the densely packed brain (López-Muñoz, 2006).

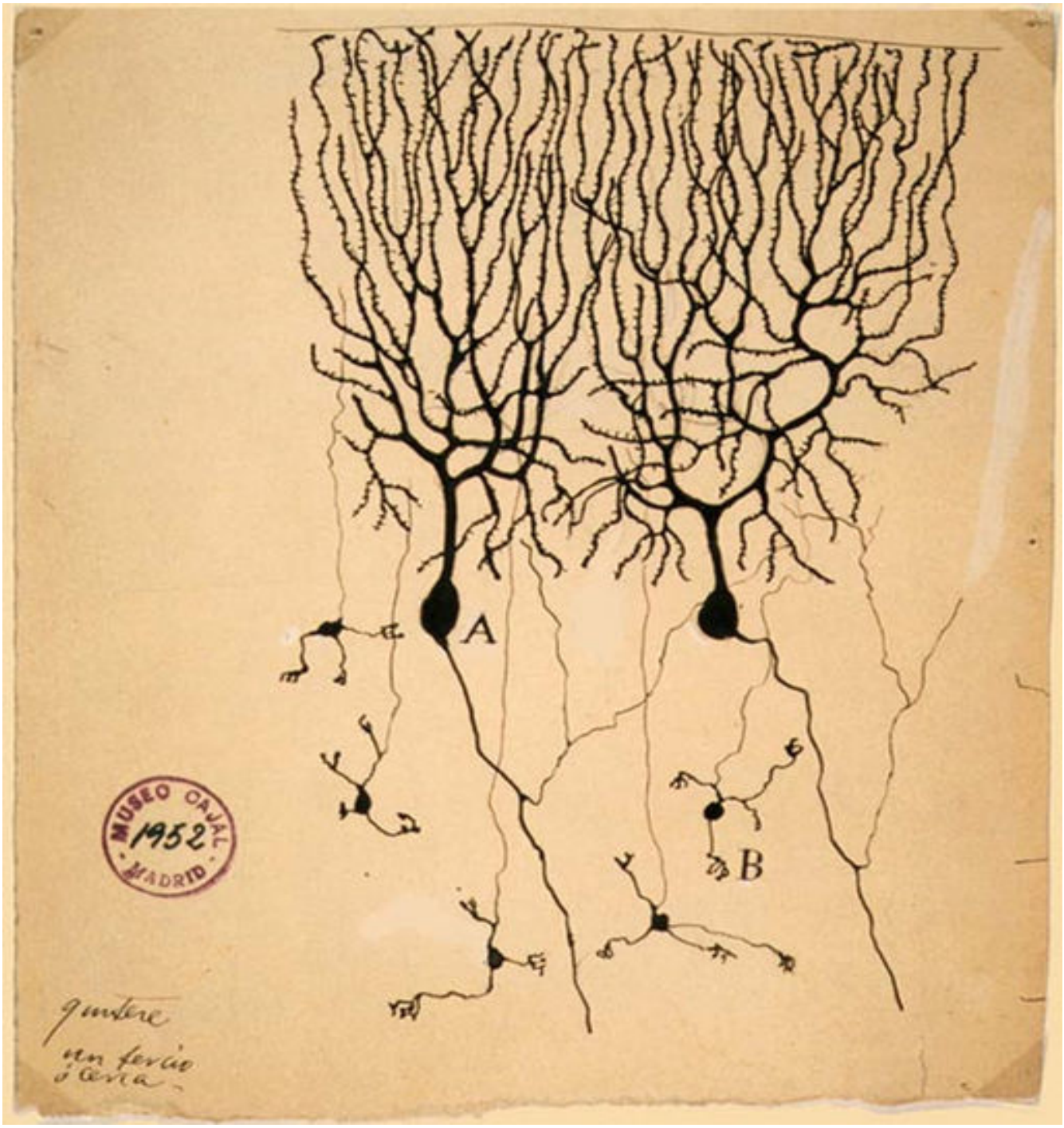


Fig1. This is an illustration of Cajal staining of Purkinje cells in the cerebellum. These cells are characterized by a very complex dendritic arborization.

Anatomy and histology

Neurons are highly specialized for the processing and transmission of cellular signals. Given the diversity of functions performed by neurons in different parts of the nervous system, there is, as expected, a wide variety in the shape, size, and electrochemical properties of neurons. For instance, the soma of a neuron can vary from 4 to 100 micrometers in diameter.

- The soma is the central part of the neuron. It contains the nucleus of the cell, and therefore is where most protein synthesis occurs.
- The dendrites of a neuron are cellular extensions with many branches, and metaphorically this overall shape and structure is referred to as a dendritic tree. This is where the majority of input to the neuron occurs. Information outflow (i.e. from dendrites to other neurons) can also occur, but not across chemical synapses; there, the backflow of a nerve impulse is inhibited by the fact that an axon does not possess chemoreceptors and dendrites cannot secrete neurotransmitter chemicals. This unidirectionality of a chemical synapse explains why nerve impulses are conducted only in one direction.
- The axon is a finer, cable-like projection which can extend tens, hundreds, or even tens of thousands of times the diameter of the soma in length. The axon carries nerve signals away from the soma (and also carry some types of information back to it). Many neurons have only one axon, but this axon may - and usually will - undergo extensive branching, enabling communication with many target cells.
- The **axon terminal** is a specialized structure at the end of the axon that is used to release neurotransmitter chemicals and communicate with target neurons.

The longest axon of a human motoneuron can be over a meter long, reaching from the base of the spine to the toes. Giraffes have single axons several meters in length running along the entire length of their necks. Much of what is known about axonal function comes from studying the squid giant axon, an ideal experimental preparation because of its relatively immense size (0.5–1 millimeters thick, several centimeters long).

Classification by action on other neurons

- **Excitatory neurons** excite their target neurons. Excitatory neurons in the brain are often glutamatergic.
- **Inhibitory neurons** inhibit their target neurons. Inhibitory neurons are often interneurons. The output of some brain structures (neostriatum, globus pallidus, cerebellum) are inhibitory. The primary inhibitory neurotransmitters are GABA and glycine.

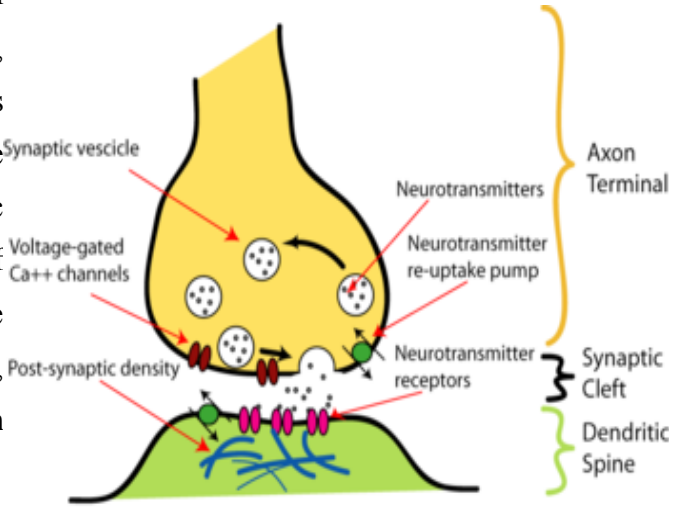
- **Modulatory neurons** evoke more complex effects termed neuromodulation. These neurons use such neurotransmitters as dopamine, acetylcholine, serotonin and others.

Glutamate is the most abundant fast excitatory neurotransmitter in the mammalian nervous system. At chemical synapses, glutamate is stored in vesicles. Nerve impulses trigger release of glutamate from the pre-synaptic cell. In the opposing post-synaptic cell, glutamate receptors, such as the NMDA receptor, bind glutamate and are activated. Because of its role in synaptic plasticity, it is believed that glutamic acid is involved in cognitive functions like learning and memory in the brain.

Glutamate transporters are found in neuronal and glial membranes. They rapidly remove glutamate from the extracellular space. In brain injury or disease, they can work in reverse and excess glutamate can accumulate outside cells. This process causes calcium ions to enter cells via NMDA receptor channels, leading to neuronal damage and eventual cell death, and is called excitotoxicity. Excitotoxicity due to glutamate occurs as part of the ischemic cascade and is associated with stroke and diseases like amyotrophic lateral sclerosis, and Alzheimer's disease. Glutamic acid has been implicated also in epileptic seizures. Microinjection of glutamic acid into neurons produces spontaneous depolarisations around one second apart, and this firing pattern is similar to what is known as paroxysmal depolarising shift in epileptic attacks.

Connectivity between neurons: Synapse

Neurons communicate with one another via synapses, where the axon terminal of one cell impinges upon a dendrite or soma of another (or less commonly to an axon). The word "synapse" comes from "synaptein" which Sir Charles Scott Sherrington and his colleagues coined from the Greek "syn-" meaning "together" and "haptein" meaning "to clasp". Chemical synapses are not the only type of biological synapse: electrical and immunological synapses exist as well. Without a qualifier, however, "synapse" by itself most commonly refers to a chemical synapse. Neurons such as Purkinje cells in the cerebellum can have over 1000 dendritic branches, making connections with tens of thousands of other cells; other neurons, such as the magnocellular neurons of the supraoptic nucleus, have only one or two dendrites, each of which receives thousands of synapses.



In a chemical synapse, the process of synaptic transmission is as follows: when an action potential reaches the axon terminal, it opens voltage-gated calcium channels, allowing calcium ions to enter the

terminal. Calcium causes synaptic vesicles filled with neurotransmitter molecules to fuse with the membrane, releasing their contents into the synaptic cleft. The neurotransmitters diffuse across the synaptic cleft and activate receptors on the postsynaptic neuron. Immediately behind the post-synaptic membrane is an elaborate complex of interlinked proteins called the postsynaptic density. Proteins in the postsynaptic density serve a myriad of roles, from anchoring and trafficking neurotransmitter receptors into the plasma membrane, to anchoring various proteins which modulate the activity of the receptors.

The human brain has a huge number of synapses. Each of the 10^{12} neurons — one billion (long scale) has on average 7,000 synaptic connections to other neurons. It has been estimated that the brain of a three-year-old child has about 10^{16} synapses (10,000 billion). This number declines with age, stabilizing by adulthood. Estimates vary for an adult, ranging from 10^{15} to 5 x 10^{15} synapses (1,000 to 5,000 thousand billion) (Drachman D (2005)). Chemical synapses allow the neurons of the central nervous system to form interconnected neural circuits. They are thus crucial to the biological computations that underlie perception and thought.

Synaptic plasticity

The strength of a synapse is defined by the change in transmembrane potential resulting from activation of the postsynaptic neurotransmitter receptors. This change in voltage is known as a post-synaptic potential, and is a direct result of ionic currents flowing through the post-synaptic receptor-channels. Changes in synaptic strength can be short–term and without permanent structural changes in the neurons themselves, lasting seconds to minutes — or long-term (long-term potentiation, or LTP), in which repeated or continuous synaptic activation can result in second messenger molecules initiating protein synthesis in the neuron's nucleus, resulting in alteration of the structure of the synapse itself. Learning and memory are believed to result from long-term changes in synaptic strength, via a mechanism known as synaptic plasticity.

Two known molecular mechanisms for synaptic plasticity were revealed by research in laboratories such as that of Eric Kandel. The first mechanism involves modification of existing synaptic proteins (typically protein kinases) resulting in altered synaptic function (Shi et al., 1999). The second mechanism depends on second messenger neurotransmitters regulating gene transcription and changes in the levels of key proteins at synapses. This second mechanism can be triggered by protein phosphorylation but takes longer and lasts longer, providing the mechanism for long-lasting memory storage. Long-lasting changes in the efficacy of synaptic connections (long-term potentiation, or LTP) between two neurons can involve the making and breaking of synaptic contacts

Long-lasting changes in synaptic connectivity (long-term potentiation, or LTP) depend on signals that are initiated at the synapse and go back to the nucleus where they serve to activate gene transcription. The products of gene transcription are sent to all synaptic terminals but only those synapses that are "marked" by the short-term process can successfully utilize those gene products, as it is shown in the following picture (Martin, 1999).

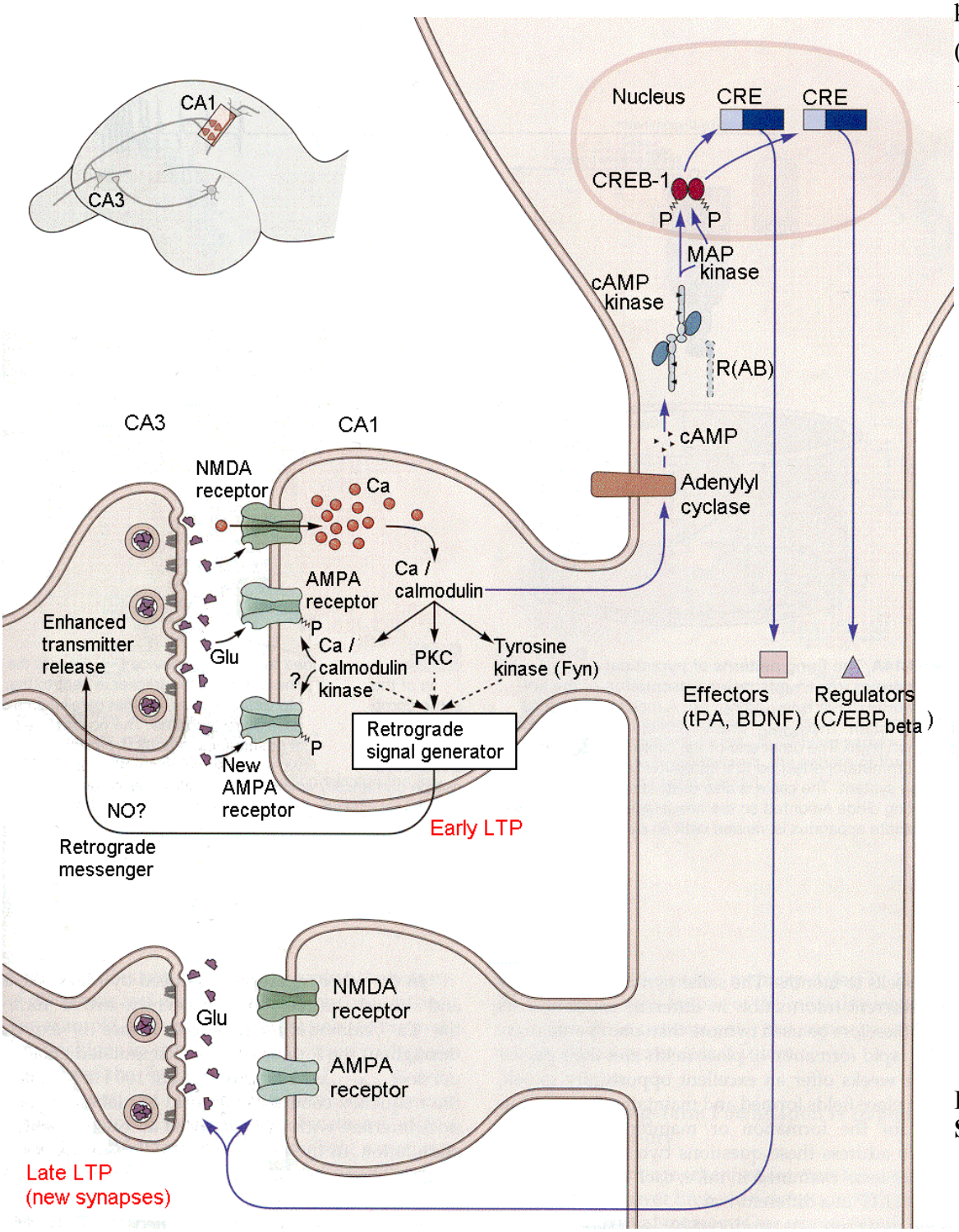


Fig2. Schematic representation of the induction of LTP in the mouse hippocampus. Glutamate is released from an axon terminal and binds to receptors on the dendritic side (NMDA and AMPA receptors). Calcium ions

enter through the receptors and activates several enzymes, Protein Kinase C and A (PKC, PKA), which modify other proteins, the so called "effectors", like CREB, which will induce the expression of genes in the nucleus of the activated neuron. These genes will be translated into proteins, that will be delivered to the marked site and will also participate in building new synaptic connections.

There are two components of this marking signal: covalent modification via an enzyme called protein kinase A (PKA), which is necessary to mark the synapse for growth, and local protein synthesis, which is required for the persistence of structural change.

Local protein synthesis

What are the molecules that stabilize the learning-related synaptic growth for the persistance of long-term memory? Si et al, (2003) in Kandel's laboratory found that a protein called cytoplasmic polyadenylation element-binding protein (CPEB), a regulator of local protein synthesis, exists in a particular form in the nervous system of *Aplysia* and stabilizes newly formed synaptic connections. We are now extending the analysis to the closest mammals homologue of ApCPEB, called CPEB3, where 3 means that this isoform has been the third to be identified among the four known at present (Theis et al, 2003).

What is the function of CPEB in the neurons? CPEB was first described as a protein able to activate translationally dormant mRNAs (ribonucleic acid messenger) in Xenopus oocytes, which it does by binding a regulatory sequence, called cytoplasmic polyadenylation elements (CPEs) within some mRNAs.

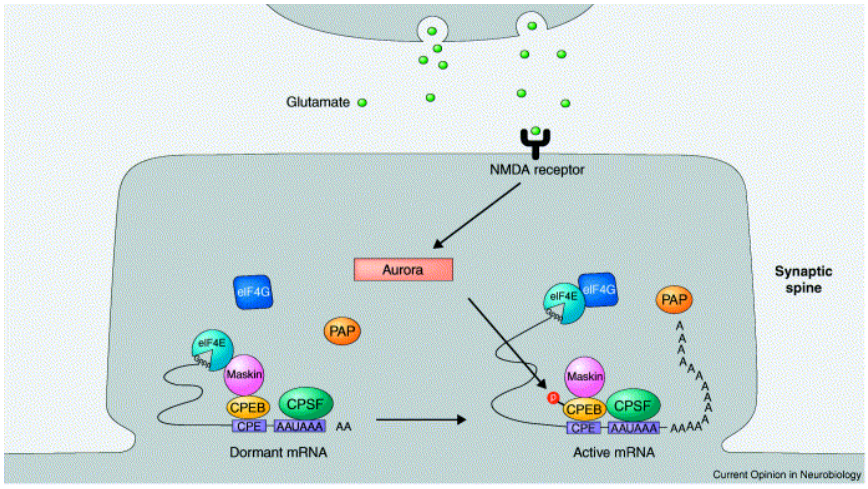
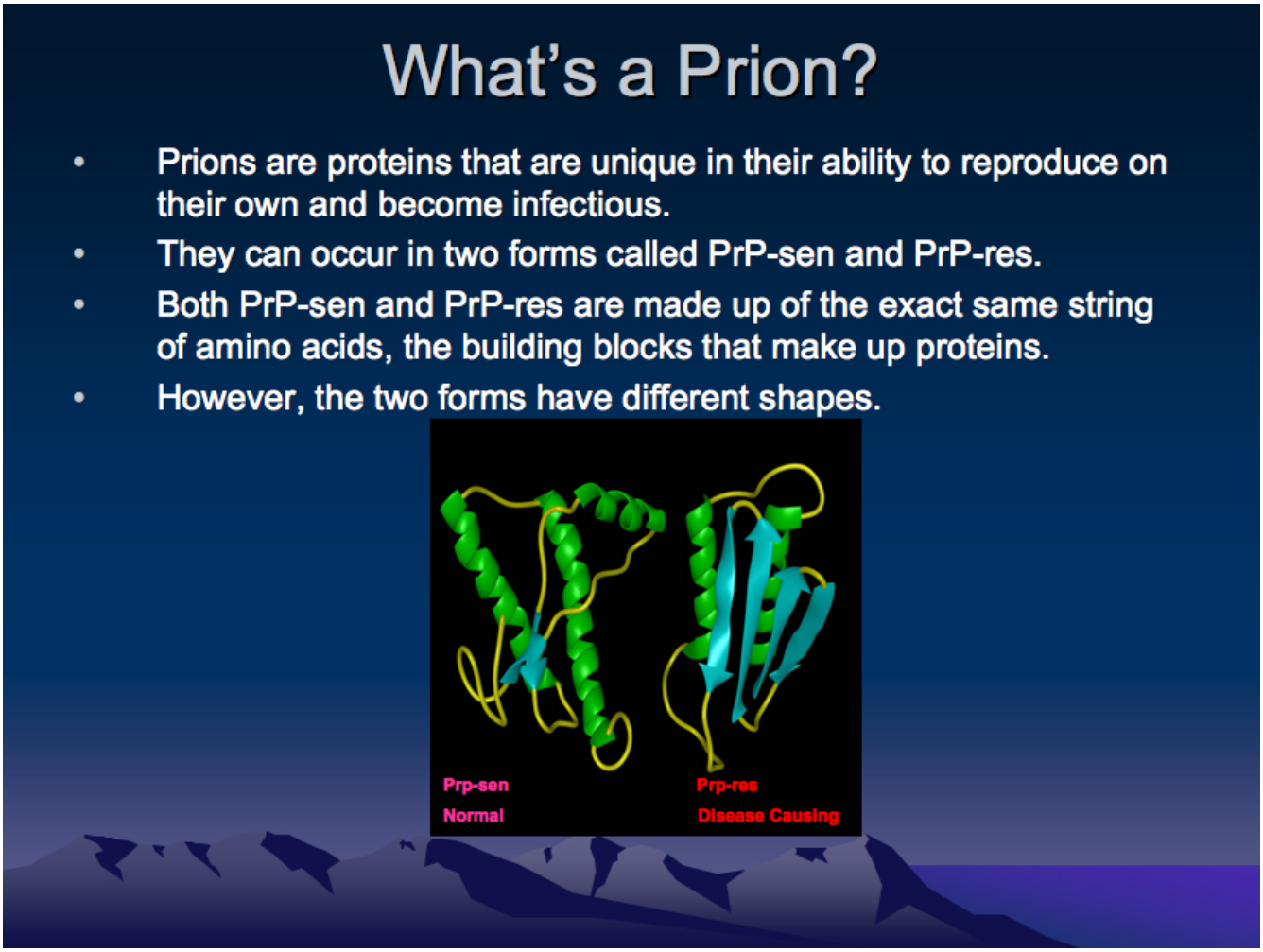


Fig3. Schematic representation of local protein synthesis regulation operated by CPEB proteins. Gluatamate activates NMDA receptors, which in turn transfer their activated state to other protein Kinase, like Aurora. Aurora phosphorylates CPEB inducing a conformational change which reduces the affinity of another protein, Maskin, for the translation initiation complex, eIF4E and eIF4G.

CPEB regulates mRNA translation through a number of mechanisms, balancing interactions with proteins that downregulate and activate translation. (Huang et al. 2003 and Richter 2001).

How can these proteins stabilize synapses? The first 150 amino-acids of ApCPEB and CPEB3 constitute a domain that is very similar to that of "prions" (pathogenic protein particles responsible for a

number of neurodegenerative fatal disorders that affect both humans, (Creutzfeldt Jacob disease) and animals (scrapie and mad cow disease) (Prusiner, 1982).



Like prions, CPEB can exist in two conformationally distinct isoforms but only one is metabolically active, the dominant form, characterized by a self-perpetuating aggregate state. In the lab we are testing the idea that these aggregates bind to dormant mRNA resident at the synapse and modify them in order to be translated and give rise to proteins that stabilize the synaptic growth. Moreover, CPEB could maintain the continuing protein synthesis that stores a memory long after the learning experience has passed, due to its prion-like, self-perpetuating qualities.

2. AIMS OF THE PROJECT

The major aim of this project is to clarify the molecular events leading to the conformational changes of CPEB at the marked synapse. But what do we know about conformational changes that happen in the prototype of prions, the so called PrP (Prion Protein)?

Over the past 30 years different hypotheses have been formulated to explain prion formation. In

the so called "nucleated polymerisation model" (Gajdusek, 1988; Jarrett and Lansbury, 1993), oligomerization of a prion protein is required to stabilize the aggregated form and allow its accumulation at biologically relevant levels (i.e able to induce the appearance of a neurological pathology). Spontaneous formation of the initial template (or seed) of prions is rare because of the weak interactions between monomeric, soluble molecules and the oligomer. However, once formed, oligomeric or polymeric seeds are stabilized by multivalent interactions. Formation of a seed may be a spontaneous event (Caughey et al., 1995; Jarrett and Lansbury, 1993) or, as seems to be the case for CPEB, it could be initiated by an appropriate stimulus such as the action of a neurotransmitter at the synapse. This stimulation could lead to an increase in the expression level of CPEB protein thus increasing the probability of a conformational change among the many CPEB molecules produced. Additional molecules could regulate the conversion process. In particular, a class of proteins called "chaperon proteins" are known to assist other proteins during their folding, and these chaperones could play an important role in the conformational change of CPEB.

In the next few pages I will show you that indeed after synaptic stimulation there is an increase in CPEB protein level in the neuron and, even more interestingly a change occur in the biochemical properties of this protein, which becomes more aggregated, thus suggesting that our initial hypotheses on the mechanism of action of CPEB might be correct. To further investigate how this change in conformation might be regulated, I started studying the role of chaperons and I found that it is possible to detect sites where CPEB and chaperons reside together, suggesting that they might physically interact.

3. RESULTS

To examine if the prion domain of CPEB causes self-perpetuation in neurons and if this is the mechanism that maintains long-term memory in neurons, I focused on the relationship between the physical, aggregated state of CPEB and the activity of the synapse. First of all I expressed a modified version of CPEB, containing a fluorescent dye tag, in neurons. This modification allowed me to observe the distribution of CPEB in neurons and also its biophysical state. Indeed the protein distributes with a homogeneous pattern when is completely soluble, whereas in an aggregated state it forms distinguishable aggregated puncta within neurons.

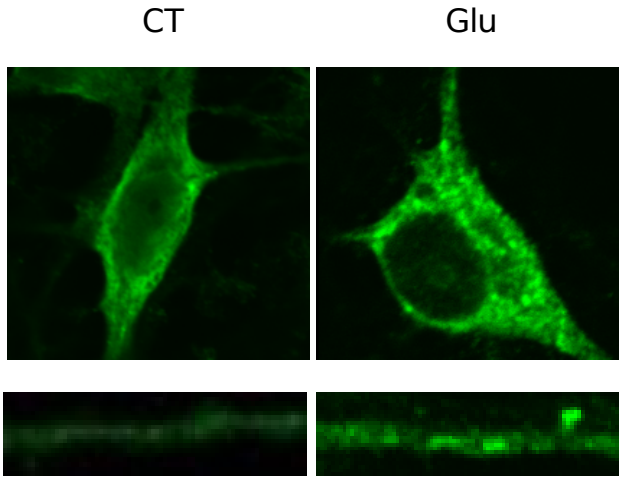


Fig4. CPEB induction in neurons stimulated with glutamate. Cells were stained with an antibody specific for CPEB3. before stimulation (Controls, CT) CPEB3 shows a diffuse pattern, while after application of the excitatory neurotransmitter Glutamate (GLU) CPEB3 forms aggregated structures, which are detectable in either the soma (upper panels) or the distal dentrites (lower panels).

Subsequently I compared the properties of CPEB (i.e the tendency to form aggregates) before and after neurotransmitter stimulation of the neuron. Protein extracts were taken from the stimulated neurons, and analyzed by a specific centrifugation assay that permits me to separate the soluble fraction of the proteins from the insoluble, aggregated fraction, in which CPEB should reside.

These biochemical analysis are supported by morphological studies examining the localization of the CPEB protein at the synapse and its association to other already known components of the translational machinery, as the main goal of this project is to study how CPEB aggregation is implicated in the regulation of new protein synthesis and therefore learning-related changes in synaptic function and structure.

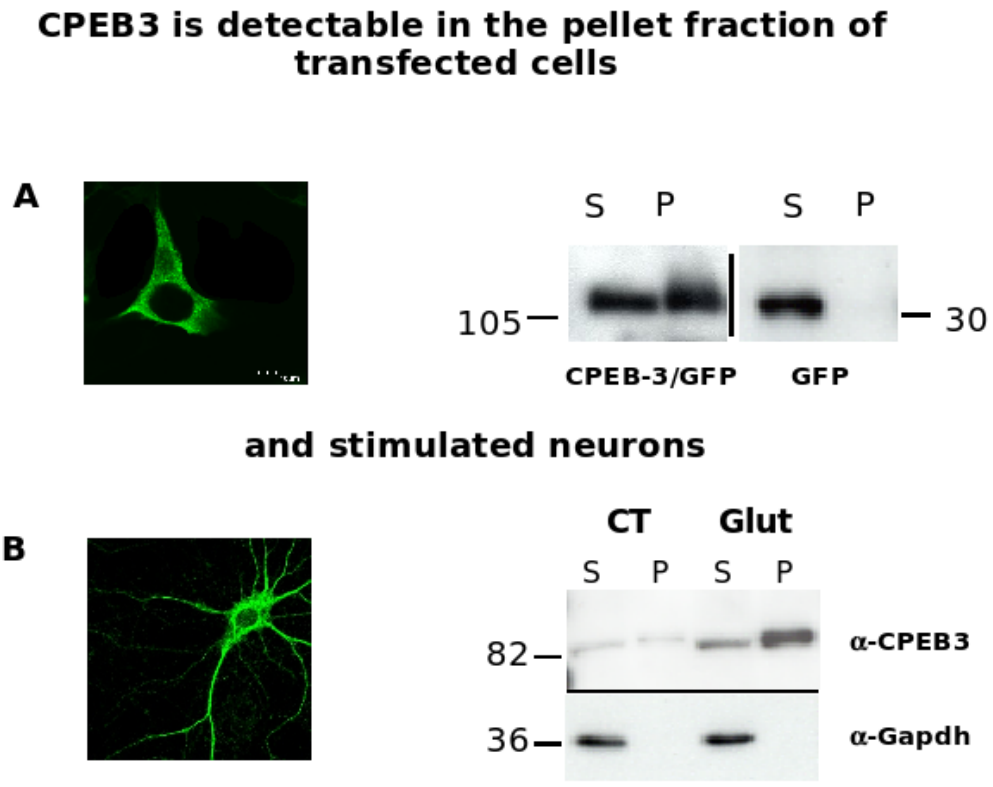


Fig5. CPEB3 possesses biochemical properties reminiscent of prions.
Proteins were extracted from (A) epithelial cells (cells transfected with DNA coding CPEB3 protein to overexpress it) and (B) neurons. The overexpression of CPEB3 is promoting the aggregation of the protein, which will partly distribute in the pellet fraction (P). Interestingly in neurons treated with glutamate there is a strong increase in the amount of protein distributing in the pellet, suggesting that neuronal activation is responsible for this shift between soluble (S) and insoluble state (P). Glyceraldehyde-3-phosphate dehydrogenase protein is used as an internal control, since it is a soluble metabolic enzyme. The numbers on the left side of the pictures represent the molecular weight expressed in kilo daltons of the analyzed proteins.

Conclusions

After one year of studies there are still many experiments to carry on in order to establish a connection between the current data derived from experiments in isolated neurons in culture and the intact animal. I have only recently started working with transgenic mice which express a modified version of the CPEB protein One aspect is of particular interest, and concerns the regulation of this aggregational process. Nobody indeed would like to have a "crazy" protein forming aggregates inside our neurons since this will turn most likely into a danger for the physiology and survival of the neuron. Therefore it will be of great interest to identify the proteins that may interact with CPEB to control the propagation of its prion state. Moreover in mammals, neuronal RNA binding proteins in addition to CPEB may also play roles in the regulation of synaptic RNAs. For instance, the fragileX mental retardation syndrome results from the lack

of an RNA binding protein believed to be present in the synapse and to play a role in synaptic plasticity (Jin and Warren, 2003). Identification of key RNA binding proteins involved in synaptic plasticity thus whets one's appetite for knowing what RNAs are being regulated, and this will be in the next future one of my projects.

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