The History of the Synapse

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Why did I choose this particular topic for my lecture rather than the history of neuroscience or the history of the neuron? Simply because I believe that every disciple has the obligation to pay homage to their mentors once in their lifetime. My formation as a neuroscientist involved three such mentors spanned across three countries. The first was Spain, where I was born, completed my medical studies, and had my first glimpse of neuroscience at the Cajal Institute with Fernando de Castro. It was him who, in 1961, advised me to spend some time abroad, and to that purpose he obtained me a scholarship from the French government, that allowed me to settle in Paris. Once in France I had the good fortune to meet Prof. René Couteaux, another generous mentor, who took care of my stay in the country. Two years later he made me a proposition to which I could only answer in the affirmative by offering me a research position in France. I got married (the best thing that happened in my life), and spent the next 57 years working on the cerebellum. The third person I want to honor and remember in this presentation is Sanford Louis Palay who was my postdoc professor during the two years I worked at Harvard Medical School in Boston. And as it turns out, all three of my mentors have made positive contributions to the history of the synapse. So, without further delay, let's dive in.

PART I

FROM THE « PNEUMA » OF ARISTOTLE TO THE « ANIMAL ELECTRICITY » OF GALVANI, PASSING THROUGH THE « ANIMAL SPIRITS » OF DESCARTES,

I won't start like many others, by going as far back as Egyptian papyruses. But I will simply remind you that, as early as the 4th century BC, the Greek

philosophers, and especially Aristotle, had already developed the dualistic concept of body and mind, or as I prefer to think about it, “Brain and Mind” (see Robinson, 1978). This concept has captured mankind’s imagination since the dawn of time. The brain, as you all know, is our interface to the outside world. It is what allows us to communicate. Our senses receive visual, auditory, and many other physical stimuli that reach the brain, and as a result we can respond, not only with physical actions such as walking or running but also with mental perceptions involving consciousness. How the brain is formed and how it works is a question we constantly ask ourselves. And it's a complicated thing to answer. The brain is something physical that can be seen and analyzed. You just need to open the cranial vault to reveal the soft, not very pliable mass filling it. But how does it work?

The first difficulty was to imagine how the brain could communicate with muscles and organs and, at the same time, take on all the responsibilities of our mental life. Aristotle (see Robinson, 1978) was the first to try to answer that question with his metaphysical approach of "pneuma", or divine breath, the driving force of the brain. Unfortunately, thanks to Thomas Aquino among others, this unscientific idea survived for a very long time, until the arrival of French philosopher, René Descartes in 1664. In his *Traité de l'homme* ("Treatise on Man"), he proposed a new concept regarding the brain and the mind. Even if his anatomical ideas were incorrect (I am referring to the mind as an extracorporeal entity, expressed through the pineal gland), functionally speaking his conclusions were close to reality. For him, the communication between the brain and the body was made through the nerves. The information generated in the brain, what he called “animal spirits”, flowed from the brain to the muscles through the nerves, that he considered being hollow. Years later, thanks to Antonie van Leeuwenhoek’ (1695) advances in microscopy it was established that the nerves coming out of the brain and spinal cord were made of unitary fibrils (the nerve fibers), and that each one appeared to have a hollow structure at their center, giving Descartes’ ideas some validation. Although the nature of the “animal spirits” remained unclear, it was obvious for Descartes that they were of a physical nature.

The real starting point in our history of the synapse took place a hundred and twenty-seven years later when Luigi Galvani (1791) published his paper *De viribus electricitatis in motu musculari. Commentarius*. It presented 10 years of observations that all started on a quiet night, when Galvani was conducting an experiment with the assistance of his wife on the terrace of his house in Bologna. Earlier that day, they had dissected a frog to its inferior limbs with a part of the spinal cord and the crural nerves still attached, and suspended it on the iron balcony by a metallic hook inserted in the spinal cord (Piccolino, 2006). When the metal of the hook and that of the balcony were in contact, the interference of both metals managed to stimulate the nerves, and they saw the frog's leg contract. This unexpected experiment pointed to electricity as being the component of Descartes' animal spirits. However, there was a later disagreement between Galvani and Volta in regard to the origin of such electricity. Was it produced by the interaction of the metals as suspected by Volta? Or, on the contrary, as indicated by Galvani, was this electricity simply created by the nervous system itself? And if yes, what components of the nervous system could generate it?

Emil du Bois-Reymond (1848), a German physiologist working in Berlin, solved the problem 57 years later. He was the first who managed to record, using a sensitive galvanometer, he developed himself, what is known today as the action potential. It clearly put in evidence that the nervous system works using electricity and that this electricity is produced by the corpuscular elements that make up the nervous system. For this reason, Galvani is recognized today as the father of electrophysiology.

HISTORY OF THE PROGRESS OF OUR KNOWLEDGE ON THE STURTURE OF THE NERVOUS SYSTEM

When an engineer receives a new machine, the first thing he wants to do in order to understand how it works, is to identify its components by examining each single piece individually. Similarly, in biology, to understand an organ's function, you must analyze it composition. This is the job of a

neuromorphologist, and the reason why it was imperative to know the structure of the nervous tissue in order to understand its functionality. But there were major difficulties in the microscopic study of the nervous tissue. The average 1.5 kg of brain tissue is made of 70% water, 21% lipids and 9% proteins. This composition results in a soft mass, difficult to manipulate and to cut into thin slices. Even when scientists tried to look at it with compound microscopes, they couldn't see anything because the sections weren't transparent enough. As a result, their ideas about the brain were very approximate, such as those of German scientist Ehrenberg (1836) and the Czech Purkinje (1837). For these pioneers the brain and spinal cord were composed of a series of “globules” from which thin “fibers” emerged, coming out of the nervous system and coursing through the whole body. Now that we know that electricity produced by these “globules” lying in the brain and spinal cord is what actually flows through those fibers, it is easier to understand the big conceptual challenges to unravel the nervous tissue’s morphological organization.

Major progress was achieved by German investigators in the 19th century. This progress has allowed us to move from the concept that every living being is composed of "tissues" as postulated by Bichat (1799) in Paris, to Rudolph Virchow's (1858) current concept of cell theory. In 1838, Matthias Schleiden, a botanist, was the first to visualize each of these cells in plants, as they have an outer cellulose membrane visible through a simple microscope, which allowed him to conclude that the entire plant is composed of cells. Almost simultaneously, Theodor Schwann (1838), who discovered the Schwann cells in peripheral nerves, said that animals were also made up of small single elements: the cells became the fundamental units of living organisms. Cell theory reached its peak thanks to Rudolf Virchow’s "Omnia cellula e cellula" (Every cell comes from a cell) principle (1858). Virchow was a great German pathologist considered to be the father of cell theory.

In 1858, the cell theory had been corroborated in almost all tissues of the body. The discovery of the cell was for biology what the discovery of the atom was for physics: a true revolution. But how was the nervous system perceived?

As previously mentioned, it is very difficult to work with the nervous system because of its physico-chemical characteristics and for this reason there was no consensus yet on its cellular organization. In the middle of the 19th century new histological techniques began to appear. The Italian Rolando (1829) was the first to use fixatives, mainly low concentrations of potassium dichromate, to harden tissues so they could be easier to handle and cut into sections to observe under a microscope. This approach led to new and important discoveries.

For example, it was used by Otto Deiters (1865) to harden the nervous tissue of the cow spinal cord allowing him to dissect the motoneurons of the anterior horn, using only needles and small pincers. He was amazed to find that the "globules" described by Ehrenberg were much more complicated than originally believed. He saw that each globule had a soma from which emerged numerous processes that branched profusely in what he called protoplasmic processes, as well as a unique and long extension he named the axis cylinder. Today, protoplasmic processes are known as dendrites, and the axis cylinder as the axon. Based on his results, Otto Deiters could then summarize that the basic components of the brain were complex structures composed by a central soma with two different kinds of irradiating processes, thus generalizing their anatomical and functional duality. In his detailed schemas, Deiters drew the stem dendrites broken about 50 μm away from the cell body. Similarly, their secondary and even tertiary branches were also broken. Deiters' schema corroborated the fact that microdissection cut the distal part of the arborizing processes.

INSERT FIGURE 1

It is also of great historical interest to note that Deiters drew triangular structures attached to the surface of the persisting dendritic branches. These triangular structures generated long thin broken tails that were interpreted as fine outgoing fibers (abgehende Fasern) proceeding from the dendrite. He considered these to be a second system of processes from which stemmed large myelinated fibers. Nowdays, Deiters' triangular structures would immediately be

identified as presynaptic boutons establishing synapses on the motoneuron dendrites. We know today, that the synaptic complex is a special type of membrane junction very difficult to brake and therefore that during the microdissection, the thin presynaptic axons which are much weaker would brake more easily than the synaptic complex. Why did Deiters interpret his observations as if there were continuity between dendritic extensions and axons? I believe that because electricity was the essential driving force of the nerve function, most neuroanatomists of that era were puzzled by the fact that if the "globules" forming the nervous system were independent cells how could the electricity flowing within these processes, jump from cell to cell?

Unfortunately, Deiters made a mistake interpreting his own material. It is Joseph von Gerlach (1871, 1872), a German professor, who was able to convert a baseless hypothesis into a trusted axiom, even though had no evidence. Gerlach formulated his reticularist doctrine in which the elements of the nervous system formed an immense syncytium, a sort of continuous network, through its processes. This erroneous concept was rapidly accepted by many students of the nervous system and became extremely popular during the second part of the 19th century. However, Cajal in his treaty "*Histologie du Système Nerveux de l'Homme et des Vertébrés*" (1909) had very harsh words in regards to Gerlach's work: *"…a theory that, for more than twenty years, we are going to see exert a disastrous influence on the direction of neurological researches, and this, in spite of all the proofs of its falsity… All the rest is false interpretations, vain affirmations, we would even say, childish*".

SANTIAGO RAMON Y CAJAL AND THE "NEURON DOCTRINE"

As previously mentioned, most if not all, important scientific advances are the result of technological progress. We were able to study the nervous tissue composition either because of the improvement of analytical tools such as microscopy, or because of the emergence of new methodologies like, for instances, new staining techniques, such as silver impregnations, immunochemical methods, or others. In 1873, the hardening of the nervous

tissue in potassium dichromate was at the origin of an extraordinary haphazard error leading to a very important discovery by Italian professor Camillo Golgi.

Following Rolando's recommendations, Golgi used potassium dichromate as a hardening fixative in his Pavia home-based small histology laboratory. One day, a small piece of nervous tissue that had been hardened in this fixative fell into an open jar of low concentration (between 1 and 2%) silver nitrate. When Golgi noticed it a few days later, instead of throwing the experiment away, he had the curiosity to take the tissue out and cut it to see what had happened to it. This is how Golgi discovered what he called the "reazione nera" or black reaction, a miraculous way to visualize single nerve cells in their entirety and the start of a new era in Neurosciences. Indeed, in the silver impregnated brain sections, the nerve cells, the components of the nervous system, turned black over a yellowish background, reveling with clarity the complete shape of individual cells with their soma, dendrites and single axon. Golgi's work during those years was very fruitful, and important advances were made. First, he corroborated the dualistic nature of nerve cell processes proposed by Deiters. He observed that dendrites, contrary to Deiters' description, end freely and are not a continuation of the axons or dendrites of other nerve cells. For Golgi, dendrites were oriented towards the blood vessels and, therefore, considered as nutrient appendices for the survival of the whole structure. However, the axons were devoid of free terminations and, as functional processes, they were establishing a continuous network first described by Deiters and by Gerlach as the syncytium of their reticularist vision. Golgi was also able to identify two main types of nervous cells. One of them had long axons that ran far away from the soma, while the other had short axons, anastomosed in the vicinity of the soma. Cajal, who was one of the first to corroborate this duality of nerve cells, proposed to name both types as Golgi cell type 1, later changed to projection neurons; and Golgi cell type 2, the interneurons.

From its inception in 1873, apart from Golgi himself, few publications mentioned his method until Cajal's first publication referencing the technique in 1888a (Cajal, 1888a). In his autobiography, Cajal (1989) called attention to the

fact that "*the wonderful revelatory powers of the chrome-silver reaction"* seemed the contrast with the *"absence of any excitement in the scientific world aroused by its discovery*". The reason for that contradiction was that the method as reported by Golgi yield inconsistent results. In 1887, Cajal was traveling to Madrid for administrative reasons, he decided to visit the psychologist Luis Simarro. Simarro who had worked in Paris with Martin Charcot in neurology at the Salpêtrière Hospital, and Louis Ranvier in histology at the "Collège de France", had learned Golgi's impregnation method, and was practicing it in his home in Madrid. He showed Cajal some of these preparations as well as Golgi's book on the fine anatomy of the nervous system published in 1885, and Cajal became fascinated with the method's potential. Once back in Valencia, he started to work tirelessly to improve the Golgi method. From 1887 to 1888, Cajal took an arbitrary method that did not do much by itself due to the inconsistency, and the incompleteness of the stained cells and improved it into a reliable technique almost devoid of artifacts. Cajal rapidly understood that myelin was an obstacle for correct impregnation and began using immature animals. In addition, he discovered that double, even occasionally triple impregnations improved not only the quality of the impregnated elements but their number, thus bringing out more cells and more details. Cajal (1888a) was then able to use this modified and improved method to study the cerebellum in young animals. I like to think that Cajal's choice of using the cerebellum for his first trial with the Golgi method is akin to winning the lottery on the first try. Indeed, it allowed him to notice the presence of interneurons in the molecular layer whose axons formed a pericellular basket around the cell body of the Purkinje cells, the only class of projection neuron of the cerebellar cortex. Three months later, Cajal (1888b) reported the parallel fibers to be the terminal fields of granule cell axons, and their terminations in the molecular layer. Finally, Cajal (1890) described fibers emerging from the white mater, which during development first wrapped the Purkinje cell body to later translocate from the soma to the dendrite wrapping around the dendritic stem of these cells, like ivy climbing on tree branches, which is why he named them "climbing fibers". These two clear and unmistakable examples are the irrefutable proof that there is contiguity and not continuity between nervous elements.

INSERT FIGURE 2

Thus, Cajal surpassed Deiters, and Golgi, in reaching the conclusion that the nerve structures are formed by independent cells that communicate with each other through what he called "intracellular articulations" and that neither anastomosis nor syncytium exist. Cajal then saw that Rudolf Virchow's cell theory, who Cajal read passionately, could also adapt to the nervous system, because the nervous system is not an exception in animal tissues, and all of them are formed by independent cells. A couple years later, Cajal had acquired a full understanding of the brain's structure. When, six years after his first publication on the cerebellum, Charles Sherrington invited him to the Royal Society to give the Croonian Lecture (1894), Cajal had already made significant advances not only in morphology but also in functional concepts that he always managed to reconcile with his anatomical discoveries. Thus, in the lecture, he clearly exposed his ideas on the polarization of nerve cells, and the orientation of nerve impulses, not only in the cerebellum but in almost all nervous centers, such as the spinal cord, the brain, the olfactory bulb, the sympathetic ganglia, the optic centers, the retina, etc. He described in his Fig.5 the complete circuitry of the cerebellar cortex, with its extracerebellar afferent axons: mossy fibers in contact with the granular cell dendrites, and climbing fibers along the soma of Purkinje cells enveloping the ascendant stem and the main protoplasmic branches. He also included a precise description of the basket cells and their axons. A very important detail in Cajal's schema are the Native-American like arrows pointing in the direction followed by the nerve impulses within the circuit resulting from applying the law of dynamic polarization developed he had developed. According to this law, nerve impulses are not random as predicted by Gerlach or Golgi. Instead, impulses in nerve cells follow a cellulipetal direction: moving towards the cell body in the dendrites while impulses in the axons follow a cellulifugal direction: moving away from the cell body. In other words, every impulse that moves across a dendrite is directed towards a soma, whereas, everything that leaves a soma through an axon goes to another neuron. Although this law is still

applicable today, it is not without exceptions. However, it is obvious that neurons are polarized cells.

Another interesting circumstance took place in 1891 when a German anatomist by the name of Heinrich Wilhelm Gottfried von Waldeyer-Hartz (1891) wrote a series of very short articles, published in the Deutsche medicinische Wochenschrift in Berlin. You probably all know his name or know him as Waldeyer, (he was ennobled by Kaiser Wilhelm II after 1916). His popularity in Neurosciences is due to his ability to provide appropriate names to already known biological entities. When Waldeyer renamed nerve cell into NEURON, Cajal wrote of the new nomenclature: "*How is it possible that this person, who wrote a summary of my work, not even in a scientific journal but in a weekly press, and had the idea to call a nerve cell 'neuron', is now the father of neural theory? All he did was christen it".*

PART II

THE ORIGIN OF THE SYNAPSE NAME AND THE CREATORS OF THE "NEURON DOCTRINE". CHEMICAL SYNAPTIC TRANSMISSION

The great breakthrough brought about by Cajal was foreshadowed by several precursors such as the pathologist August Forel (1887), who was able to envision the "neuron doctrine" by showing that postlesional atrophy was never diffuse but remained confined to selective brain circuits depending on the lesion. Another precursor, the embryologist Wilhelm His (1886; 1890), demonstrated that "every nerve fiber originates as the outgrowth of a single cell" during development. One of the first problems raised by the "neuron doctrine" was to determine the way neurons communicate with each other. For Cajal, it was clear that it was done through some specific zones of cell contact that he named "nervous articulations". Charles Sherrington was the one who was able to combine morphology and function. He was a great physiologist that analyzed in details the spinal cord reflexes. Most of his work was summarized in his well-known book "The integrative action of the nervous system" published in 1906, where he developed his new ideas on nerve activity.

In 1937, in a letter to neurobiologist John Fulton, Sherrington explained how he came up with the term “synapses” to name Cajal’s “nervous articulations”. He explained that some years earlier, Michael Foster had asked him to contribute a chapter on the nervous system to his physiology treatise named “Textbook of Physiology”, which was published in 1897. Sherrington knew that sensitive information reaches the cord through the posterior horn, and that when it reaches the motoneurons, there is a very short delay in the response, which was explained as the time taken by the nervous impulse to move from one neuron to another, and which later will be designated as synaptic delay. At the same time, a change in the direction of the impulse occurs, which instead of going towards the soma, now moves out leaving the spinal cord, through the motoneuron axon towards the muscles. Then the synapses exert a valvular effect, and the nerve impulse can only travel in one direction, from presynaptic to postsynaptic neuron. Sherrington decided that such an important phenomenon deserved its own name. At first, he proposed to call it "syndesm" which means "connection". But then he explained that "*He consulted his Trinity friend Verrall, the Euripidian scholar about it, and Verrall suggested "synapse", and as that yields a better form of adjectival form, it was adopted for the book ...* ". That is how the synapse made its entrance into neurosciences in 1897.

The nature of the synaptic transmission: electrical or chemical?

But what is the mechanism for this transfer of activity? Is it electric or chemical? The controversy between proponents of the electric versus the chemical nature of the synaptic transmission led to a long disagreement that today is, jokingly remembered as "the War of Soups and Sparks" (Valenstein, 2006), in which the sparks were championed by electrophysiologists, while the soups were by pharmacologists. Historically speaking, one of the first champions among the tenants of the electrical nature of synaptic transmission was Du Bois-Reymond (1848), the first to record an action potential. But after 30 years he changed his mind, and considered that, at the motor end plate, the synaptic mediator should be something chemical, either lactic acid or ammonium (1877).

Sherrington himself, when defining synapses, gave them two important characteristics: their valvular effect, and the synaptic delay. Both speak in favor of a chemical transmission. Others essential discoveries in the field of chemical synaptic transmission took place at the beginning of the 20th century. The first of them was Elliot's discovery of the presumptive action of adrenaline as a neurotransmitter presented in 1904 and published on 1905. This discovery was made simultaneously with the paramount concept of "receptor" proposed by Elliot's mentor, Langley (1905; 1906). This concept was based on Langley's observations on the effects of nicotine and curare on skeletal muscle, corroborating and extending the intuition of Claude Bernard (1857) half a century before. Little by little, the concept of chemical transmission took over.

After years of fight, a German scientist, Otto Loewi (1921) reported the simplest and the most compelling evidence in favor of chemical transmission. He dissected the vagus nerve and the heart of a frog, put them in vitro under perfusion and electrically stimulated the nerve. This stimulation slowed down the heartbeats. Then, he isolated another frog's heart, this one devoid of its vagal innervation, but perfused it with the fluid used on the first vagal stimulated heart. The second heart also slowed down its rate. He called this phenomenon "vagusstoff", or vagus substance, which makes the heart slow down. This simple and clear experiment showed without a doubt, that something of a chemical nature, produced by the stimulated nerve, changed the way the heart worked. Therefore, Loewi was able to convince everybody that the synaptic transmission between de vagus nerve and the heart is chemically mediated. Years later, Henry Dale and collaborators (1930, 1936) were able to show that the "vagusstoff" was the acetylcholine, and that this molecule was the neurotransmitter of motor axons innervating striated muscles, and of all the parasympathethic nervous system. In a long article, Dale and Gaddum (1930) concluded: "The evidence… supporting the view that the vasodilator effects of parasympathetic nerves and of sensory fibers stimulated antidromically, and the contractures of denervated muscles accompanying these actions, are due to the peripheral liberation of acetylcholine." In 1936, Dale shared the Nobel Prize with Otto Loewi "for their discoveries relating to the chemical transmission of nerve impulses".

INSERT FIGURE 3

But it is not until John Eccles (1949) that the defenders of the chemical transmission finally won the war. Eccles had been a fierce supporter of the electrical nature of the synaptic transmission until he started working on synaptic input to a cat's spinal motoneurons using intracellular recordings via glass micropipettes. With this new technique, he was able to record inhibitory postsynaptic potentials that were not compatible with the electrical mode of synaptic transmission. Therefore, he changed his mind, and became a champion for the chemical transmission cause. After these results, it was soon generalized that both peripheral and central synapses use chemical transmission for their functioning. The triumph of the chemical neurotransmission brought as a consequence the concept that the independence and unity of the neuron was not only anatomical and electrophysiological, but also biochemical. In such a way that a given neuron releases the same neurotransmitter through all of its axon terminals. This biochemical unity was known as the "Dale principle" as named at the time by John (Eccles et al. 1954). Today, the concept of the "biochemical unit" cannot be totally accepted, because the "Dale principle" does not apply to all synapses, as demonstrated by Tomas Hökfelt and collaborators (1980). With the discovery of monoamines and neuropeptides as potential neurotransmitters it became evident that co-localization of a classical fast acting neurotransmitter with monoamines and/or neuropeptides was not only possible, but even quite frequent.

MY MASTERS IN THE HISTORY OF THE SYNAPSE

I) Fernando de Castro (1896 – 1966)

Now it is time to introduce, as I mentioned at the beginning, the contributions of my mentors to the formulation and support of the synapse. Let start with Fernando de Castro. Don Fernando as we called him, with whom I began to work at the Cajal Institute from 1957 to 1961, when I was a medical

student in the Complutense University in Madrid. De Castro is well known in the history of Neurosciences for his demonstration of the chemosensory nature of the carotid body (1926, 1928). He was one of the last disciples of Cajal, and he had been working since 1916 on sympathetic ganglia and vegetative nervous system when he started to worry that the "neuron doctrine was being questioned once more. There were people who believed in the reticular theory and who were trying to promote the existence of a reticular terminal plexus, not necessarily in the entire nervous system, but certainly in the autonomic nervous system.

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In a letter to José Luis Rodríguez-Candela, who was one of the heads of the Higher Council for Scientific Research in Spain (CSIC) at that time, Fernando de Castro wrote: "*The work of Cajal and partly that of his school remains impressing and suggesting research topics in many countries of great scientific concern (except Bonn, center and crucible of reticularism or fibrillarism), especially since the use of electrophysiological techniques*". He specifically mentioned Bonn because Philipp Stöhr (1891–1979), who was a professor of anatomy at the University of Bonn, was waging a crusade against the "neuron doctrine" denying the existence of synapses in the whole vegetative nervous system. He believed that instead of synapses, nerve processes built up a network of anastomosing fibers. The reason de Castro was so concerned with a coming back of the reticular views was not only because they were in opposition with the "Neuron Doctrine", the beloved discovery of his master, but also because they were denigrating his own work. In a book compiling the best work done in neurocytology at the time and edited by Penfield in 1932, "Cytology and Cellular Pathology of the Nervous System", there were two chapters written by de Castro: the vegetative nervous system and dorsal root ganglia. In the vegetative nervous system, what de Castro saw was the existence of preganglionic axons entering the ganglia, climbing around the dendrites of the multipolar ganglionic neurons, and providing numerous "en passant" boutons, which established synaptic contacts with the somata and dendrites of the latter. He called these fibers,

climbing fibers, due to their morphological analogy with those reported by Cajal in the cerebellum on Purkinje cell dendrites.

Stöhr's denial of the existence of synapses in the whole vegetative nervous system is in complete opposition to the interpretations of de Castro. For Stöhr these pericellular nests and climbing-like fibers would be nothing else but fragments of the syncytium that would fill up the spaces located between the somata of the nerve cells. Philipp Stöhr was very influential. His chapters in the handbook of histology of Möllendorf (Stöhr, 1957) were used by many teachers in many Universities throughout the world defending and spreading wrong ideas for years. Even at the Medical School in Paris in 1962, where I attended lectures on the Nervous System, some of the teachers where still supporting the idea that the autonomous nervous system was not neuronal but reticular. For Fernando de Castro, whose early years of scientific life had been devoted to patiently studying both dorsal root and autonomic ganglia, this dissemination of wrongful information was a disregard for his work.

Also, in Paris where Jacques Taxi (1961), a collaborator of René Couteaux, and the person who introduced me to electron microscopy, carried out the first study on the sympathetic ganglion's ultrastructure in frogs. In his monumental work "*Contribution à l'étude des connexions des neurons moteurs du système nerveux autonome*" (Contribution to the study of motoneuron connections in the autonomic nervous system, Taxi 1965), there is a complete study on both ganglion synapses (Chapter II), and the innervation of the smooth musculature (Chapter IV). Out of the many important conclusions of this impressive work, I'd like to focus on the one denoting the presence of free endings in the autonomous system, both at ganglionic level and in its terminal region of innervation of smooth muscles. This corroborates the work of Cajal and de Castro and underlines Stöhr and Boeke erroneous concepts.

But the main contributions of de Castro in favor of the presence of synapses in the sympathetic ganglia were not only the result of his morphological studies on normal material, but mostly of his experimental work.

First, in 1930 he reported that 12 to 36 hours after sectioning or crushing of the preganglionic nerve, most of its terminations had degenerated, without any noticeable changes in the structure of the postsynaptic elements. At the same time, by means of physiological studies, de Castro established that a physiological degeneration corresponded to this morphological degeneration because the electrical stimulation of the peripheral segment of the sectioned cervical trunk did not generate passage of nerve impulses. Finally, he demonstrated that during the regenerative period, the growing preganglionic axons were capable of developing new pericellular nests and climbing-like fibers, re-establishing synaptic transmission with postganglionic neurons.

A second set of arguments aimed at reinforcing the validity of the previous studies, was provided by the examination of heterologous regeneration. Following Langley's experiments (1898; Langley and Anderson, 1904) on crossed anastomosis between somatic or autonomic nerves with the superior cervical ganglion, de Castro (1930, 1932, 1934, 1937, 1950) showed that centrifugal nerves' fibers (vagus, hypoglossus, phrenic) anastomosed to the distal segment of the superior cervical sympathetic ganglion can regenerate, and establish new synaptic contacts with the ganglionic neurons. One of his most famous anastomoses was the cranial trunk of the vagus to the cranial end of the cervical sympathetic trunk in the cat. The regenerating fibers entered the ganglion and reinnervated the denervated ganglion neurons, reproducing the characteristic pericellular apparatus and climbing-like fibers. This heterologous reinnervation produced a functional recovery establishing a completely new type of reflex. When he inserted a balloon in the stomach and inflated it, he obtained a mydriasis of the pupil ipsilateral to the anastomosis, which was an indirect proof of the formation of new synaptic contacts. In addition, if after the establishment of this new reflex, the preanastomotic vagal trunk was sectioned, the synapses present in the ganglion degenerated and the new reflex disappeared, thus testifying of its vagal origin. Years later, Bruno Ceccarelli et al. (1971), with a similar experiment, were able to visualize with electron microscopy new synaptic contacts, mainly axo-dendritics in the ganglion, and their degeneration after transection of the pre- anastomotic vagal nerve.

Another well-known advocate of the neuron in the first half of the 20th century was János Szentágothai (see in Gulyas and Smogyi, 2013), an anatomy professor in Budapest. He was, like de Castro, also shocked by the fact that around 1930, both Philipp Stöhr in Bonn, and Jan Boeke in Utrecht were rebuffing the "Neuron Doctrine". Boeke (1921; 1949) denied the existence of synapses to the contact points between sympathetic fibers and heart muscle fibers. For him, the sympathetic nerve's distal end of the striated heart muscle did not end near the muscle, but rather through a system of fine terminal neurofibrils, which he called "periterminal plexus". The periterminal plexus entered the muscle cytoplasm to the contracting region, where it directly stimulated the myosin provoking the muscle contraction. Therefore, for Boeke the muscle contraction was not the result of a synaptic transmission but of the contiguity between the conducting neurofibrils of the "periterminal plexus" of the nerve, and the contracting material. Boeke finished his 1921 paper with some triumphalist statements that I feel compelled to share: "*This may furnish a solid base for further study in this direction, and for the present-day histologist it is another proof of the inadequacy of the old cell theory, which regards the cells as independent self-sustaining units, and of the wonderful harmony in which all those units combine and act together to build up and sustain the higher organism, the individual*".

Stöhr and Boeke's influence was extremely great, even in Spain, Cajal's home state, it was possible to find followers of such nefarious theories. The best-known Spanish reticularist was Dr. Vicente Jabonero. This histologist and pathologist was a military doctor, member of the "blue division" that accompanied German troops in 1942 on the Russian campaign. Last but not least, he was also a member of OpusDei. He was a good morphologist, very much under the influence of German science, who started working in 1946 on the autonomous nervous system. In the fifties, in one of his first articles he presented his concept of the autonomous nervous system's organization. For Jabonero (1951), while neuronalists (he mentions only a few but important ones: Szentagothai, Nageotte, de Castro, Hillarp) wrongfully accept the lemmoblastic

significance of conductive syncytial protoplasm, and consider that the axons circulating within this protoplasm are independent; many others like him (here the list is longer but the scientists are much less known, with the exception of Stöhr, and Boeke) have proven that these fibrils are not independent, but anastomosed within a very large plexus. According to Jabonero, the central nervous system, and even the sympathetic and parasympathetic ganglia, are all organized in the neuronal way, with synapses interconnecting with neurons. The major difference occurs with the efferent vegetative pathways, which form a distal nervous syncytium, corresponding to the fundamental sympathetic plexus of Boeke and to the terminal reticulum of Stöhr. In conclusion, in Jabonero's words: "*There is always a "plexiform synapsis of distance*" and the influx is transmitted with the aid of a chemical mediator which extends in the spaces of the tissue."

I'd like to add a personal anecdote in homage to Vicente Jabonero. In 1963, when I was making my debut as a speaker, I was invited to in a symposium at the University of Leuven. I was presenting a histochemical work I was doing at the Medical School in Paris on: "Coordinated metabolic systems for the enzymological study of the nervous system" (Wegmann, Sotelo, 1963, published in the compilation of articles of the symposium, in a Belgian dermatology journal). The meeting was organized by Professor Ernest Van Campenhout, who was a reticularist. Vicente Jabonero was the main speaker. A small revolution took place, when, against all expectations, he honestly, confessed that his hypothesis on the "plexiform synapsis of distance" was wrong. Indeed, in 1961, Jabonero and collaborators reported that the use of the Champy-Maillet technique (1959), (a fixing and staining technique developed by Marc Maillet based on Champy's older procedure, consisting in substituting the potassium osmium iodide by the zinc osmium iodide) had allowed them to better visualize the amyelinic nerve fibers. With this technique, Jabonero (1963) was able to observe that after partial lesion of a sympathetic ganglion, only part of the plexus degenerated, instead of the entire plexus, as should be the case for his "plexiform synapsis of distance". Therefore, the trophic principle sustained by Cajal in his "neuron doctrine" was maintained in the terminal region of autonomous

innervation. Jabonero's confession eliminated the last reticularistic vestiges from Spain.

II) René Couteaux (1909 – 1999)

Among the scientists that fought erroneous reticularistic ideas, my mentor René Couteaux deserves special mention. In 1933, René Couteaux started his research on the structure and development of the neuromuscular junction, the model used by electrophysiologists to study synaptic transmission and dedicated his work life to it. He was one of the first to argue the reticular misconceptions about the neuromuscular junction, and to obtain indirect evidence of the independence of the nerve fiber and the muscle fiber. In 1933 he started his research working on the structure and development of the neuromuscular junction. After his early work Couteaux 1938, 1941), he was convinced that the junction was the result of the close interweaving between the terminal nerve fiber and the muscle cytoplasm. However, it was clear to him that the relationship between nerve fiber and muscle cytoplasm could not be studied with silver impregnations alone, and that a deeper analysis of the muscle cytoplasm, at the level of the junction, was urgently needed. To this aim, and since the junction was supposed to contain numerous mitochondria, he decided to use a vital dye –Janus Green B- (introduced by Leonor Michaelis in 1900 and known to stain mitochondria). With this dye, Couteaux (1944, 1946, 1947) managed to visualize a labyrinthine set of gutters, bonded by a thin membrane, and equipped with perpendicularly oriented lamellae. He called this ensemble the "subneural apparatus".

INSERT FIGURE 5

The first results showing the special arrangement of muscle cholinesterase in mammals were obtained through the collaborative biochemical work of René Couteaux and Davis Nachmansohn (1938, 1940). Due to the special anatomical disposition of motor-end plates in the mammalian internal

gastrocnemius muscle, very rich in some zones and devoid in others, they were able to show that the vast majority of the enzymatic activity was present in zones rich in nerve terminations. This explained the very rapid inactivation of the acetylcholine from the neuromuscular junctions. After Koelle introduced the histochemical method to locate acetylcholinesterase activity (Koelle and Friedenwald, 1949), Couteaux and his collaborator Jacques Taxi (1951, 1952) were able to locate this activity at the subneural apparatus, as previously suspected. Similarly, using double staining –silver impregnation and cholinesterase histochemistry- it was also possible to corroborate that the terminal branches of the motor-axon were funneled within the tunnels formed by the gutters of the subneural apparatus. More importantly, these double stained preparations allowed for the first time to prove that the nerve and the muscle plasma-membranes were not touching but separated by a clear space.

III) Sanford L. Palay (1918 -2002)

I would like to point out again that most if not all scientific advances arise from the emergence of a new technique. As such, the arrival of the electron microscope gave the "neuron doctrine" renewed momentum. One of the limitations of light microscopy was the impossibility to visualize the cell membrane. Since 1935, we had a partial understanding of its constitution thanks to the publication of James Danielli and Hugh Davson biochemical model of the cell membrane. It was described as composed of a central bi-layer of phospholipids interspersed between an outer and an inner layer of globular proteins, and having high electrical resistance. This knowledge was corroborated and expended by electron microscopy. In animal cells, the membrane is about 10 nm thick, and therefore is not visible with light microscopy. J. David Robertson (1957, 1958) working with frog's peripheral axons was one of the first to describe the neuronal membrane -that he coined "unit membrane" as" two pairs of parallel dense lines separated from one another by a light space about 25 Å wide. Each dense line is about 25 Å thick so that each of the pairs makes a unit about 75 Å across". In other terms, the plasma membrane was defined as a three-layered structure, with two external dense layers and a central light one,

consisting of the fluid phospholipid. Singer and Nicolson (1972) modified the concept of membrane structure with their lipid-globular protein mosaic model, where molecules are free to diffuse within the membrane. Today we know that lipids are not uniformly distributed along the membrane and that there are regions rich in cholesterol molecules, named “rafts”. Moreover, it has also been established that the membranes, mainly through membrane-associated proteins, are able to influence the cytoskeleton and affect cell physiology. Today, the membrane can be considered as a fluid system inside which molecules can move. For instance, in the case of the synapses, receptors and channel molecules are constantly moving. In a clear summary of their work on movement of receptors within the postsynaptic membrane, Daniel Choquet and Antoine Triller (2013) concluded: “*Receptor movement in the plane of the plasma membrane by thermally powered Brownian diffusion movement and reversible trapping by receptor-scaffold interactions has emerged as the main mechanism to dynamically organize the synaptic membrane in nanoscale domains*”.

The era of electron microscopy shined a light on the "neural doctrine", the work of Cajal and Sherrington and the triumph of the synapse. And with the electron microscopy era we enter the domain of Sanford L. Palay, my master in Boston during my years (1965-1967) of postdoctoral fellowship at the Anatomy Department of the Harvard Medical School. Sandy Palay was a pioneer in electron microscopy of the nervous system. He managed to correctly fix the tissue to obtain preparations of excellent quality. Better tissue preservation allowed for better study of the material, and more accurate conclusions. In 1955, Palay published the first electron microscopy study of the neuron and the central synapse in vertebrates’ central nervous system while working at the Rockefeller Institute with George Palade (1954; 1955). The study was performed in the abducens nucleus of the medulla oblongata. This was an obvious choice because the fixation was done “in situ” with a buffered solution of osmic acid, and the abducens is a superficial nucleus, which can be directly fixed by dripping osmic acid over the brain surface to avoid most of the artifacts that would be produced if a deeper nucleus had been chosen. This work was published almost simultaneously with the work of Eduardo De Robertis and Stanley Bennett

(1955), in frog sympathetic ganglia and earthworm nerve cord neuropile. However, the quality of the images in de Robertis's publication was definitely inferior than in Palay's. In the former, many regions of the neuronal membrane were broken, leaving doubts about the presence or not of intercellular communications between the two partners of the synapse. Nevertheless, conclusions of both studies were rather similar, and the holes in the membrane have been described as an artifact. In any case, the subsequent use of aldehydes as fixatives, and the perfusion fixation following the protocol reported by Palay and collaborators (1962) were able to suppress many of the fixation artifacts, and to clearly show that pre- and postsynaptic elements were bounded by unit membranes, and separated from each other by a synaptic cleft of about 20 to 30 nm in diameter. Finally, David Robertson (1956) also reported that at the level of the lizard myoneural junction, the axonal membrane is separated from the muscle membrane, corroborating Couteaux' results with the Janus Green B dye. These results all show that there is no contiguity because the presynaptic part is separated from the postsynaptic part by an enwrapping unit-membrane, and by a more or less narrow extracellular space, or synaptic cleft. This provided the nail in the reticular coffin, a triumph for Cajal's "Neuron Doctrine".

Specialization of synaptic membranes: the synaptic vesicles and the "synaptic complex"

The neuronal membranes involved in the establishment of the "synaptic complex", as Palay (1958) called the portion of the plasma-membranes directly responsible of the chemical synaptic transmission in central synapses, are highly specialized, and each subdomain has a very different structure. The presynaptic one is specialized for the release of the neurotransmitter, whereas, the postsynaptic one is equipped with the receptor proteins required for the efficiency of the neurotransmission. The synaptic complex was first considered as an evolved junctional attachment plate, differentiated for chemical transmission. Due to the paramembranous electron-dense cytoplasmic material undercoating the cytoplasmic face of the synaptic complex, they were described as regions with thicker membranes, separated from one another by a 20 to 30

nm extracellular space, the synaptic cleft. The electron-dense material is generally distributed asymmetrically, being more prominent in the postsynaptic membrane, where it is called "subsynaptic web" due to its filamentous aspect (de Robertis, 1961). Gray (1959) considered that electron microscopy could reveal the binary character of excitation and inhibition with which synapses function. Synaptic complexes with excitatory function have thicker paramembranous differentiations, and thicker synaptic clefts than ininhibitory synapses. Excitatory synapses were called "Gray type 1" and inhibitory "Gray type 2".

INSERT FIGURE 6

In the presynaptic element the most characteristic organelle is the presence of numerous vesicles, the so-called: synaptic vesicles. The most numerous ones are the “small clear vesicles” or “agranular vesicles”. They have a diameter ranging between 30 and 50 nm. In aldehydes fixed material some presynaptic terminals have spherical vesicles while they are flattened in others. However, in osmium fixed material all agranular vesicles exhibit a rather spherical shape. This change in shape has allowed to correlate morphology and function, because in some regions of the central nervous system, where it is possible to recognize the origin of the terminal (for instances in cerebellum climbing fibers and basket cell axons) and their excitatory or inhibitory nature, it has been shown that excitatory synapses contained spherical vesicles, and conversely inhibitory ones are filled with pleomorphic vesicles (Uchizono, 1965, Bodian, 1966). This relation between functional dichotomy (excitation, inhibition) and structural dichotomy of small synaptic vesicles (rounded, flattened) has been further consolidated by the high frequency of coincidences between one type of vesicular shape and one type of synaptic junction. Thus, most asymmetrical or Gray type 1 synapses contain rounded vesicles, whereas most symmetrical or Gray type 2 ones are filled with flattened vesicles. This assumption has been confirmed by immunogold immunocytochemistry of GABA (van der Want and Nunes-Cardoso, 1988).

Ultrastructural basis for identification of pre- and postsynaptic

membranes

A cluster of synaptic vesicles is accumulated facing a distinct region of the presynaptic membrane characterized by the presence of triangular electron-dense protrusions attached through their basis to the presynaptic membrane. Georges Gray (1963) using the ethanolic- phosphotungstic acid (PTA) technique, a selective staining that reveal at their best the paramembranous and intermembranous synaptic material highlighting pre- and postsynaptic differentiations was able to describe such protrusions, named "presynaptic dense projections" (PDPs). Concerning presynaptic differentiations, in sections parallel to the plane of the membrane, where its face side is visible, the PDPs are disposed in a regular array. The aggregation of the vesicles and the array of PDPs is called the "presynaptic vesicular grid". This grid is the ground on which the ready to be released pool of synaptic vesicles leans, where they have free access to the axonal membrane at the open spaces between the PDPs. The grid is, therefore, the only membranous domain in which the membranes of the synaptic vesicle can be in direct contact with the presynaptic membrane, and where they can fusion, through a process of exocytosis, with the axonal plasma-membrane, for the release of the neurotransmitter into the synaptic cleft. This process was fully described in the motor end plate, by Couteaux and Pecot-Dechavassine (1970). They referred to the membrane zone containing the presynaptic grid as the "active zone". It is important to note that the presynaptic grid is a rather dynamic structure and that the synaptic functioning can distort its geometrical arrangement. Antoine Triller (Triller and Korn, 1985) working with synapses established by unmyelinated club endings on the Mauthner cell of teleost, was able to either inhibit them by cooling the preparation or to stimulate them by perfusing the fish with a high-KC1 Ringer solution, changing this way the amount of vesicle openings. This active process of increasing the number of vesicular membrane fusion to the axonal membrane might provoke the distortion of the grid demonstrating that it is dynamically involved in the secretory process.

INSERT FIGURE 7

Freeze-etching analysis of the presynaptic membrane and of exocytose

At the end of the sixties, a new method was introduced that allowed for a more precise study of the membrane (Moor et al., 1969). It has been extremely useful to better understand the difference between the opposed membranes at the synaptic complexes. This method of "freeze-etching" combined with electron microscopy allowed for the analysis of the protein constitution of membranes of cells and organelles. The tissue was placed in a high-vacuum freeze-etching device (Balzers BA 360M) without fixative and freeze rapidly to prevent ice formation. The tissue was then fractured with a steel knife. Under these conditions, the fracture occurred at the middle of the phospholipids bilayer, artificially exposing the surface of the outer leaflet of the inner or protoplasmic part of the membrane (P face), and the inner leaflet of the outer or extracellular part of the membrane (E face). Etching was performed on the fractured membrane, and platinum and carbon were evaporated from the tips of pointed carbon electrodes, in the high vacuum machine to obtain a precise molding of the fractured specimen surface. The specimen was then removed from the Balzers machine, the biological material eliminated, and the fine carbon-platinum replica transferred with a grid to the electron microscope for study. This procedure helped to develop modern concepts of how biological membranes are organized. Because it fractures the membranes between the two layers of phospholipids, but contours the proteins, which constitutes the intramembrane particles (IPs), leaving their trace in the replica, it allowed for the analysis of their dynamics on a millisecond time-scale. IPs protrusions were present on the E-face, and complementary dimples were detected on the P-face.

The freeze-etching method has been useful in clarifying the specific organization of pre- and post-synaptic membranes. The presynaptic membrane can be recognized by the presence of some large IPs (8 – 10 nm) on the E-face with complementary dimples on the P-face that have been identified as calcium channels. The IPs are in close relationship with crater-like depressions, which correspond to the empty spaces between the PSPs, and are traces of the fusion of

vesicles and axonal membranes during the exocytosis. Konrad Akert and collaborators (Tokunaga et al. 1979) called these holes "vesicles attachment sites" (VAS). Therefore, the association of the VAS and large IPs at the presynaptic grid defines the morphological characteristics of "active zones". The functional heterogeneity of neuronal membranes assumes a structural heterogeneity. Nevertheless, ultrastructural examination of postsynaptic membranes is rather disappointing. The uniform three-layered unit membrane appears identical to the presynaptic membranes and to the neuronal membranes outside the synaptic complex. Only a distinct cytoplasmic differentiation marks the presence of the "subsynaptic web" (see above), an electron-dense undercoating beneath all the extension of the postsynaptic membrane opposite the presynaptic grid. With the freeze fracture method, an interesting feature was noticed, the presence of IPs protruding into the E-face aggregated into macular plates and matched by an array of complementary pits in the P-face (Landis and Reese, 1974). This salient feature only occurs in Gray type 1 synapses, in other words in excitatory synapses, probably corresponding to proteins of glutamatergic receptors (Landis and Reese, 1974).

The vesicular hypothesis: quantal release of neurotransmitters

As we have discussed, synaptic transmission is chemically mediated, and acetylcholine is the neurotransmitter for the motor end plate. But how is the transfer, from the depolarization wave in the presynaptic axon membrane, to the depolarization or hyperpolarization in the postsynaptic neuron or effector cell performed? In other words, how is the neurotransmitter released to diffuse within the synaptic cleft, to reach the postsynaptic receptor? Bernard Katz and collaborators' (Paul Fatt, José del Castillo, and Ricardo Miledi) answered this essential question with their work on frog neuro-muscular junction during the fifties and the sixties. The discovery of the "miniature end-plate potentials" (Fatt and Katz, 1952) occurring after spontaneous electric discharges with amplitude in the order of 1% of the normal end-plate response, provoking the spontaneous release of acetylcholine, allowed Katz and collaborators (Katz and Miledi, 1965a;

1965b; 1968) to understand the quantal mechanism of neurotransmitter release. A few years later del Castillo and Katz (1955) were able to formulate the vesicular hypothesis, which assumes that packets of standard size neurotransmitters, the quanta, are stored in the synaptic vesicles, and released after stimulation. René Couteaux's electron microscopic work and his morphological analysis of the acetylcholine release from the synaptic vesicles confirmed this theory.

Due to the vesicular release of quantal acetylcholine, the exocytotic process that takes place progressively during synaptic activity transiently increases the length of the axonal membrane. These newly added patches of membrane reach the lateral ends of the axon terminal, where an inverse process takes place. The membrane, coated by a monolayer of clathrin molecules, is incorporated into the axoplasm through an endocytotic mechanism. This constant process of addition and retrieval of vesicular membrane to the axonal membrane is known as vesicle recycling. Paradoxically, the process of chemical synaptic transmission could be matched, as Rodolfo Llinás says, to a process of growth without growth.

Chemical synaptic transmission in the absence of synaptic complexes: Paracrine secretion. The synapse as a functional concept

Jacques Taxi was a pioneer at studying the autonomic nervous system with electron microscopy, especially with regard to the ganglia and their terminal plexus. In his long and excellent thesis (Taxi, 1965), he corroborated the presence of axon terminals and synapses in the frog sympathetic ganglia. Furthermore, he described the presence of a band of electron-dense material located a short distance under the postsynaptic web in 10 to 40% of these synapses, the so-called Taxi's "subsynaptic formation". He showed that one to seven days after cutting the preganglionic fibers, the presynaptic axon terminals followed a process of Wallerian or retrograde degeneration, and were engulfed by astrocytic processes. However, the postsynaptic web and subsynaptic formations remained unchanged. These results confirm the independence of the two nervous elements forming the synapse. In 1964 and 1965,

during my stay in Taxi's laboratory, I learned the electron microscopy's techniques. When I moved to Boston for my postdoctoral training, I also used the frog sympathetic ganglia as my biological material. I had decided to prolong the surviving time between the section of the preganglionic fibers, and the fixation of the ganglia to determine if after the total disappearance of the degenerative debris, the postsynaptic elements remained intact or not. Ten to twelve days after transection of the preganglionic fibers, most of the degenerating axon terminals disappeared, but the postsynaptic differentiation and subsynaptic formation remained unchanged. The preganglionic axon and the ganglionic neuron were indeed two distinct cellular elements. These results were published in 1968, a year after the death of my mentor de Castro, and I dedicated the paper to his memory (Sotelo, 1968a).

INSERT FIGURE 8

Taxi's (1965) electron microscopic study on how postganglionic autonomic fibers innervated the smooth musculature has also been beneficial to the study of the synapse. It made it obvious that even the finest nerve fibers never leave the fascicle to glide individually between the smooth muscle cells. Nevertheless, the ultrastructure of the beaded fibers located on the surface of the vessels or around smooth muscle cells revealed that each bead corresponded to a synaptic bouton. These boutons contained synaptic vesicles and sometimes clustered against the membrane in an "active zone", and located opposite the smooth muscle cells (fasciculated innervation). In other instances, the bouton is somewhat distant from the muscle cell, overpassing the 500 Å of motor end plates. In any case, the opposed muscle membrane is neither thicker nor undercoated by postsynaptic differentiations. In other words, the autonomous innervation of peripheral targets does not establish morphological "synaptic complexes", allowing for the identification of synaptic contacts. However, this type of synaptic transmission can be physiologically revealed (see for instances Lam et al., 2016; Manchanda et al., 2016). Therefore, since there can be synaptic transmission in the absence of synaptic complexes, it is obvious that "synapse" is not a morphological concept but a functional one. A similar situation has been reported in the central nervous system where monoaminergic innervation (Beaudet and Descarries, 1978), particularly the serotonergic one in the cerebral (Descarries et al., 1975) or cerebellar cortices (Beaudet and Sotelo, 1981), is

established by axon terminals of which 90% are devoid of synaptic complexes. They are called non-junctional synapses whereas the few that did make synaptic complexes, retained their junctional synapses name. But how could this kind of transmission occur? The answer is simple; it is the result of paracrine interactions, starting with the exocytotic release of the neurotransmitter content present in the synaptic vesicles of the presynaptic bouton. In our case the monoamines are released into the extracellular space, where they progressively diffuse in all directions across a distance that can reach 10 or 15 microns and activate all the receptors along the way. We described this years ago in what we named "global systems" while reporting on transplantation of embryonic neuronal progenitors into adult cerebellum (Sotelo and Alvarado-Mallart,1986).

Role of astrocytes in chemical synaptic transmission: the tripartite synapse

This part of the story cannot be complete without a few brief comments on the role of glial cells, especially astrocytes, in chemical synaptic transmission. First of all, it is well established that glial cells are numerically speaking as important as neurons. Previously, results pointed to a somewhat surprising and quantitative conclusion that in the cerebral cortex, astrocytes were much more numerous than neurons (O'Kusky and Colonnier, 1982), although this conclusion has not been corroborated (Azevedo at AL., 2009). This quantitative argument has been widely and erroneously used over the past two decades by some researchers working in the field of neuroglia to defend their work. The underlying idea was that if there were so many of these cells, it was because they were of paramount importance, although this importance was not yet fully understood, they proposed that their work could help unravel it.

INSERT FIGURE 9

Cajal himself developed many years ago the concept that astrocytes are integral part of the neuronal chemical transmission. By looking at the cell density and distribution of astrocytic processes all around the cell bodies of neurons in the

Amon horn of the hippocampus, Cajal (1895) imagined that the transition between consciousness and unconsciousness, as in the passage from wakefulness to sleep, would be the result of a sliding mechanism of a fine astrocytic process between pre- and postsynaptic elements, preventing the synaptic transmission. A kind of electrical insulator if you will. Whereas, in reverse, the astroglial cytoplamic finger will retract freeing the synaptic space, passing from the unconscious to the conscious state. Of course, this unorthodox concept remained hypothetical, and was soon abandoned, but it does emphasize the high density of thin astrocyte lamellae that surround the cellular bodies and dendrites of neurons. Thus, almost half a century later, Fernando de Castro (1942; 1951), while working on spinal cord with two of Cajal's favorite methods (reduced silver impregnation, and gold sublimate technique), reached the conclusion that his reduced-silver preparations, were free of tissue retraction. Nevertheless, there was a small free space between the axon terminals and motoneuron cell bodies whereas in his astrocytic impregnated preparations, the whole soma of motoneurons was covered by astrocytic cytoplasm. His conclusion was that a thin process of glial cytoplasm could be interposed between the pre- and the postsynaptic elements. With this conclusion he eleborated the new concept of the glia and the astrocyte became an active element in the chemical synaptic transmission, being the "vector element of the synapses". This theory was embraced by René Couteaux who was always troubled by the significance of the presence of glial processes at the motor end plate from what he called teloglial cells (personal communication). Although electron microscopy has demonstrated the falsity of this hypothesis, de Castro's idea that neuroglia has a primordial function in the maintenance of nervous activity, remains a reality today.

We know thanks to the pioneer work of Steve Kuffler (Orkand et al., 1966) that astrocytes act as K+ electrodes to extracellular K+, and are able to respond to neuronal activity with small membrane depolarization. Another important advance was achieved when Alfonso Araque (Araque et al., 1999) published his work announcing the "tripartite synapse". Electrophysiological as well as biochemical and pharmacological data has been gathered in the past 20 years to create a body of evidences that unequivocally demonstrate the active role played by astrocytes in the synaptic functioning. First, it has been broadly confirmed that these glial cells are equipped with most of neurons' neurotransmitter receptors and ion channels (Schipke and Kettenmann, 2004). Therefore, they can take direct action in nervous function,

and through the release of gliotransmitters they can even modulate synaptic transmission, and contribute to synaptic activation and synaptic gain (see in Papouin et al., 2017, even if D-serine –the supposed gliotransmitter- could be released also from neurons). Nowadays, we finally can say that glial cells contribute in an active way to the synaptic transmission.

PART III

THE REVENGE OF THE ELECTRIC MODE OF SYNAPTIC TRANSMISSION.

The struggle between supporters of electrical versus chemical synaptic transmission did not end with the ultimate triumph of the chemical transmission defenders. Electrophysiological advances similar to those that helped to highlight the presence of chemical synapses were useful to prove that electrical transmission was also possible. In this case, the transmission takes place by means of a passive spreading of the neuronal depolarizing current, through the synaptic membranes, which causes a very fast (about 0.1 msec) postsynaptic excitatory potential. This way of synaptic transmission requires that the membranes of the areas through which the current passes be of very low electrical resistance. In addition, the electrotonic spread of the depolarizing current is in general bidirectional (with exception of the so-called rectifying electrical junctions). These two properties, together with the absence of fatigability, underlie the main functional features of this mode of synaptic transmission: fast speed and synchronization (see in Bennett, 1997). The first detections of electrical transmission took place in the nervous system of invertebrates (Furshpan and Potter, 1957; 1959; Watanabe and Grundfest, 1961; Hagiwara and Morita, 1962; Levitan et al., 1970; Spira et al., 1976), quickly followed by their finding in nonmammalian vertebrates (Washizu, 1960; Bennett et al., 1963; Furshpan, 1964). It is this historical schedule that allowed Eccles (1964) to propose that electrical transmission was a rare kind of synaptic mediation observed almost exclusively in phylogenetically primitive forms.

The first studies, attempting to establish a morpho-functional match between an electrical transmission pathway and its ultrastructural correlate, pointed to five-layered junctions where the plasmalemma of the two apposed nerve elements fused, occluding the extracellular space (the tight junction of Farquhar and Palade, 1961; 1963), as the low resistance pathways required between neurons for electrotonic coupling, (Bennett et al., 1963). Therefore, for almost four years we lived with the idea that tight junctions were low-resistance pathways. Even if, also in 1963, David Robertson had the first indication that "thigh junctions" were not the morphological substrate of electrotonic coupling. Indeed, in his ultrastructural study of the club endings on teleost Mauthner cell dendrite (Robertson, 1963), he used both osmium tetroxide (OsO_4), and potassium permanganate ($KMnO_4$) as fixatives. In the latter case, in perpendicular sections of the synaptic complexes the two opposing membranes are –as in osmium fixed material- separated one from another by a narrow cleft of about 100 to 150 Å, at the center of the cleft there was a beaded band of electron-dense material with a periodicity of 90 Å. In "in face" views, in sections tangential to the membranes, this center had the appearance of a honeycomb composed of small hexagonal cavities separated from center to center by 95Å. The most surprising observation was the discovery of a dense spot of less than 25Å in diameter occupying the center of each of these cavities. When Lucio Benedetti and Peter Emmelot (1965) centrifugated liver homogenates to isolate the plasmalemma and then negatively stained it with PTA to examine it by electron microscopy, they found similar hexagonal array containing central pits. This indicated that these membranes were tightly packed, but not fused. All these preliminary observations paved the way for Jean-Paul Revel and Morris Karnowski (1967) final discovery. By using liver and heart muscle tissue blocks impregnated with lanthanum to label the extracellular space, they were able to show that what had been labeled as tight junctions by Marilyn Farquhar and Georges Palade (1963) were in reality two different types of junctions: Truly tight junction or "zonulae occludentes" (not lanthanum permeable) with fusion of adjacent membranes, and gap junction or "zonulae communicantes" (lanthanum permeable). In the latter there is no membrane fusion, and the external leaflets of both membranes remain separated by a minute gap (gap that

is at the origin of their name). More importantly, in frontal views the lanthanum improves the visibility of the honeycomb structure, with its hexagonal organization and its central spot also permeated by lanthanum. The thickness of the whole junction was ~125 Å. All this data suggested that these structures were the result of a close intermembrane apposition and not of a membrane fusion. Revel and Karnovski (1967) concluded that since the cardiac muscles were electrotonically coupled (van der Kloot and Dane, 1964), like hepatic cells (Penn, 1966), and particularly the electrical synapses of the teleost's Mauthner cell (Furshpan, 1964), and since in all these cases, the cells share a similar type of hexagonal microarrays membrane junction, these "gap junctions" should be considered to be the real low resistance pathways allowing electrotonic coupling.

INSERT FIGURE 10

When fixed by aldehydes perfusion, postfixed in osmium tetroxide, and en bloc stained with uranyl acetate, the gap junction between neurons in the central nervous system are in high contrast with the unit membranes and easily identifiable. They appear as plaques of very different sizes where the junctional membranes run straight and parallel. In perpendicular sections to the membranes, gap junctions exhibit a heptalaminar configuration, due to the closeness of the junctional membranes, which narrows the extracellular space to a minute gap of about 20 Å width. An almost constant feature of the interneuronal gap junctions is the presence of a cytoplasmic semi-dense band undercoating the whole length of the inner membrane surface at the gap junctional plaque.

As previously mentioned, freeze-etching provides a more detailed image of the membrane structure at the gap junction level. In replicas, these junctions appear as polygonal lattices of integral transmembrane proteins providing particles that protrude on the P-face of the junctional membranes, and complementary lattice of on the E-face (Revel et al., 1971; Makowski et al., 1977).

X-ray diffraction and electron microscope measurements show that these intramembrane particles (IPs) are 8 nm in diameter, and arranged in a crystal-like manner, forming hexagonal arrays, with a center-to-center separation of about 9 nm. This arrangement is symmetrical in both membranes of the junction, thus the IPs on one P-face are mirrored with those on the other P-face. In both membranes, the IPs protrude into the extracellular space, where they touch each other by their distal ends. Since each of these particles has a central aqueous pore of about 12 Å, the IPs establish channels that extend along both membranes of the junction. Each particle forms a hemi-channel, and the two docked particles forming the channel are named "connexon" (Caspar et al., 1977). Therefore, gap junctions are intercellular channels connecting two linked neurons, where ions [cations being slightly more permeable than anions (Neyton and Trautmann, 1985)], and molecules, up to 1 kDa molecular weight, including second messengers, can circulate (Loewenstein, 1966; Simpson et al., 1977). However, since the connexons gating is under strict pharmacological regulation (Peracchia and Girsch, 1985), they cannot be considered passive pathways. This last statement is essential to avoid considering that connexons could be equated to intercytoplasmic bridges, as it is the case in a syncytium. Consequently, one cannot assume that the presence of connexons is in opposition to the "Neuron Doctrine", contrarily to what many have said, even among supporters of the neuron (Bennett, 2002) or being used to promote a new comeback of some reticularistic ideas.

From the protein to the channel: connexins and connexons

Daniel Goodenough's modified method of isolation of liver cells' gap junctions to obtain bulk quantities (1974) was key in the identification of their proteins. In polyacrylamide gel electrophoresis, he identified proteins peaks of 34,000 and 18,000 daltons, and proposed to call them "connexins" (Cx). The first gene for connexins was cloned by Paul (1986), but today the Cx gene family is rather large (37 genes in zebrafish with 16 mammalian Cx, Zoidl et al., 2008). Moreover, Cx are not the only protein able to create low resistant pathways in vertebrates gap junctions. Twenty years later a new gene family was found, the

pannexins (Panchin et al., 2000). They are a shorter gene family with two genes abundantly expressed in the central nervous system, pannexin 1 (Px1) and Px2 (Bruzzone et al., 2003). Mostly by the work of Daniel Goodenough (1974) it became clear that the hemichannels were hexameres of Cx, and that a full connexon was built up by 12 molecules of Cx. Connexins are not cell specific and frequently the same Cx is expressed in a variety of tissues. Specificity is also most frequently excluded among cell types in nervous tissue. Thus, Cx26, Cx30, and Cx43 are considered mostly astrocytic connexins, whereas Cx29, Cx32, and Cx47 are oligodendrocytic ones. Moreover, connexon channels can be heterotypic (not all connexons formed by the same connexins) or asymmetric (connexons with heterogeneity of connexins between the coupling partners). This is mandatory for the numerous gap junctions found between oligodendrocytes and astrocytes. A special case should be done for neurons. Of note is Federico Cicirata and collaborators (Condorelli et al., 1998)'s pioneer work aimed at cloning the gap junctional protein by working on samples taken from rat brain areas with the richest amount of electrical synapses of the whole CNS. According to previous morphological studies, among these areas, the inferior olivary complex (Sotelo et al. 1974; Sotelo and Korn, 1978) was the most appropriated. With this approach, they cloned a new connexin, the Cx36, which has become the neuronal connexin of record.

When looking at the neuronal elements that can be involved in establishing electrical synapses, it can be said that gap junctions in neurons are ubiquitous. They have been reported in all locations of the neuronal membrane: axosomatic, axodendritic, axoaxonic, somatosomatic, somatodendritic, and dendrodendritic gap junctions do exist (Sotelo and Korn, 1978). It is interesting to remark that the coupling of giant Deiters' neurons in the rat is not very strong. Moreover, no one has ever reported that gap junctions were present between cell bodies, or dendrites, or even dendrites and cell bodies. Where are the low resistance pathways where the coupling is done? In electric fishes it has been clearly shown that interneuronal coupling may take place by way of presynaptic axons (Pappas et al., 1975; Fig.16 in Sotelo et al., 1975). It has been concluded that the coupling between giant cells of Deiters was also done by way

of presynaptic fibers (Korn et al., 1973).

One of our topics has been on corroborating or disavowing Sir John Eccles (1964) in his desire to consider electrical synapses as a remnant of the phylogenesis, only present in the less evolved forms of the animal kingdom. For this purpose, electron microscopists started to carefully search for gap junctions between neuronal elements, somata, dendrites, axons and of course axosomatic and axodendritic locations. The race for this research began in the late 1950s, a few years earlier than my beginnings as an electron microscopist. It was just after Ed Furshpan and David Potter (1957; 1959)'s publications demonstrating the reality of electric transmission in invertebrates. The occurrence of electrical synapses in non-mammalian CNS was also fully documented, particularly in fishes (David Robertson and Mike Bennett, see above), and in amphibians (Washizu, 1960). The only "terra incognita" open for discovery was the mammalian brain. After my arrival in Boston in 1965, the opportunity to work alongside Jean-Paul Revel, Ed Furshpan, and Steve Kuffler was very significant to me. During my last months in Paris, working in Jacques Taxi laboratory, I switched from the frog's sympathetic ganglia to its spinal cord, and I was fortunate enough to find large plaques of what resembled tight junctions between large dendritic profiles in the ventral horn. When talking about it with Steve Kuffler, he was kind enough to share with me Alan Grinnell's unpublished work on the frog spinal cord showing new electrophysiological data on the motoneurons electrotonic coupling. Grinnell's paper would appear a few months later (Grinnell, 1966). Four years later, back in Paris, we finally published our work, describing the large gap junctional plaques between motoneurons dendrites, but also the presence of "mixed synapses" between large axon terminals and the dendrites of the motoneurons in the intermediate gray matter (see below) (Sotelo and Taxi, 1970).

My research topic in Sanford Palay's laboratory had been the ultrastructural study of the lateral vestibular nucleus (LVN) of the rat, whose connectivity (Brodal, 1964; Brodal et al., 1962), and microchemistry (Hamberger and Hyden, 1949; Hyden and Pigon, 1960) had been analyzed a few years earlier.

Sandy proposed this topic to me because he considered that the study of the cerebellum (my favorite subject) was already very documented with his personal work, and that the vestibular system would be a good substitute for somebody who like me had studied the spinal cord. This is because the LVN provides a direct link between the cerebellum and the spinal cord. In 1966, after spending six months in the dark gazing at the small screen of an RCA electron microscope, I was happy enough to find something resembling tight junctions in my ultrathin sections of the lateral vestibular nucleus. They were not of course between astrocytic processes, where they are frequent, but between large axon terminals and neuronal cell bodies. Excitedly, I went to see Palay to show him the pictures of my finding. I will always remember his answer: "*one picture is only promising, but far from proving the existence of electrical synapses in the rat brainstem. Among other things, it might be the result of a fixation artifact. Now, go back to the microscope, and when you have found at least 20 of them, come back to me and we shall discuss it*". It took several more months to fill my collection, but finally he accepted the idea that electrical synapses were present in the mammalian brain. I did have time to quickly send a very short abstract to the French meeting of electron microscopy, describing my results (Sotelo and Palay, 1967). A year later, I presented my first electron micrographs of interneuronal electrical junctions (even if still called them "tight junction") in the mammalian central nervous system at the 4th European Regional Conference on Electron Microscopy held in Rome (Sotelo, 1968b). The full paper came out two years later (Sotelo and Palay, 1970).

Only one paper describing the occurrence of mammalian electrical synapses was published before 1967, in the time between their discovery in crayfish (Furshpan and Potter, 1959) and our work in the lateral vestibular nucleus of the rat. John Dowling and Brian Boycott (1966) reported tight junctions in the primate retina along the axosomatic junctions of bipolar terminals and ganglion cell somata in the inner plexiform layer, and also between adjacent cones and rods in the outer plexiform layer. They concluded their study by saying: "*areas of fusion between the plasma membranes are seen,*

suggesting that such axosomatic junctions could be electrical" . Another paper came from the laboratory of Manolo Larramendi (Hinrichsen and Larramendi, 1968) reporting tight junctions between the somata of neurons in the mouse mesencephalic fifth nucleus. The latter was published the same year as our presentation in Rome (Sotelo, 1968b) where we reported the presence in the rat's LVN of large axon terminals with interneuronal gap junctions and synaptic complexes side by side (see mixed synapses below).

The electrophysiological correlation between interneuronal gap junctions and electrotonic coupling in mammals has been quite rare, due to the technical difficulties to perform simultaneous pair intracellular recording in a live animal. The paired recording requires to stimulate intracellularly one of the neurons and to record its depolarization as well as the immediate depolarization of the coupled second neuron, as response to the first stimulation. An indirect but simpler way for testing the presence of electrical transmission was developed (see in Bennet, 1964). It consisted of the antidromic stimulation of the axons of the Deiters' neurons in the vestibulospinal tract, at the ipsilateral ventral funiculus of the spinal cord. The tract was stimulated at the C3 level with increasing subthreshold stimuli. Under these circumstances, even if the neurons are coupled, an impulse will not propagate actively to the neighboring ones if the coupling ratio is low; the action currents set up by this impulse will however produce a small depolarization in the nearby cells. Consequently, if the threshold of the impaled cell's axon is high relative to that of a significant proportion of the neurons in the nucleus, antidromic stimulation of graded intensities will produce in that cell graded subthreshold depolarizations, called "graded antidromic depolarizations" (GAD) or "short latency depolarizations" (SLD). Therefore, intracellular recording of short latency depolarization either after graded stimulation of the trigeminal nerve (Baker and Llinas, 1971) for neurons in the mouse mesencephalic fifth nucleus, or by graded antidromic stimulation producing graded antidromic depolarizations (GADs) -of very short latency preventing chemical transmission- (Korn et al., 1973) for giant Deiters neurons of the LVN, testify for electrical transmission of the impaled neurons with another neuron located in close vicinity. Since we have not found the presence of gap junctions between somata or dendrites or between dendrites

and somata, there is no other possible way to explain the coupling than to consider that it takes place through an axon, as it is the case in electric fishes (see above, Pappas et al. 1975, Fig. 16 in Sotelo et al., 1975).

A less accurate method, but with high possibility to disclose electrotonic coupling, is the "dye-coupling", with highly fluorescence dyes of molecular-weight less than 1kDa, particularly the Lucifer Yellow that can be traced even after fixation and embedding. Once injected intracellularly, this dye can diffuse through the connexon-channels of the gap junctions, into a large number of neighboring coupled neurons, providing a quantifiable method for determining the extension of the coupling. A beautiful example of the possibilities offered by this method is the study of the turtle retina of Hersch Gerschenfeld (Gerschenfeld et al., 1982) on the number of fluorescent horizontal cells in the coupled network, and the regulation of the connexons permeability induced by the dopamine chemical neurotransmitter (Piccolino et al., 1984). Today, there are easier methods for determining the presence and extension of electrical synapses in the different brain centers. For instances, at light microscopical level, immunohistochemical labeling with antibodies directed against neuronal connexins mainly Cx36, marks the location and density of gap junctions, and double immunohistochemistry with a neuronal marker, details their neuronal belonging (Belluardo et al., 2000). At the ultrastructural level, the Freeze-Fracture Replica Immunogold Labeling (FRIL) helps to analyze the gap junctional plates, as well as some other structures in the nearby membrane, for instance synaptic complexes, to identify mixed synapses (see below) (Nagy et al., 2004). All these new technologies have made possible to conclude that the electrical transmission is not restricted to the primitive forms of the animal kingdom, but that it is present in all the species analyzed up to now, including humans. Therefore, today we can say that electrical synapses (gap junctions between neurons) are abundantly present in many different regions of the central nervous system, of invertebrates as well as vertebrates, including mammals (Nagy et al., 2019).

The presence of gap junctions between neurons in the vertebrates CNS

generated a debate among some neuroscientists who considered that this discovery partially invalidated the Cajal's neuron doctrine. The connexons channels have been interpreted as micro interneuronal passive bridges, establishing intercytoplasmic connections organized in a syncytial manner. This interpretation was somewhat provocative to the supporters of the Cajal's "neuron doctrine", since it can be considered that cytoplasmic continuity does indeed occur in the CNS. Every single time that I have been harassed by these arguments, I repetitively provided the same response: The connexon is not a passive tunnel but that, on the contrary , has a proper chemical regulation controlled by intracellular (second messengers, Ca2+, pH) and extracellular signals (mainly neurotransmitters and nucleotides), that govern its opening and closure accordingly to a specific pharmacology. In conclusion, the gating behavior of the connexins dictates the properties of the gap junctions, which are different from those expected for a syncytium

MIXED SYNAPSES

One of the last points that I should like to emphasize is that chemical and electrical transmissions do not always exclude each other but, in precise morphological situations, both operate between the same two neurons, and even throughout the same axon terminal, in cases where chemical synaptic complexes and electrical gap junctions are located at the same synaptic interface between an axon terminal and a neuronal cell body or a dendrite. We (Sotelo and Palay, 1970) named this last type of anatomical situation as "mixed synapses".

INSERT FIGURE 11

Historically speaking, the occurrence of a dualist mode of synaptic transmission, was shown by the neurophysiologists Bob Martin and Guillermo Pilar (1963) on the ciliary ganglion of birds. They found that at many of the synapses chemical transmission was accompanied by electrotonic coupling. The electrical synapses were not rectifying, meaning that they were bidirectional. They concluded that in the ciliary ganglion the presynaptic boutons are very large, forming long calices covering the hilar region of the ganglionic cell bodies. This large covering

could provide a low cleft resistance to allow the coupling. This given explanation was necessary because in the first electron microscopic analysis of the chick caliceal synapses (de Lorenzo, 1960), pre- and postsynaptic membranes had no fusion plaques, and any narrowing of the synaptic cleft was observed. For years this dual mode of synaptic transmission was considered to be an exception in the field of electrical transmission. Nevertheless, in a later study by Dario Cantino and Enrico Mugnaini (1975), gap junctions were found nearby synaptic complexes. By using the freeze etching approach, these investigators calculated that the mixed synapse had a chemical preponderance (only 1,8% of the surface area used in synaptic complexes is occupied by gap junctional connexons). In conclusion the electrical part of the synaptic connection may only enhance the liberation of neurotransmitter at the chemical junctions. For Cantino and Mugnaini (1975) "*the electrical coupling between the preganglionic fiber and the ciliary neuron may be of resistive nature*".

Such mixed synapses pose a difficult functional problem: do the boutons with mixed synapses have a dual transmission mechanism? Mixed synapses have already been described in the central nervous system of electric fishes (Bennett et al., 1976), in the granular layer of the frog cerebellum, as well as in the frog spinal cord (Sotelo, 1969; Sotelo and Llinás, 1972; Sotelo and Taxi, 1970) and in the lateral vestibular nucleus of the rat (Sotelo and Palay, 1970). In the latter, we advanced a series of functional speculations for the mixed synapses. We concluded that they should be related to more subtle effects than simply electrotonic coupling, emphasizing a kind of cooperative action between the two modes of synaptic transmission. But in none of these instances, the dual mode of synaptic transmission has been corroborated by electrophysiological methods.

In addition to the publication of Martin and Pilar (1963) described above, some few studies have approached the question of the dual (electrical and chemical) synaptic transmission in "mixed synapses". Among the few studies on the electrophysiology of mixed synapses that appeared before the nineties there was not complete agreement on their dual transmission capacity. Thus, for Mike Bennett in most of the morphological mixed synapses in electric fishes (Pappas & Bennett, 1966; Bennett et al., 1967; Korn et al., 1973) no physiological evidence of the

existence of a dual transmission mechanism was found; the only mode of transmission they observed was only electrical. However, for neurocytologists, it was discouraging to accept the idea that specialized structures commonly related to a specific function may sometimes be devoid of functional significance. This uncertainty was partially dispelled in another publication of which we are coauthors (Korn et al., 1977) on toadfish LVN, where mixed synapses were also numerous. In this case, graded stimuli applied to the vestibular nerve evoked graded short latency depolarizations (electrical transmission), together with long latency excitatory postsynaptic potentials (chemical transmission) in these presumed efferent neurons to the labyrinth. The work of Alberto Pereda and collaborators during the nineties (see references in Pereda, 2014), dedicated to the understanding of the functional characteristics of mixed synapses in an excellent model, the teleost's Mauthner cell, opens the way to the understanding of the dual transmission in some mixed synapses. Already in our pioneer paper, where we described the "mixed synapses" in a mammalian brain, we offered a series of tempting explanations on such side-by-side presence of synaptic complexes and gap junctions, concluding that chemical and electrical transmission were not exclusive one for another, and that they can combine "*underlie more subtle and refined kind of neuronal interactions that has been hitherto contemplated*" (Sotelo and Palay, 1970). The Alberto Pereda study reported that the active zones of the synaptic complexes do indeed release amino acid involved in chemical transmission that also interacts with electrical synapses. High-frequency activation of "large myelinated club endings" or simply "club endings" leads to long lasting potentiation of both modes of transmission that can be blocked by NMDA receptor antagonists. Moreover, glutamatergic synapses regulate the traffic through the connexons channels modifying the gap junctional conductance, highlighting that electrical synapses are able of activity-dependent plasticity. It is obvious that the electrical synapses, at the club endings synapsing on Mauthner cells, are very plastic, and can follow several types of dynamic regulatory control (Pereda et al., 2004).

CHEMICAL SYNAPTIC MODULATION OF ELECTROTONIC COUPLING

Besides the mixed synapses other co-operative interactions between electrical and chemical synapses have been described. They concern a specific mechanism allowing the increase or the decrease of the electrotonic coupling of neurons. The

main one is undoubtedly the neuromodulatory role of neurotransmitters. Although a large part of the gathered knowledge on this topic has been obtained in invertebrates (Spira and Bennett, 1972; Spira et al., 1976; Carew and Kandel, 1976), here only one example taken from cat inferior olivary complex will be presented. A relatively large number of neurotransmitters have been identified as active modulators of gap junctional coupling, the most important ones being: dopamine (Piccolino et al., 1984), noradrenaline (Zsiros and Maccaferri, 2008), serotonin (Rörig and Sutor, 1996), histamine (Hatton and Yang, 1996) and, of course, glutamate and GABA (see references in Pereda, 2014).

INSERT FIGURE 12

In inferior olive of mammals, a central region containing abundant interneuronal gap junction (it was used by the team of Federico Ciccirata to clone the Cx36, the connexin of mammalian electrical synapses), an anatomical situation favorable for the chemical synaptic modulation of the interneuronal gap junctions is present. The vast majority of gap junctions are restricted to specialized anatomical arrangements of the neuropil, the inferior olivary glomeruli. In the cat (Sotelo et al., 1974), the glomerulus is composed of a central core of slender dendritic processes, arising from different olivary neurons surrounded by a peripheral ring of excitatory (glutamatergic) and inhibitory (GABAergic) axon terminals (Sotelo et al., 1986). The axon terminals forming the peripheral synaptic coverage establish synaptic complexes with the central dendritic processes. Gap junctions are frequently linking the central dendritic elements. Usually, the same axon terminal has formed active zones with the two dendritic protrusions linked by the gap junction. The glomerulus is isolated from the remaining neuropil by thin astrocytic lamellar processes. This strategic arrangement of chemical and electrical synapses suggests that the chemically inhibitory synapses may exert a shunting effect on the electrotonic transmission, by increasing conductance of their membranes, during activation and transmitter release, producing a functional amputation of the electrotonic coupling. Vice versa, when the excitatory synapses are activated, the released glutamate, could increase the coupling ratio between the linked neurons (Llinás et al., 1974).

In conclusion, the morphological results discussed here emphasize the fact that interactions between the two mechanisms of synaptic transmission can take place in vertebrates, including mammals. The possibility to combine electrical and chemical transmission for the modulation of synaptic effectiveness, at least in some neuronal networks, would increase the number of possible operational states of the networks. The main problem still to solve would be to understand the behavioral significance of the interactions of chemical and electrical synapses.

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LEGENDS FOR FIGURES

FIGURE 1.
Fig.1A: Cajal drawing to illustrate Gerlach's erroneous reticular concept regarding the organization of the nervous system, taking the spinal cord as an example. Note that both dendrites and the axons are anastomosed forming a large and unique network.

Fig.1B: Schematic representation of the cerebellar cortex used by Golgi in his Nobel lecture. As Raviola and Mazzarello (2011) pointed out, in this drawing the dendrites of the basket cells remained free, since for him the dendrites were not conductive elements but only had nutritional use. However, the axons in the granular layer are extensively anastomosed, much more so than in his earlier publication of 1873. This over-exaggeration was intended to defend his reticular position.

FIGURE 2.
Fig.2A: Golgi impregnation of the molecular layer of a two months-old guinea pig cerebellum. (A) Basket cell axon; (B) soma and dendrites of a basket cell; (c2) descendant axon collaterals of the basket cells forming the pericellular baskets around the Purkinje cell perikarya; (C_1) climbing fiber; (d) Purkinje cell.

Fig.2B: Adult human cerebellum illustrating a climbing fiber around the dendritic tree of a Purkinje cell.

Fig.2C: Climbing fiber climbing over the Purkinje cell dendritic tree. Purkinje cells are labeled by using anticalbindin immunohistochemistry, and the olivocerebellar fibers were visualized by biotinylate dextran amine anterograde axonal tracing, in an adult rat cerebellum.

Fig.2D: Schema of the parasagittal section of the cortex of a cerebellar folium with its three layers: A) molecular, B) granular and C) white matter axis. a) Purkinje cell with a dendritic tree spanning on the sagittal plane of the molecular layer. b) basket cells and their axons, forming the pericellular nests of nervous twigs around the cell bodies of Purkinje cells; n) climbing fibers, climbing on the proximal segment of the Purkinje cell dendrites ; o) Purkinje cell axon and its recurrent collaterals; e) stellate cells of the molecular layer; g) granular cells with their ascending axons dividing in T into the molecular layer to form the parallel fibers; f) Golgi cell; h) mossy fibers; J) Bergmann glia; and m) fibrous astrocyte.

FIGURE 3.
A and B: Superior cervical ganglion of a normal cat. C: Human sympathetic lumbar ganglion of a 38-years-old man.

Fig.3A: Semi-schematic drawing of a synapse of the sympathetic ganglion. a) Preganglionic fiber; b) axon of the sympathetic neuron (postganglionic fiber); g) neuroglial cells (gliocytes) surrounding the neuron's cell body and its dendrites. The preganglionic fiber branches within the ganglion, and their branches through either “en passant” or terminal buttons establish synaptic contacts (arrows) within the cell body and the dendrites of the ganglion neuron.

Fig.3B: This more complex de Castro's drawing also illustrates the presence of terminal synaptic buttons belonging to a preganglionic fiber, synapsing on the surface of the dendrites (arrow heads) and the cell body (arrow) of the ganglion neuron. Cajal reduced silver impregnation.

Fig.3C: Drawing of a portion of human ganglion cells, with preganglionic (*a*) and intraganglionnic endings (*d*) over dendritic bushes (b, g), a protoplasmic process forming collaterals(*c*) and a pericellular dendritic nest over one of the dendritic bushes (*f*).

FIGURE 4
De Castro. Experimental studies. Anastomosis of the preganglionic tract with the pneumogastric nerve.

Fig.4A) Portion of a sympathetic ganglion with its regenerated afferent fibers originating from the vagus nerve, and in connection with the intrinsic ganglion cells. Vago-sympathetic cross anastomosis 30 days after surgery. a) Large regenerated nerve fiber. b) ganglion cell dendrite showing two terminal synaptic buttons in contact with its surface (arrows). Several other buttons appear in connection with the cell body of neurons (arrow heads). Cajal reduced silver method, fixation in a somniphene mixture

Fig.4B) Scheme of the bineural reflex arc, constituted in the crossed vago-central afferent anastomosis with the sympathetic ganglion, and direction of the nervous impulses (direction of the arrows), originated by excitation of the peripheral receptors of the vagus. The portion integrated by the sensitive protoneuron, in red, and that corresponding to the sympathetic motor neuron, in blue. Peripheral receptors: (1) bronchi and lungs; (2) cardio-aortic zone; (3, 4) esophagus and stomach. Afferent route: (6) peripheral trunk of the vagus; (7) nodose ganglion; (a) anastomosis; (8) sympathetic ganglion with synapses constituted by the regenerating sensitive fibers. Efferent route: (9) postganglionic neurites terminated (10) in the hairs; (11) pupil and nictitating membrane, and (12) ear vessels.

Fig.4C) Thick neurites (a, b) belonging to afferent roots in a sympathetic ganglion (111 days after the crossed anastomosis). Note the morphological diversity of terminal apparatus, originated in collateral expansions, destined to model the new interneuronic synapses. Method: somniphene, reduced silver.

Fig.4D) Reflex activity in the operated sympathetic system caused by mechanical distension of the stomach by means of a balloon, previously introduced, at a pressure of 45 mm Hg; Notice the extreme dilatation of the right pupil (mydriasis), the side of the anastomosis.

FIGURE 5
Fig.5A, B, C) Subneural apparatus of Couteaux stained with Janus B Green and fixed with ammonium molybdate. Only the subneural apparatus is stained. The nerve branches (not visible) occupy the axis of the synaptic gutters, which are lighter. The lamellae of the subneural apparatus outcrop only by their edge with the surface of the end-plate. Arranged parallel to each other and very tight, they give the impression of a

continuous layer at first glance. Muscle of the pelvic plate of the Grey Lizard (X 1900).

Fig.5D, E, F) Histochemical detection of cholinesterase activity (thiocholine method) in cross-sections of the subneural apparatus showing the continuity of the lamellae under the sub-neural gutter. Mouse end-plate.

Fig.5G) Cholinesterase activity at the level of the subneural lamellar structure of the frog neuromuscular junction.

FIGURE 6.
Sanford L. Palay and the synapse under the electron microscope

Fig. 6A) Photographic portrait of Sandy Palay in 1986 (personal belonging)

Fig. 6B) Electron micrograph of two axon terminals synapsing upon a dendrite in the neuropil of the abducens nucleus. The dendrite, contains mitochondria and endoplasmic reticulum profiles. The synaptic buttons contains mitochondria and numerous synaptic vesicles. The arrow-heads point to the two synaptic complexes. Notice the apparent increase in thickness of both pre- and post-synaptic membranes, that are clearly separated by an intrasynaptic cleft approximately 200 A wide. The nervous system is not a syncytium but is formed by individual separated cells. X34,000.

Fig. 6C) Molecular layer of the rat cerebellum immunostained with anti-GABA antibody. Two buttons are establishing synaptic contacts with a small dendritic profile (den). Both the small dendritic profile and the larger of the two synaptic buttons are marked with colloidal gold particles, indicating their belonging to GABAergic inhibitory neurons. This micrograph intends to illustrate the fact that synaptic complexes of Gray type 2 (arrow-head), together with the presence of pleomorphic or flattened synaptic vesicles belong to inhibitory synapses (I), while buttons with Gray type 1 synaptic complexes (arrow) together with the presence of round vesicles are the excitatory (E) button distinctive.

Fig. 6D) Electron micrograph of a section oriented parallel to the synaptic membrane treated with the “bismuth-iodide block impregnation combined with uranyl acetate and lead hydroxide contrast” of the subfornical organ of the cat, that provides a strong coloration of electron-dense synaptic differentiations. In this en face view of the presynaptic vesicular grid, the cluster of dense bodies form the nodal points of the hexagonal network with its interconnected tiny filaments, leaving place to the free spaces (some of them are labeled with an asterisk) where the synaptic vesicular membrane can be in direct contact with the axolemma, and to fuse with it for the exocytotic release of neurotransmitter.

FIGURE 7.

René Couteaux and synaptic vesicle recycling: exo- and endocytosis.

Fig.7A) Electron micrograph of a frog’s motor end plate illustrating the terminal of

the motor axon, with its synaptic vesicles, and the location of two active zones (between the black and the white arrow-heads). The latter are identifiable by the disposition of two parallel rows of synaptic vesicles along the central presynaptic dense projection. Note how the active zone are strategically located facing the subneural folding.

Figs.7B, C) Tangential sectioning of the deep face of the motor nerve terminal of frog neuromuscular junction. These electron micrographs illustrate the spatial ordering of the synaptic vesicles at the level of the active zones. They form double rows of vesicles bordering the presynaptic dense projection, the denser material between the vesicles' row (see in B).

Fig. 7D, E) Exocytotic opening of the synaptic vesicles into the synaptic cleft at the level of the active zone of the frog neuromuscular junction. The arrow marks the presynaptic dense projection. The arrow-heads point to synaptic vesicles with omega shapes, at different steps of their fusion with the axonal membrane and opening to the extracellular space.

Fig.7F) Photographic portrait of René Couteaux (Personal belongings).

Fig. 7G) Schematic representation of the retrieval cycle of synaptic vesicles in a terminal synaptic button of a neuromuscular junction. Vesicles, budding from the smooth endoplasmic reticulum, are conveyed to the axonal membrane in the active area (long arrow), where they will open to the synaptic cleft. Within the axonal membrane, the vesicular membrane is transported to the lateral region of the axon terminal (curved arrow), where -recovered with a coating of the filamentous protein clathrin- it is endocytosed and delivered again to the lamellae of smooth endoplasmic reticulum (shorter arrow) to be retrieved.

Fig. 7H) Lateral zone of the motor axon terminal showing the retrival of the vesicle membrane in a phase of endocytosis (arrow-head). Note the covering with catrine protein of the internal part of the membrane.

FIGURE 8.

Paracrine interactions between axonal buttons and postsynaptic targets. The innervation of the rat smooth muscle in the autonomous nervous system, and serotonergic innervation in the cerebellum.

Fig. 8A, B, C): These three electron micrographs illustrate the noradrenergic innervation of the vas deferens' smooth muscle of the rat. A) Appearance of a nerve fiber still covered with a very small Schwannian cap (arrow-head). B) Varicosity between two very closed muscle cells. C) Very long contact between a nerve fiber and a muscle cell. Note first that the axon varicosities contain small granular vesicles, as corresponds to noradrenergic nerves. Secondly, the distance between the varicosities and the muscle cells is variable, some times rather large (see in A). Finally no active zones or postsynaptic differentiations are observed. The three micrographs have the

same magnification.

Fig. 8D) Radioautograph of tritiated serotonin illustrating a labeled varicosity exhibiting the cytological features characteristic of a "non-junctional" axonal button of 5-HT cerebellar afferents. Mitochondria, large granular vesicles, numerous elongated vesicles and discrete profiles of smooth endoplasmic reticulum are embedded in a moderate electrondense
axoplasmic matrix. Note the absence of synaptic differentiation between this terminal and the
surrounding structures, x 55,000.

FIGURE 9.
Drawings in support of de Castro hypothesis conferring to astrocytes the important role of vector elements in synaptic transmission. (de Castro, 1941).

Fig. 9A) Spinal cord stained with the Cajal's gold sublimate method to impregnate astrocytes. (a) A protoplasmic astrocyte in the anterior horn in the neightborhood of a motoneuron (b) sends a large amount of its processes to cover the surface of the cell body and dendrites, leaving practically no open space for a direct contact with the impinging afferent fibers. (d) Higher magnification of the glia covering of the dendritic surface.

Fig. 9B) In the spinal cord impregnated with the Cajal's reduced silver, presynaptic buttons appear separated from the neural plasmalemma by a narrow pale colored space. Since de Castro was confident that in his preparations there was not srinkage, this pale space was considered to be filled by the astrocytic processes. (c) At higher magnification, this small drawing illustrates the three components of the synapse. 1) neuronal surface; 2) glial mantle; 3) presynaptic axon terminal. This hypothesis was discarded with the first glimpse of the synapses organization with the electron microscope.

Fig.9 inset) Photograph of Fernando de Castro, during the conjoint meeting of the Spanish and French societies of Anatomy, held in Madrid in 1964.

FIGURE 10
Electrical versus chemical transmission. The gap junction:

Fig. 10A) Axodendritic gap junction in the cerebellum of the viper. A mossy fiber (MF) establishes a gap junction with a granule cell dendrite (GD). Note the presence of a semi-dense material undercoating the internal membrane surface of the gap junction (arrows), and the attachment plaques (AP) at the boundaries of this electrical synapse. X98,000.

Fig. 10B) Electron micrograph of a gap junction in the LVN of the rat, in a material treated with colloidal lanthanum to delineate the extracellular space. Note the beaded aspect of the central dense stratum. The beads are repeating at a period of about 90 Angstroms.

Fig. 10C) e Freeze fracture of the rat cerebellum illustrating two gap junction plaques (GJ) between the plasma membranes of two apposed molecular layer interneuronal dendrites in the lower half of the molecular layer. The fracture plane of the upper gap junction shows the P face (P), which contains numerous diffused intramembrane particles arranged in a crystal-like pattern. The lower gap junction shows part of their particles also in their P face, but most of them appear as complementary pits within the membrane E face (E).

Fig.10D) Schematic model of electrotonic coupling by way of presynaptic fibers. Two post- synaptic neurons A and B are shown. A presynaptic fiber branches and synapses with both cells through gap junctions (dark areas), thus bridging them electrically. The action currents set up by a spike in one neuron (A) flow through the presynaptie bridge to the second cell (B) were a depolarizing potential (G.A.D.) is thereby generated.

Fig.10E) A single axon terminal in synaptic contact with the cells bodies of two small neurons (N). Gap junctions (arrows) are present at both synaptic interfaces. This anatomical arrangement correlates with electrotonic coupling between neurons by the way of a presynaptic axon.

FIGURE 11.

Mixed synapses in the lateral vestibular nucleus (LVN) of the fish

Fig. 11A) LVN of the toadfish *Opsanus tau*. A primary vestibular axon establish a mixed synapse on the cell body of a giant vestibular neuron. At the synaptic interface three classes of junctional complexes are present: (1) an active zone of a chemically transmitting synapse (AZ); (2) an attachment plaque (AP); and (3) a gap junction (GJ). Notice the absence of synaptic vesicles in the axoplasm close to the gap junction. X44.000

Fig. 11 B) Freeze-fracture replica showing the axonal membrane exposed on its P-face, whereas the somatic membrane shows its E-face. Large intramembrane particles, aggregatesin the typical crystalline of the gap junction (GJ) are present in the axonal P-face. At the bottom of the micrograph there is an area of the axonal P-face characterized by the presence of large particles associated with an array of dimples (area marked by the arrow-heads) which corresponds to the vesicles attachment sites of the active zone (AZ) X66.000

FIGURE 12

Regulation of the electrical transmission by chemical synapses: the inferior olive

Fig. 12: Electron micrograph of an inferior olivary glomerulus in a rat injected, 5 days before fixation by intracardiac perfusion, with an axonal anterograde flow marker (WGA-HRP) in the dentate cerebellar nucleus. After sectioning and revelation of the HRP activity with diaminobenzidine, the thick sections were embedded in Araldite

and ultrathin sections were processed for immunocytochemical demonstration of GABA with the immunogold reaction. The double-labeled sections were analyzed with an electron microscope. One GABA-immuno- reactive dentato-olivary terminal (marked by the HRP reactivity, white asterisk) with a glomerular location, establish symmetrical synaptic contacts (arrow-heads) with dendrites linked through a gap junctions (thin arrows). The two-coupled dendrites, in addition to the dento-olivary button also receive synapses from an unlabeled terminal. The synaptic complexes of the latter are of Gray type 1 (larger arrows), and together to the fact that it is not labeled by the colloidal gold, assures that it is an excitatory terminal. Therefore, the coupled dendrites are under the regulation of inhibitory and excitatory chemical synapses.

Fig.12 Upper insert: Schematic representation of an inferior olivary glomerulus. In the central core, small dendritic branches and spiny appendages, emerging from thicker branches (IOD), are joined by gap junctions (arrowheads). The central core is surrounded by synaptic terminals (ST), which establish contact with the core elements. (B) Illustrates the path of coupling current between two IO neurons. (c) Indicates hypothetical function for the synaptic junction at the glomerulus. When the synapses are activated, the conductance change produced by the synaptic transmitter action on the postsynaptic membrane produces a shunt at the glomerular level which reduces the coupling coefficient between the cells, since the current tends to be lost across the shunt.

Fig. 12 Lower insert: High magnification of a gap junction jointing two dendritic appendages in the central core of an inferior olivary glomerulus of the cat, illustrating its seven-layered structure.