Role of N-Caderin and SAP97 in dentritic spine morphology and synapse formation

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ROLE OF N-CADHERIN AND SAP97 IN DENDRITIC SPINE MORPHOLOGY AND SYNAPSE FORMATION

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On croit connaître la raison pour laquelle on s'enrôle en guerre, mais c'est quand on ne croit plus en la guerre que l'on sait que la raison était mauvaise. La guerre trouble l'esprit et l'âme et ne laisse personne indemne. On en revient honoré d'une croix plantée sur le poitrail, de fer ou de bois...

Ma thèse fut ma longue guerre de salon dans cette vie facile et privilégiée qu'est la mienne. Je m'y suis lancé rêvant de nouvelles expériences, de nouvelles connaissances, de gloire, et cela avec la conviction du devoir et de l'appartenance à une grande chose. J'en suis revenu désillusionné. Néanmoins, ce fut pour moi un merveilleux laboratoire de vie. J'ai pu découvrir énormément de choses sur ma personne, sur les autres et bien entendu sur le monde formidable et fondamental des neurosciences. Je suis donc content de ces cicatrices qui au moment où j'écris ces lignes me font encore un peu mal. Mais je sais qu'elles dessinent déjà certaines des constellations qui forment mon univers personnel.

Je choisi donc un chef, ce fut le Professeur Dominique Muller. Un chef diablement intelligent ayant une intuition hors-norme dans le domaine qui est le sien. Un chef avec lequel je n'ai pas toujours été d'accord, mais tout le monde sait bien que le chef a toujours raison. Voilà pourquoi, comme simple soldat, on n'a que peu le choix de ne retourner au front pour couper du bloc. Mais il n'y a que ceux qui se sont battus qui gagnent, et bien qu'endommagé, finalement, une petite parcelle de savoir a été gagnée sur le champ de l'inconnu! De plus, Dominique m'a donné beaucoup de liberté pour faire des expériences en dehors du labo. Ce fut un chef disponible, efficace et qui finalement m'a donné les moyens nécessaires pour parvenir à mes fins. C'est pourquoi, je suis reconnaissant d'avoir pu me battre et apprendre sous sa longue vue. Merci!

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Et finalement, j'aimerais remercier toutes ces personnes que j'aime et qui m'aiment. Ma famille, Mam, Pap, Franch, Dav, Pietr. Mes amis d'enfance, Dastier, Jérôme, Nils, Philippe, Thibault. Mes amis de Genève, et ceux d'ici et d'ailleurs. Mes amis du département de Neurosciences. Les jumpistes. Et ceux qui sont un peu tout à la fois et qui m'ont superbement soutenu et aidé ces dernières années. Merci à eux du fond du coeur.

I would like to warmly thank all the people that contributed to this work and all the people that supported myself during the years of my thesis work.

"The facts remain and theories pass away"

Santiago Ramòn y Cajal, 1894

"The brain, the most beautiful masterpiece of nature"

"Our mind thinks that nothing can set a limit to its knowledge, but when it withdraws to its own habitation it is unable to give a description of it, and no longer knows itself"

Niels Stensen (1638-1686)

Résumé de mon travail de thèse

Un objectif central de mon travail de thèse a consisté à étudier la diversité morphologique des synapses excitatrices des épines dendritiques de neurones, et de comprendre la relation entre la morphologie et la fonction au niveau des synapses. Pour ce faire, j'ai utilisé le modèle des cultures de tranches organotypiques d'hippocampe de rat qui m'a permis de pouvoir tester différentes approches pour marquer les neurones du CA1 de l'hippocampe avec des marqueurs fluorescents. Le marquage de neurones a rendu possible la comparaison de la morphologie des synapses observées en microscopie confocale avec celles observées en microscopie électronique. Un autre aspect technique important de ma thèse a été la collaboration avec Joël Spaltenstein pour l'élaboration d'un programme informatique dans le domaine de la reconstruction 3D. En effet, les images sériées prises en microscopie électronique doivent être alignées pour pouvoir être utilisées pour la reconstruction tridimensionnelle. Nous avons élaboré un nouveau programme qui a grandement facilité l'alignement des images.

Les synapses contiennent une multitude de protéines qui interagissent entre elles pour constituer la diversité morphologique des synapses. Parmi ces molécules, j'en ai choisies deux : N-Cadherin, liée à la famille des Cadhérines, ainsi que SAP97, lié à la famille des MAGUKs. Pour en comprendre la fonction, j'ai joué sur la modulation de leur expression, à savoir que j'ai soit diminué ou augmenté leur fonction par la sur-expression de leurs formes mutées ou sauvages (WT), et cela dans des cellules pyramidales du CA1 (système biolistique de transfection).

Les épines dendritiques ainsi que les synapses ont des morphologies qui dépendent du développement et de l'activité. Dans mon travail de thèse, j'ai, comme sus-mentionné, focalisé mon attention sur 2 molécules importantes après avoir vérifier la concordance entre les observations en microscopie confocale et celles en microscopie électronique.

Les travaux préliminaires à l'étude de SAP97 et N-Cadherin ont montré une concordance entre les observations de microscopie confocale et celles obtenues en microscopie électronique. Dans des conditions normales, 94% des épines possèdent une densité postsynaptique, et seulement un petit pourcentage de protrusions, 6%, couramment classées comme des filopodes, en sont dépourvus.

Dans le cas de SAP97, j'ai observé qu'une sur-expression induisait des épines dendritiques multi-innervées. Ces épines faisaient des synapses avec plusieurs boutons axonaux, cela avec un pourcentage de plus de 40%. Ce résultat pouvait être inhibé par le traitement des cultures avec du L-NAME qui est un blocker de la NO synthase. Une étude récente faite par Irina Nikonenko a démontré que PSD-95, également une MAGUK protéine, peut aussi induire des épines multi-innervée par l'activation de la NO synthase. La libération de NO dans le milieu extra-cellulaire semble être le facteur trophique menant au recrutement de boutons axonaux. En plus du phénomène d'épines multi-innervées, une sur-expression de SAP97 a provoqué une augmentation moyenne du volume des épines ainsi qu'une augmentation de la taille moyenne de la surface des synapses. La morphologie produite par la sur-expression de SAP97, avec ou sans traitement de L-NAME, s'est traduite par l'enroulement des épines autour des boutons présynaptiques. Ce phénomène soulève plusieurs questions quant au rôle de SAP97 non-seulement au niveau de la synapse, mais également son rôle dans le façonnement de la morphologie de l'épine tout entière.

N-Cadherin est connue pour altérer la maturation des synapses et interférer avec la plasticité synaptique. En sur-exprimant une forme mutante de N-Cadherin (Δ390 N-Cadherin), j'ai également observé, en microscopie confocale, des épines dendritiques ayant un aspect immature, les épines étaient pour la-plupart allongées avec de petites têtes. Mes observations en microscopie électronique ont permis de montrer que ces épines immatures formaient des synapses dans leur grande majorité. Cependant, le roulement (*turnover*) de ces épines était plus grand qu'à la normale. D'un autre côté, la sur-expression de la forme sauvage de N-Cadherin a induit une augmentation du volume de l'épine. Ce qui suggère que N-Cadherin a un rôle important dans la maturation des épines et des synapses.

Le résultat principal est que ces molécules de la PSD affectent de manière différente la morphologie des synapses en affectant de manière distincte au moins 4 paramètres: la taille de l'épine, la forme de l'épine, la taille de la PSD et le nombre de contacts. Ce résultat indique des rôles spécifiques de chaque molécule à la synapse. Il y a cependant aussi des traits communs. Les molécules d'échafaudage semblent avoir un effet prédominant sur la dimension de la PSD. Sur exprimer une de ces molécules probablement augmente l'expression de nombreuses autres protéines de la PSD, dont notamment NOS, ce qui expliquerait la formation de multi-innervations. Par contraste, la sur-expression de N-Cadherin ne produit pas ces effets, même si elle favorise une sur-dimension de l'épine. L'épine est plus grosse, la PSD est plus grande, mais la forme de l'épine reste normale, ce qui suggère que N-Cadherin régule plus probablement l'organisation générale de la synapse et peut-être sa stabilité comme suggéré par des analyses confocales.

En conclusion, l'analyse 3D de l'organisation structurelle des synapses se révèle être un outils très utile et puissant pour comprendre de manière plus précise la fonction des protéines synaptiques.

CONTENTS

INTRODUCTION

1. From the Antiquity to Cajal

The “physical nature” of the messages used to exchange information in an organism has long remained a labile notion. ¨Neuro¨ investigators passed two thousand years of speculations about the nature of communication signals between the “centre of perception” (thought to be either the heart or the brain) and the “effectors” (skeletal muscles, which were more easily identifiable). Despite a vivid argumentation that led to the neurone doctrine of Santiago Ramòn y Cajal, it took five supplementary decades before the speculated point of connection between neurons, the synapse, was definitely observed thanks to electron microscopy.

1.1. Aristotle

Aristotle, 384–322 B.C., maintained that the heart was the central organ of perception rather than the brain as postulated by Plato. In addition, he suggested the existence of a fifth element (besides air, fire, water and earth), the ether. The ether was bathing the entire universe, hence belonged also to heavens, and it was taken into the body during breathing and it conferred the vital pneuma once it was transformed by the heart. This pneuma was then distributed to the peripheral organs by the blood vessels. Therefore, this vital pneuma, the so called signal, was considered as having a dense nature.

1.2. Galen

Galen (129–201) supposed that the vital pneuma passing trough blood vessels was transformed in the brain and converted into psychic pneuma. From the brain the psychic pneuma traveled outwards along nerves.

It is only with Luigi Galvani (1737–1798) that a clear demonstration has been made about the particular property of electrical signals that can be conducted along nerves, hence revealing the physical nature of the messages between the brain and the skeletal muscles.

1.3. Leopoldo Nobili, Carlo Matteucci, Emil Du Bois-Reymond, Hermann von Helmoltz

Leopoldo Nobili (1784–1835), Carlo Matteucci (1811–1865), Emil Du Bois-Reymond (1818–1896) and Hermann von Helmoltz (1821–1894) performed experiences that brought evidences of the transient electrical changes (the action potentials) which had a finite velocity and were conducted along the nerves.

1.4. Golgi and Cajal

Thus, when Golgi and Cajal entered the field (fig.1), they already were aware of the notion of electricity as a physical signal passing along nerves and allowing the exchange of information between neurons.

Fig.1 Camillo Golgi & Santiago Ramon y Cajal

Above: Camillo Golgi and Ramon y Cajal portraits.
Below: Golgi and Cajal in their labs respectively.

2. Synapse: From the debate to the conclusion

The synapse as a concept is the consequence of the neurone doctrine that is based on the idea of the existence of contacts between cells, which was opposed to the reticular concept of Golgi.

2.1. The Reticular, Neuronal and Neurofibril models

Three models of nervous system organization were in vogue at the end of the 19th century (fig.2). A ferocious competition of ideas prevailed for few decades between the tenants of these three models. We yet know how much the winner model shaped the neuroscience field for the subsequent century (Agnati et al., 2007). Golgi and followers were tenants of the reticular model. In this model, nerve cells and their extension anastomosed with each other into a continuous protoplasmic network in such a way that nervous current could flow freely in every direction like water in a maze of pipes. In the neurone doctrine, the nerve cells are individual units, tightly associated, but essentially discontinuous with each other. The axonal terminal makes an intimate contact but does no fuse with the dendrite of a different cell. This implicated for Cajal that the nerve impulse propagation was fixed in direction in what he called the ¨ law of dynamic polarization ¨. The propagation goes from the dendrite to the cell body and then to the axon. More consensual, the neurofibril model proposed by Apáthy (1863-1922) and Bethe (1894–1895, 1896) took account of the tiny linear structures that were observed to run throughout the neuronal network. It says that neurons are individual units, although keeping the conducting system as a continuous network constituted by neurofibril threads through cells, axons, dendrites and even across the extracellular space (Frixione, 2008).

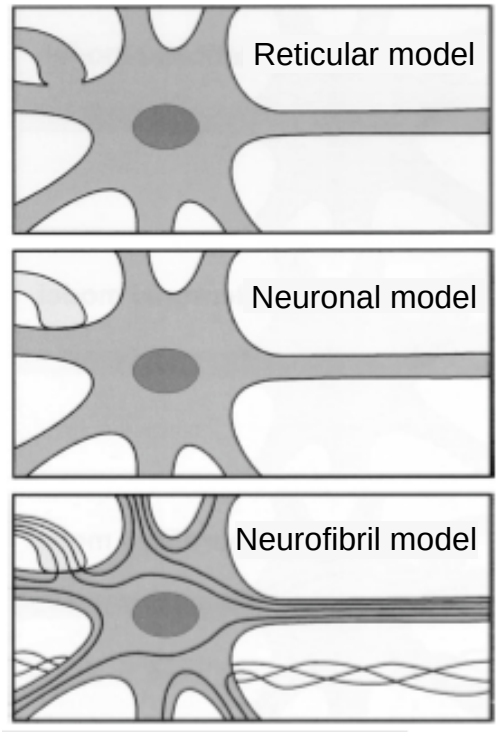


Fig. 2 The three models in neuroscience early 20th

Above: The reticular model of Golgi considers the neuronal network as a unique reticulum.
Middle: The neuronal model of Cajal (mainly accepted nowadays) says that the neuronal network is constituted by neurons that are juxtaposed and connected by contacts between axon terminals, and either the dendrites or neuronal bodies.
Below: The neurofibril model of Apáthy and Bethe is a sort of mix of the two previous models. Neurons do not form a continuous network, but neurofibrils make a continuous network throughout the neuronal network.
Adapted from (Frixione 2008)

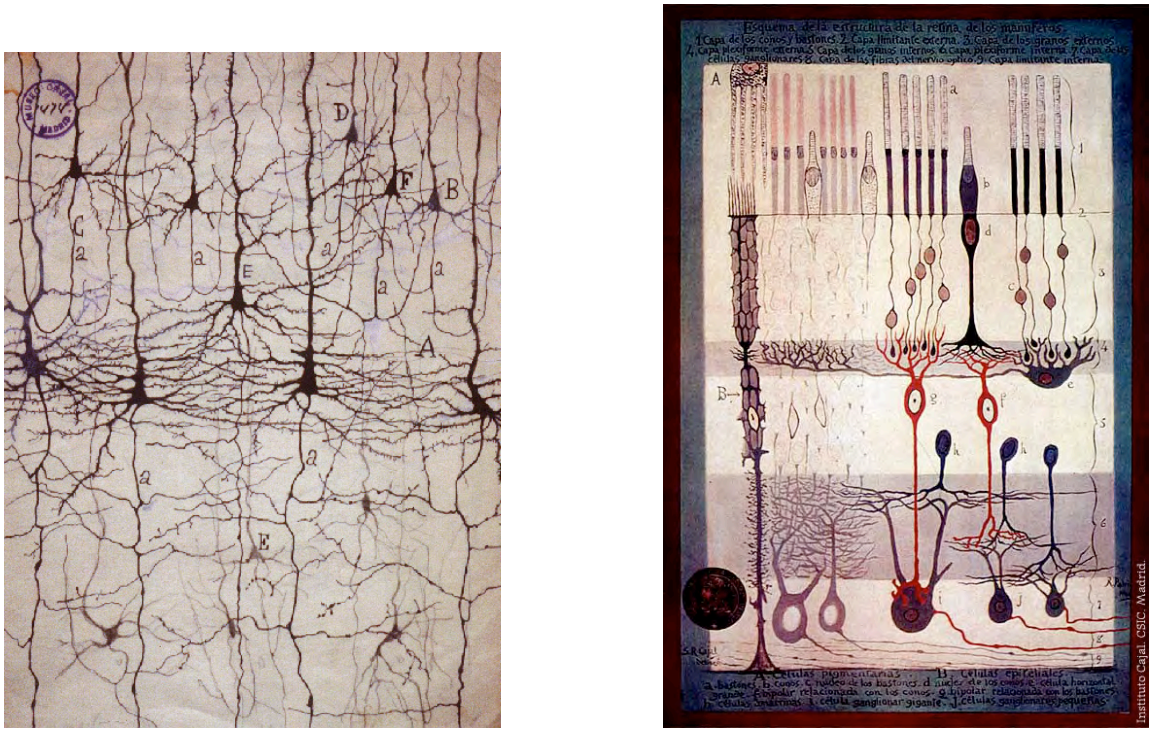


Fig. 3 Neuronal network drawing by Cajal

Left: Drawing of Ramon y Cajal of pyramidal neurons from his observations of silver-nitrate stained neurons (Museo Cajal, Madrid).
Right: Cajal's drawing (1900) of mammalian retinal neurons. Notice that cells have different shapes and individual specificities that would hardly match with the Golgi's reticular model (Instituto Cajal, Madrid).

2.2. The synapse

The point of connection between neurons, which was evoked in Cajal's neurone doctrine, received a name when Charles Scott Sherrington(1857-1952) was asked to write a chapter on the nervous system for the Textbook of Physiology (1897), by Michael Foster (1837–1907). Notice that the term ¨synapse¨, introduced then to explain the delay observed in the spinal reflex at the junction between neurons, was relying on any clear morphological data:

" *So far as our present knowledge goes, we are led to think that the tip of a twig of the arborescence is not continuous with but merely in contact with the substance of the dendrite or cell-body on which it impinges. Such a special connection of one nerve cell with another might be called a 'synapse'. The lack of continuity between the material of the arborization of one cell and that of the dendrite (or body) of the other offers the opportunity for some change in the nature of the nervous impulse as it passes from one cell to the other* ¨.

Sherrington provided a functional explanation of the structural postulates of Cajal, joining the anatomical and physiological concepts in a single entity. This set the fundamental ideas of what subsequently became known as the concept of neurotransmission. From a semantic point of view, the term "synapse" was not the first chosen by Sherrington, who suggested, as an explanation for the internal contact

zone, the term syndesm. However, Foster, pushed by Sherrington himself, consulted a doctoral student at Trinity College named Verrall, who proposed “synapse”, which literally means “joining together”.

2.3. The Neuron Doctrine

The Cajal’s discoveries, resulting of his studies on the grey matter of the cerebellum, published in 1888 and 1889, are summarized in four laws that revolutionized the ideas of the time on the histological structure of the nervous system. They represent the bases of the neuron doctrine:

1. *The collateral and terminal ramifications of all axis-cylinders end in the grey matter, not by means of a diffuse network, as argued by Gerlach, Golgi and the majority of neurologists, but by means of free arborizations, disposed in a variety of forms (pericellular baskets or nests, climbing branches, etc).*
2. *These ramifications apply themselves intimately to the body and dendrites of the nerve cells, establishing a contact or articulation between the receptor protoplasm and the final, tiny axonic branches.*
3. *Given that the body and dendrites of neurons are applied narrowly (to) the final tiny roots of the axis-cylinders, it is necessary to accept that the body and the protoplasmic processes participate in the chain of conduction, that is, that they receive and propagate the nervous impulse, in contrast to the opinion of Golgi, for whom these cell segments would play a merely nutritive role.*
4. *The exclusion of substantial continuity between cell and cell leaves the way open for the opinion that the nervous impulse is transmitted by contact, as in the articulations of electrical conductors, or by a kind of induction, as with induction coils.*

Historical milestones in the development of the neuron doctrine

1836	First microscopic image of a nerve cell (Valentin)
1838	First visualization of axons (Remak)
1855	Consolidation of cell theory (Virchow)
1862	First description of the neuromuscular junction (Kühne)
1865	Description of types of processes of nerve cells (Deiters)
1871	Definitive postulate of the reticular hypothesis in the structure of the nervous system (Gerlach)
1873	Introduction of silver-chromate technique as staining procedure (Golgi)
1886/1887	First discrepancies with reticular theory (His, Nansen, Forel)
1888	Birth of the neuron doctrine: the nervous system is made up of independent cells (Cajal)
1889	Dissemination of neuron theory at the German Anatomical Society congress (Cajal)
1891	The term “neuron” is coined (Waldeyer). Defence of the physiological significance of the “diffuse nerve network” theory (Golgi)
1892	Laws of dynamic polarization of neurons (Cajal)
1897	Concept of synapse (Sherrington)
1903	Introduction of silver nitrate as staining technique (Cajal)
1904	*Textura del Sistema Nervioso del Hombre y de los Vertebrados*: coming-of-age of the neuron theory (Cajal)
1906	Nobel Prize: Cajal and Golgi. *The integrative action of nervous system*: modern neurophysiology is born (Sherrington)
1913	Studies on degeneration and regeneration of the nervous system (Cajal, Tello, Perroncito)
1921	Nobel Prize: Sherrington
1933	*Neuronismo o reticularismo*: Cajal’s scientific testament
1954/1955	Ultrastructural confirmation of synapses (Palay, Palade, De Robertis, Bennett)

Fig. 4 Key dates that led to the neuron doctrine

The neuron doctrine is the expression of a century of research in neuroscience. Puzzle pieces of this doctrine have been assembled by Cajal after his smart observations. Nevertheless this doctrine triggered many debates and polemics. The final confirmation came in 1954-1955 after Golgi and Cajal had passed away.

This led to the famous Neurone doctrine which contains four tenets

1. The fundamental structural and functional unit of the nervous system is the neuron.
2. Neurons are discrete cells which are not continuous with other cells.
3. The neuron is composed of 3 parts, the dendrites, axon and cell body.
4. Information flows along the neuron in one direction, from the dendrites to the axon, via the cell body.

Notwithstanding the many arguments reinforcing its doctrine, Cajal had no clear confirmation of his ideas when he passed away in 1934. Indeed, the neuroscientist community had to wait until the emergence of the electron microscopy to have an irrefutable observation about the existence of the synapse, and therefore of the neurone doctrine.

However, it is worth noting that the discontinuity of the neuromuscular junction was observed by a histochemical staining method with light microscopy several years before the observation of the synapses at the electron microscopic level by Palay, De Robertis, Bennett and Robertson (DE and BENNETT, 1955; PALAY and PALADE, 1955; ROBERTSON, 1956). Around 1940, Couteaux was the first histologist who brought a morphological basis to the synapse, regarded until that time only as a physiological entity.

During the german occupation of Paris, Couteaux continued to work on the neuromuscular junction. He even succeeded in staining, with Janus Green B, a specialized element of the postsynaptic membrane. He observed the ‘synaptic gutter’, a depressed sarcolemma of concave shape beneath the nerve terminal, and the ‘subneural apparatus’, a lamellar structure attached to the sarcoplasmic side of the synaptic gutter. These discoveries gave a substantial credit to the neuron doctrine and defined the morphological bases of the concept of synapse (Couteaux et al., 1946; Couteaux et al., 1953).

3. The hippocampus: a cortical neuronal network model

A major feature of the brain is its complexity. To unravel this puzzled system many ways have been taken by the neuroscientific community. Reductionist approaches revealed often necessary to be able to examine specific aspects of brain functions. This has been particularly the case for studies of learning and memory mechanisms, which have for years mainly concentrated on the hippocampus, its circuitry and the properties of synaptic transmission and plasticity expressed at various excitatory connexions in the network. An important reason for this interest, justifying this type of approach, was the discovery and description of the memory problems of H.M., a patient that underwent bilateral removal of both hippocampi. The loss of learning capacity revealed in this patient stimulated an entire field of research for decades and made of H.M. one of the most studied patient in the literature. Because of this importance, I summarize below the main observations and conclusions obtained from these studies.

3.1. Tribute to H.M.:

He died at the age of 82 on December the 2nd 2008, he stopped working at the age of 27 in 1953, but he unintentionally opened the modern era of memory research. Dramatic seizures resulting from a bicycle accident at the age of 7 or 10 led to a radical surgical intervention. Since 1953, he has never been able to remember a single day of his life occurring after the moment when Dr. William Scoville had removed bilaterally his hippocampi and parahippocampal gyri (anteriorly). Henry Molaison was the famous person hidden behind the well known H.M. case.

Brenda Milner was finishing her doctoral research at McGill University under the direction of Donald Hebb. At about that time she first visited H.M. She saw that the epilepsy was gone, but he was suffering from a severe memory impairment. H.M. was forgetting daily events nearly as fast as they occurred, despite any apparent intellectual loss or perceptual disorder. H.M. perceived his state as: ¨ *like waking from a dream...every day is alone in itself* ¨. The first observations and testings were reported in the very often cited work (WB and Milner, 1957). H.M continued to be studied for five decades by Brenda Milner, her former student Suzanne Corkin and their colleagues.

Fig. 5 Henry Molaison (1926-2008)

Henry Molaison, known as the H.M. case, suffered from anterior amnesia after the removal of a part of his temporal lobe.

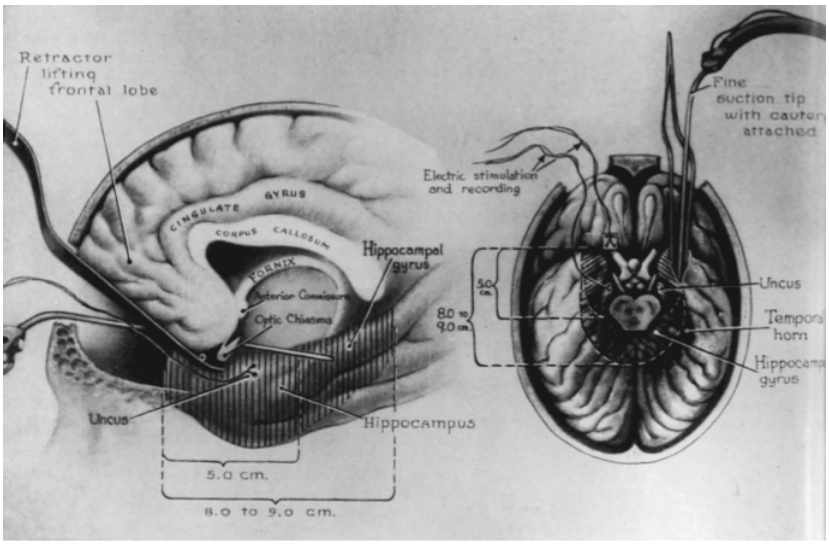


Fig. 6 Surgery anatomical figure

Left: Drawing of the surgical performance on the temporal lobe of H.M. (sagittal view).
Right: Area removed bilaterally from the medial temporal lobes demonstrating 5 cm as well as 8 cm removals through supra-orbital trephines (ventral view) (Scoville WB and Milner, 1957).
Recent IRM studies showed that H.M. Lesion was 5 cm caudally from temporal lobe (not 8 cm) leaving the posterior parahippocampal gyrus largely spared.
Adapted (Scoville WB and Milner, 1957).

MRI studies made in 1997 (Corkin et al., 1997) revealed that H.M. lesions were less extensive than described originally by the surgeon (fig.6-7). Posterior parahippocampal gyrus was largely spared as the surgeon cut extended a little more than 5 cm caudally from the temporal lobe (and not 8cm) as described in (WB and Milner, 1957). Damage limited to the hippocampus usually induce memory impairments far less important than those of H.M.. The seriousness of H.M. memory impairment was due to his bilateral damage including the hippocampi and the parahippocampal gyri (anteriorly). Memory impairment more severe than H.M.'s are observed when lesions run from the hippocampi and anterior parahippocampal cortex but also to the posterior parahippocampal cortex (patients E.P. and G.P. ;(Kirwan et al., 2008).

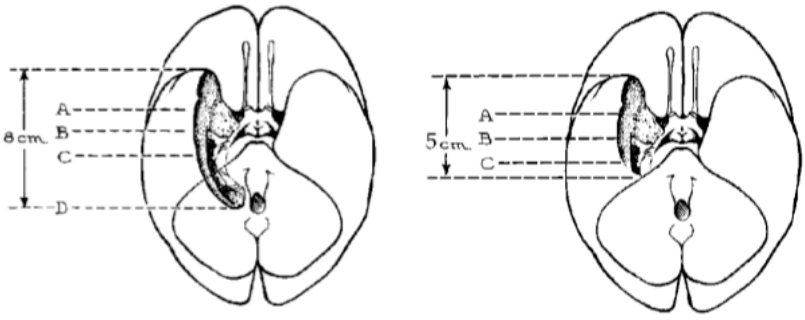


Fig. 7 H.M. surgery lesions

Left: Diagram showing the surgeon's estimate of H. M.'s medial temporal lobe resection.

Right: An amended version of the original diagram indicating the extent of the ablation based on the MRI studies reported here. The rostrocaudal extent of the lesion is 5 cm rather than 8 cm, and the lesion does not extend as far laterally as initially pictured.
Adapted from (Corkin et al., 1997).

Concluding the 1957 Scoville's article, the following statement introduced the premises that would, after several studies on monkeys and neuroanatomical studies, become the medial temporal lobe memory system {Squire and Zola-Morgan, 1991, Science, 253, 1380-6}.

¨It is concluded that the anterior hippocampus and hippocampal gyrus, either separately or together, are critically concerned in the retention of current experience. It is not known whether the amygdala plays

any part in this mechanism, since the hippocampal complex has not been removed alone, but always together with uncus and amygdala.¨ from: (WB and Milner, 1957).

The medial temporal lobe memory system consists of the hippocampus and adjacent, anatomically related cortex, including entorhinal, perirhinal, and parahippocampal cortices (Squire and Zola-Morgan, 1991).

H.M. was able to retain information, like digits, for a few minutes unless his attention and continual rehearsal was active. But, when he would change the orientation of his attention, information would disappear. Furthermore, when informations (like faces or designs) were complex and difficult to rehearse, they would quickly disappear. These findings helped to distinguish between immediate memory and long-term memory and they brought insight into the way the brain organizes its memory functions.

Motor skills tests carried out on H.M. demonstrated that he was able to reproduce the task with learned abilities, but he could not remember that he had already done the test. This suggested a distinction between two broad classes of knowledge, the declarative and the procedural (understood as skill-based knowledge that develops gradually but with little ability to report what is being learned) (Cohen and Squire, 1980; Squire, 2009). Other preserved learning abilities discovered in patients during the following years led to the idea of multiple memory systems. This includes declarative memory, which is what is usually meant when the term ¨memory¨ is used in everyday language and refers to what can be consciously described. This is the kind of memory that was impaired in H.M., and is dependent on the medial temporal lobe. Non-declarative memory regroups additional memory properties including systems that support skill learning, habit learning, simple conditioning, emotional learning as well as priming and perceptual learning. The basal ganglia, the cerebellum, the amygdala and the neocortex are of special importance for these kinds of memory (Squire, 2009).

According to Squire and Zola, the constituents of the medial temporal lobe memory system, presumably because of their widespread and reciprocal connections with neocortex, are essential for establishing long term memory for facts and events, which is known as declarative memory. Therefore, the medial temporal lobe memory system seems to be central in the connection of the scattered storage sites of the neocortex that represent a whole memory (Squire and Zola-Morgan, 1991). Interestingly, this role appears to be temporary, because as time passes after the learning point, the memory in the neocortex slowly becomes independent of medial temporal lobe structures (Squire and Zola-Morgan, 1991).

<u>The very specific surgery on H.M. helped:</u>

-To precise functional neuroanatomy: Damage limited to the hippocampus induces weaker mnesic impairment than bilateral lesions comprising the hippocampi and the parahippocampal gyri (anteriorly). Impairment is more severe when lesions include the posterior portion of the parahippocampal gyri.

N.B. Amygdala and uncus have to be considered in mnesic impairment as they are removed during such intervention.

-To understand the role of the medial temporal lobe memory system as a sorting or connecting system.

-To distinguish between declarative memory and non declarative memory, as well as to differentiate between immediate memory and long-term memory.

Henry Molaison discreetly passed away, and we can guess that before he died, he forgot to ask the neuroscientist community to take care of his memory and legacy.

3.2. Anatomy of the hippocampus (rat)

In 1564, Giulio Cesare Aranzi (1529/1530–April 7, 1589) coined the term "hippocampus" in a small book entitled *De humano foetu liber* (Rome, 1564).

The hippocampus is a part of the forebrain located in the medial temporal lobe. It belongs to the limbic system. The hippocampus is a folded and laminated structure (fig.8). In mammals, the hippocampus can be considered as a relatively simple formation compared with the other cortical zones and it is the major part of what is referred to as the archicortex. The hippocampus can be considered as a primitive and simple cortex consisting primarily of one basic cell type, the pyramidal neurons, associated with its surrounding interneurons. Pyramidal neurons are condensed in one layer of a three-layered structure, whereas neocortex can be divided in six layers. The interposed *subicular area* was divided by Lorente de No (1934) into *parasubiculum*, *presubiculum*, *subiculum* and *prosubiculum* based on cytoarchitectonic characteristics.

The hippocampus itself is divided into two major tangled C-shaped sectors: the dentate gyrus , the *fascia dentata*; and the hippocampus proper, the *cornu ammonis* (CA). Following P. Andersen suggestion, the *fascia dentata* has a buried blade and an exposed blade. Cajal (1911)

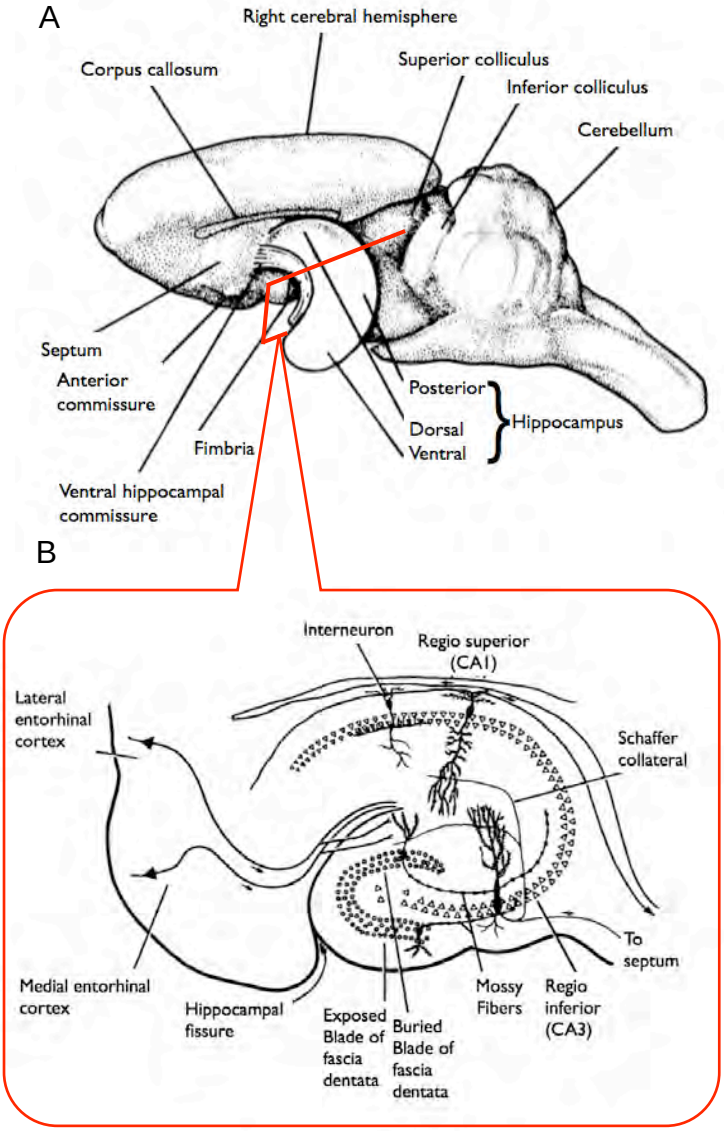


Fig. 8 Rat hippocampus

A: Left rat hippocampus drawing showing mid-line structures, all other forebrain structures have been removed.
B: Schematic diagram of an horizontal section of the hippocampus. Adapted from J O'Keefe & L. Nadel 1978.

and later Blackstad (1956) further divided the hippocampus proper on the basis of differences in cell morphology and fiber projections. They identified a *regio superior* and a *regio inferior.* The *regio superior* contains medium-sized pyramidal cells that meet on one border with the *prosubiculum*, the opponent border is followed by the semi-circle of the *regio inferior* that abuts in the burried blade of the facia dentata. The *regio inferior* contains giant pyramidal cells. Lorente de No further divided the hippocampus proper into four fields, CA1-4. The *regio superior* took the name of CA1 and the *regio inferior* was subdivided into CA2 and CA3 (fig.9); he designated the scattered cells inside the hilus of the fascia dentata by CA4; notice that these cells are not lined up like the pyramidal cells of the CA3, but they are comprised in the hippocampus proper because of their pyramid-like characteristics, because they receive the mossy fibers input, and because they send axons into the fimbria.

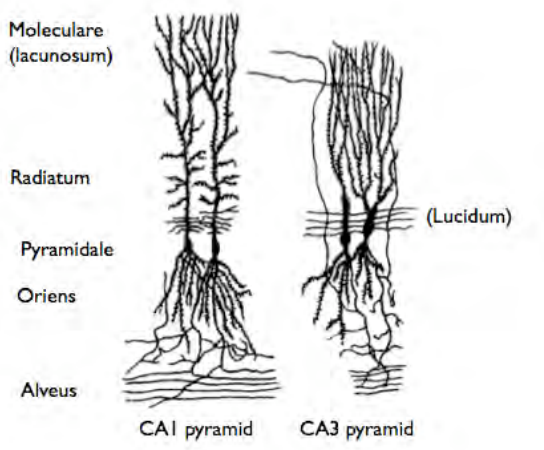


Fig. 9 CA1 & CA3 cells with associated strata

CA1 show smaller cell-bodies than CA3 cells.
Strata:
Alveus: Pyramidal cells' axons, afferents to the Hippocampus.
Oriens: Basal dendrites of pyramidal cells.
Pyramidale: Byramid cell-bodies
Lucidum(CA3)***:*** Inputs of mossy-fibers
Radiatum: Proximal segments of pyramid's dendrites.
Moleculare: Distal segments of pyramid's dendrites.
Adapted from J. O'Keefe & L. Nadel 1978.

The architecture of the hippocampus proper is very regular, it is a sheet of large neurons the cell-bodies of which are condensed in one layer. Basal and apical dendrites spout from the cell-body in opposite directions. Axonal input fibers make roughly right angles with dendrites on which they make *synapses en passant*. The hippocampus proper is divided in six (CA1) or seven (CA3) sub-layers that are defined by a particular feature of the pyramidal cells or their afferents. From the ventricular surface, the sub-layers can be observed as follows:

The *stratum oriens* located between the *alveus* and the pyramidal cell bodies host the basal dendrites of the pyramidal cells, some basket cells, and afferents from the septum. Deep to the *stratum oriens* is a thin, fiber-containing stratum called the *alveus* which contains pyramidal cells' axons that are directed towards the fimbria or the subiculum, together with afferents to the hippocampus that also travel in the *alveus.* The *stratum pyramidale* mainly contains the cell-bodies of the pyramidal neurons. The *stratum radiatum* contains the proximal segments of the apical dendrites, and the *stratum lacunosum/moleculare* contains the distal segments of the apical dendritic tree. The *stratum lucidum*, limited to the CA3 cells, is an additional layer observed between the pyramidal cell-bodies and the *stratum radiatum that receives the mossy-fibers inputs.* The laminate feature of the layers comes from the axonal afferents that cross the dendrites.

3.3. Connectivity

The wiring diagram of the hippocampus is traditionally presented as a tri-synaptic loop (fig.10). The major input is carried by axons of the perforant path, which convey polymodal sensory information from neurons in the layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory

synaptic contact with the dendrites of granule cells in the dentate gyrus: axons from the lateral and medial entorhinal cortices innervate the outer and middle third of the dendritic tree, respectively. Granule cells project, through their axons (the mossy fibers), to the proximal apical dendrites of CA3 pyramidal cells which, in turn, project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections. In addition to the sequential tri-synaptic circuit, there is also a dense associative network interconnecting CA3 cells with each other on the same side. CA3 pyramidal cells are also innervated by a direct input from layer II cells of the entorhinal cortex. The distal

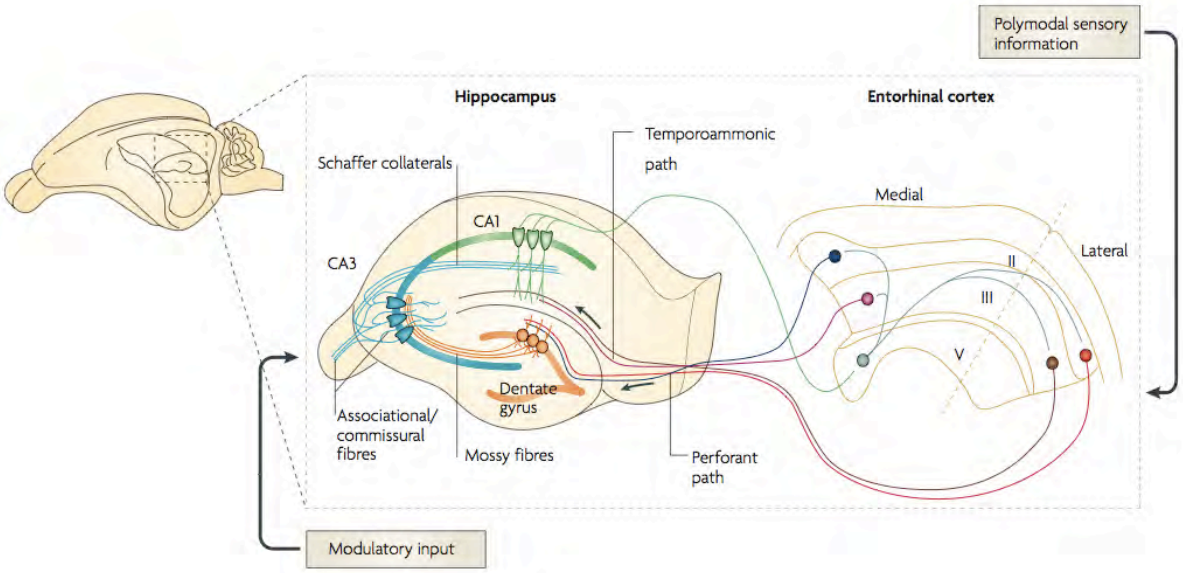


Fig. 10 Hippocampal circuit

Circuit of activity flow in the hippocampal formation. Trisynaptic loop with the **perforant path** bringing polymodal sensory information from neurons in **layer II** of the **entorhinal cortex** to the **dentate gyrus** (DG). DG **granule cells** project, through their axons (**the mossy fibers**), to the proximal apical dendrites of **CA3** pyramidal cells. CA3 cells, in turn, project through**Schaffer collaterals** to ipsilateral **CA1** pyramidal cells. **CA3** also project to contralateral **CA3** and **CA1** pyramidal cells through commissural connections.
A dense associative network interconnects **CA3** cells on the same side. Cells from the **entorhinal cortex** of the **layer II** innervates the **CA3** neurons, whereas **layer III** projects on the **distal apical dendrites of CA1** neurons.
Adapted from (Neves et al., 2008)

apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex. The hippocampus is also home to a rich diversity of inhibitory neurons (ANDERSEN et al., 1964b). The main type of inhibitory cells is the basket cells with cell-bodies situated in the *stratum oriens* and in the *stratum pyramidale* (Schwartzkroin et al., 1990). They synapse on the cell-body of pyramidal cells. Chandelier cells innervate the initial segment of the pyramidal axons, whereas astrocytes are located near the apical and basal pyramidal dendritic segments. These 3 types of inhibitory neurons, using GABA as neurotransmitter, are responsible for the inhibitory mechanisms designated as anterograde and recurrent (ANDERSEN et al., 1964a).

Extrinsic modulatory inputs from the brain stem also innervate the *subiculum*, including the projections from *locus coeruleus* (noradrenergic), *ventral tegmental area* (dopaminergic), and *raphe nuclei* (serotoninergic). Among them, cholinergic fibers sprouting from the septum innervate mainly the *stratum oriens* of the CA3

region and the hilus cells of the *facia dentata* (Crutcher et al., 1981; Amaral and Kurz, 1985). The theta rhythm may be due to the slow depolarization of the pyramidal cells induced by these cholinergic fibers. In addition, glutamatergic/aspartategic projections to the hippocampus and to the septal complex coming from the supramammillary area are involved in the generation/regulation of the theta rhythm (Kiss et al., 2000). The medial raphe nuclei also contribute to the innervation by sending serotoninergic fibers through the *fimbria*, the *fornix* and the *cingulum* to the *stratum lacunosum* of the CA1 and CA3 regions and partially in the *fascia dentata* hilus, the *stratum oriens* and the CA3 *radiatum* (Moore and Halaris, 1975). The noradrenergic fibers from the *locus coeruleus* present a third extrinsic modulator that in this case specifically innervate the *stratum lacunosum* of the CA1 and CA3, the *fascia dentata* hilus and the *stratum lucidum* of the CA3 (Lindvall and Bjorklund, 1974; Swanson and Hartman, 1975). Several evidences suggest that the serotoninergic and noradrenergic systems have an inhibitory impact on the pyramidal neurons (Biscoe and Straughan, 1966; Segal and Bloom, 1974).

3.4. Developmental aspects of the hippocampus

A remarkable feature of the hippocampus is its double C-shaped highly laminate structure. The hippocampal structure begins its development as a narrow strip of cortical tissue trapped along the medial wall of the hemisphere, therefore its expansion can only occur in the ventricle. The embryonic hippocampus start by pushing laterally before to double back underneath itself. Bayer and Altman suggested that the C-shaped f*ascia dentata* comes from the invagination of the hippocampus into the granular cells along the successive stage of development (Bayer and Altman, 1974). A special feature of hippocampal development is that the *fascia dentata* develops far after the rest of the hippocampus, other regions of the brain and even continues to develop after birth.

Most of the hippocampal pyramidal cells of rat brain are formed during prenatal embryonic development (Angevine, 1965; Altman and Das, 1966). The pyramidal cells of ammon's horn are formed during the 16th to the19th days of embryogenesis but are preceded by the large interneurons of the *stratum lacunosum-moleculare* which are formed on days 15 and 16 of embryonic life. The granule cells of the superficial layers of the *stratum granulosum* are formed during the 20th, 21st, and 22nd days of gestation (Hine and Das, 1974). The neuro- and morphogenetic gradients throughout the different pyramidal and granular cells may explain the laminar structure of the adult hippocampus (John O'Keefe & Lynn Nadel,1978) (Bayer, 1980a; Bayer, 1980b). The first connections between the axons and the dendrites follow the rule of the first come first served. Indeed, the first afferents (commissural contacts) contacts reach the proximal portions of dendrites, and the later afferents (enthorinal) join on distal portions of dendrites. However, this connecting hierarchy follows some kind of specificity. As it has been shown in different studies, the empty space left in the case of lesions of the commissural system or part of the perforant path in new born rats is not exploited by the remaining fibers of the perforant path. Instead, the remaining perforant path fibers stay on the original dendritic portions (Hjorth-Simonsen and Jeune, 1972; Lynch et al., 1973; Matthews et al., 1976a; Matthews et al., 1976b). Further works explained that the associative ipsilateral fibers were impinging on the vacant sites let by the previous commissural fibers (Zimmer and Hjorth-Simonsen, 1975).

4. Synapses

In 1888, as seen in the previous chapter, Ramon y Cajàl brought the idea that the contacts between axons and dendrites or soma could be the point of nervous influx transmission. The term synapse (from the greek *sunapsis* : junction point) was further proposed by Sherrington in 1897 to define the contact zones implicated in the transmission of information.

4.1. Types of synapse

Commonly the synapses are divided in electrical and chemical synapses.

4.1.1. The electrical synapse

Electrical synapses have been discovered by Edwin Furshpan and David Potter who described the rectifying synapse of crayfish (Furshpan and Potter, 1957). Further works by Michael Bennett showed that electrical synapses were not rectifying; their junctional resistance was constant (Kandel, 2000). The electrical synapses are made of two membranes continuously joint by gap-junctions. Each gap junction (aka nexus junction) contains numerous gap junction channels which cross the membranes of both cells (fig.12-13) (Gibson et al., 2005). With a lumen diameter of about 1.2 to 2.0 nm (Bennett and Zukin, 2004; Hormuzdi et al., 2004) the pore of a gap junction channel is wide enough to allow ions and even medium sized molecules like signaling molecules to flow from one cell to the next (Kandel et al., 2000; Hormuzdi et al., 2004), thereby connecting the two cells' cytoplasms. As ionic current flow can occur freely between the two cells, electrical transmission via the intercellular channels can be bidirectional. In fact, it is the distinctive reciprocity of the stimulus supported by electrical but not chemical neurotransmission that, together with the transfer of sub-threshold potentials favoring synchronous activity, may well be one of the advantages of electrical synapses. For instance, electrical synapses are observed between retinal neurons making this region a popular model for electrical synapses studies (Hormuzdi et al., 2004).

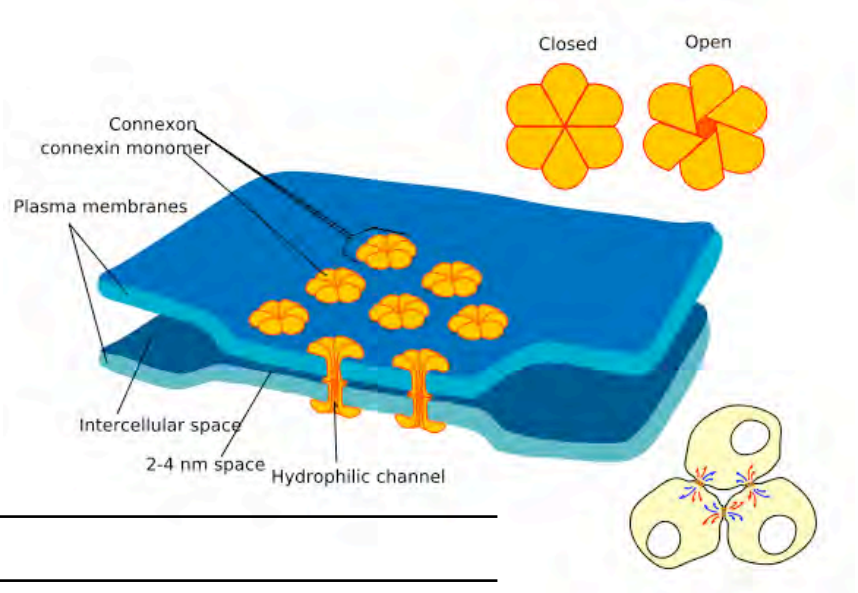


Fig. 12 Electrical synapse

Electrical synapse can be assumed as electrical gap-junctions that allow ions passing from a cell to another through 1.2 to 2.0 nm large pores.
Drawing by Mariana Ruiz 2006 wikipedia

4.1.2. The chemical synapse

Roughly, chemical synapses are composed by two neuronal elements facing each other across an extracellular space called synaptic cleft. More precisely, and according to the structural definition, a synapse consists of a presynaptic membrane bearing a presynaptic active zone, a region specialized for transmitter release and vesicle exocytosis, a synaptic cleft and a postsynaptic membrane exhibiting a dense region, the postsynaptic density (PSD), which is the site of receptors. Synapse diameter is between 0.2 and 1 micron. Neuronal elements such as axons, cell bodies and dendrites are thoroughly combined to form a large panel of synapses (Gray, 1959) (Peters, The Fine Structure of the Nervous System: Neurons and Their Supporting Cells New York Oxford University Press 1991). Adult pyramidal cell hosts between 5000 and 15000 synapses according to the estimations made on the number of spines on the dendrites of individual pyramidal neurons from layers 2/3 and 5 of the visual cortex of the rat (Larkman, 1991). 80 to 90% of contacts are made on dendritic spines, the remaining contacts finish on dendrites or on cell bodies. A synapse can be considered as the smallest biological unit for information processing. Information carried by a nervous electrical influx is translated into a chemical message, the neurotransmitter release. This transmitter diffuses trough the synaptic cleft to stimulate postsynaptic receptors and generate a new electrical signal. The vast number of synaptic components and proteins are designed to ensure and tune this process resulting in amazing propensity for plasticity of synaptic functions. Bi-directional and reversible alterations in synaptic efficiency make possible the dynamic storage encoded information in the neurons. Therefore synapses are key figures of brain plasticity and information modulation in the neuronal network.

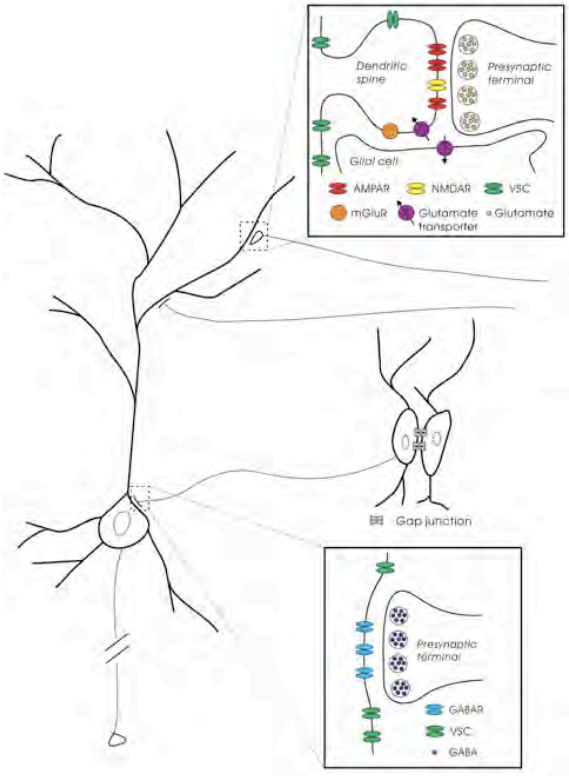


Fig. 13 The three types of synapse

Above: Type I synapse with presynaptic vesicles facing glutamate receptors beyond the synaptic cleft.
Middle: Electrical synapse with gap-junctions forming the contact through which ions flow.
Below: Type II synapse with presynaptic vesicles facing GABA receptors beyond the synaptic cleft.
Adapted from (Kim and Linden, 2007)

4.1.2.1. Types of chemical synapse

According to Edward Gray's electron microscopic observations, synapses can be classified as type I (excitatory) (fig.28) and type II (inhibitory) (fig.29) synapses (Gray, 1959). They are also classified as asymmetric or symmetric (type I or II, respectively), depending of their ultrastructural characteristics (Colonnier, 1968):

-The presynaptic part (synaptic vesicle's shape and turbidity)

-The postsynaptic part (length of the apposed membranes, membrane thickenings and synaptic cleft).

Type I synapses represent 84% of cortical synapses. They contain round synaptic vesicles in presynaptic projections boutons. They have a wide synaptic cleft (20nm), a large active zone and a related postsynaptic thickening of the membrane, a PSD. Type II (inhibitory) synapses have flattened, more scarcely distributed vesicles in the presynaptic boutons. The synaptic cleft is narrow (12 nm) compared to the type I. They also have smaller actives zone and a PSD with significantly thinner postsynaptic microdomain.

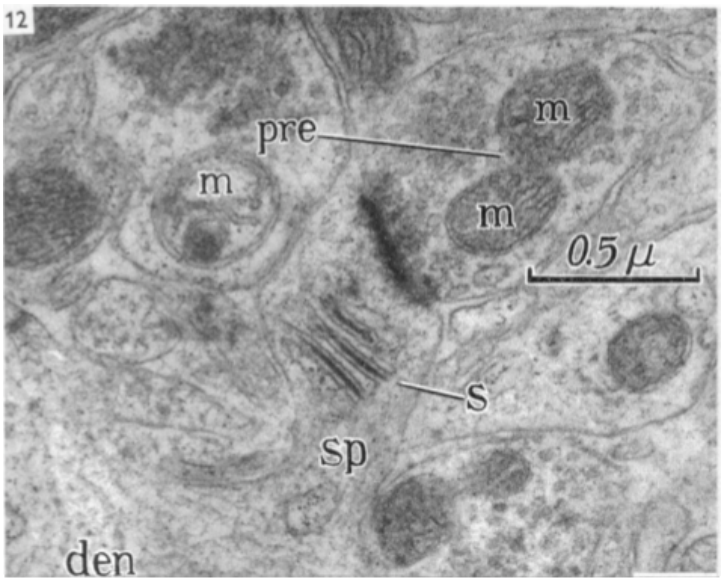


Fig. 28 Type I synapse

Type I synapse is considered as an excitatory synapse also referred as asymmetric synapse as dark densities are only present on the postsynaptic side of the synapse.
(**s**:dendritic spine)(**pre**:presynaptic), (**m:**mitochondria)**,(sp**:spine apparatus)(**den**: dendrite)
Adapted from (Gray, 1959) that is the original published description of type I synapse.

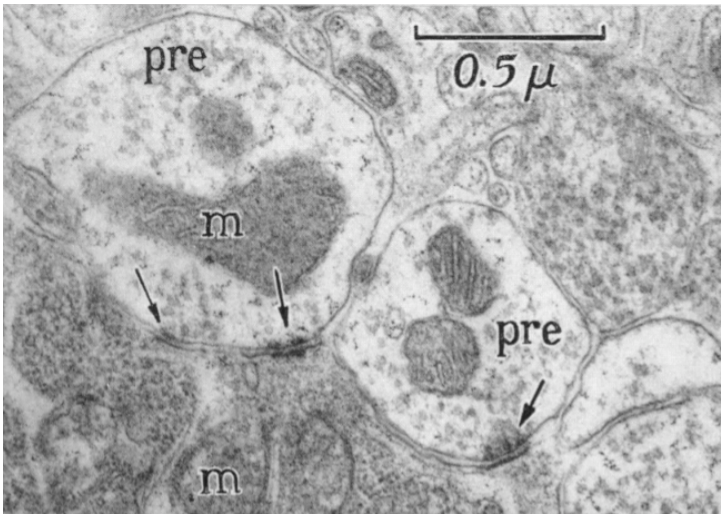


Fig. 29 Type II synapse

Type II synapse is considered as an inhibitory synapse also referred as symmetric synapse as dark densities are present on both sides of the synapse.
Adapted from (Gray, 1959).

4.2. Structural Characteristics of axo-dendritic synapses

Several approaches have been used to characterize the different elements of the neuropil that are observed either with light microscopy or with electron microscopy. The gross components of the central synapse comprise the axonal bouton and the dendritic spine (fig.14):

-**Axonal boutons** are swellings on axons that contain the cellular machinery for neurotransmitter release, including neurotransmitter filled vesicles. The presynaptic site facing the postsynaptic site is called the **active zone** (presynaptic description will be limited to the active zone in the following chapter).

-**Dendritic spines** are protrusions sprouting from a neuronal dendrite. Beside the so-called filopodia, a usually long and thin protrusion without a **postsynaptic density** (PSD). The postsynaptic density observed on electron microscopy images (EM) looks like an electron-dense thickening, usually on dendritic spine heads or on dendritic shafts, and is apposed to the presynaptic active zone. PSDs contain the receptors and their associated scaffold proteins. Dendritic spines have different morphologies that are believed to be correlated with their degree of maturation (Kasai et al., 2003; Ethell and Pasquale, 2005; De Roo et al., 2008a; De Roo et al., 2008b; De Roo et al., 2008c).

Dendritic spines are protrusions bearing a head at the tip. The major criteria for description are the absolute or proportional size of the head and the presence or absence of a constricted spine neck. EM studies show a continuum between these categories.

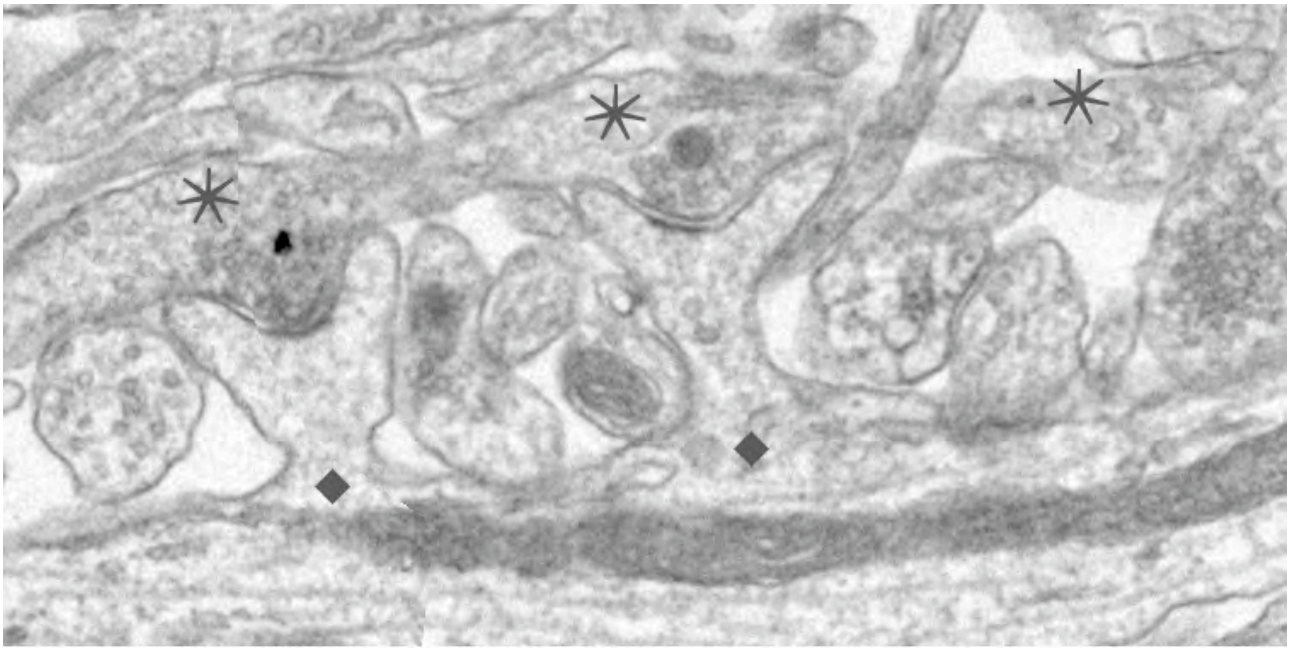


Fig. 14 Two synapses between two related en passant boutons

Boutons are axon varicosities (stars). Facing the postsynaptic density, presynaptic vesicles of the active zones are visible in both boutons.
Two dendritic spines making synapse with presynaptic boutons from the same axon. These two CA1 dendritic spines are sprouting from the same dendrite (diamonds), spine length are approximatively 1 µm.

4.2.1. Ultrastructure of the active zone (presynaptic)

The term “active zone” was coined in 1970 by Couteaux and Pecot-Dechavassine (Couteaux and Pecot-Dechavassine, 1970) during their ultrastructural studies of partially contracted frog muscles (fig.15). They observed that the outline of open synaptic vesicles occurred immediately adjacent to the presynaptic dense bands. Therefore, they designated these dense bands: “les zones actives.” Subsequently, similar observations were made in other types of synapses, like for instance the mammalian central nervous system. In cortical neurons, axons form mainly *en passant* boutons, which are enlarged ovoid structures containing vesicles, active zones, and the machinery for maintaining synaptic transmission. The *en passant* term comes from the fact that axons pass by the dendrites with which they make contacts, and then they continue their way contacting the same or other dendrites further away (fig.14).

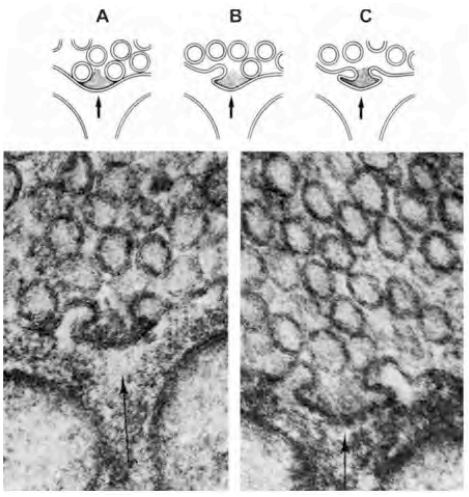


Fig. 15 Active zone

Above: (a),**(b)**,**(c)** scheme of vesicle merging with the cell membrane respectively.
Below: EM images of vesicles merging with the extracellular space.
Adapted from (Couteaux and Pecot-Dechavassine, 1970)

Morphologically, active zones are defined as sites of synaptic vesicle docking and fusion, and physiologically they are defined as sites of neurotransmitter release. On the basis of these definitions, the active zone is dissected into three morphologically and functionally distinct components:

1. The plasma membrane is juxtaposed to the PSD, and this is the place where synaptic vesicle fusion occurs.
2. The cytoskeletal matrix is immediately internal to the plasma membrane where synaptic vesicles dock.
3. The electron-dense projections extend from the cytoskeletal matrix into the cytoplasm, and on this matrix the synaptic vesicles are tethered. All active zones comprise these three components, though they may vary in appearance.

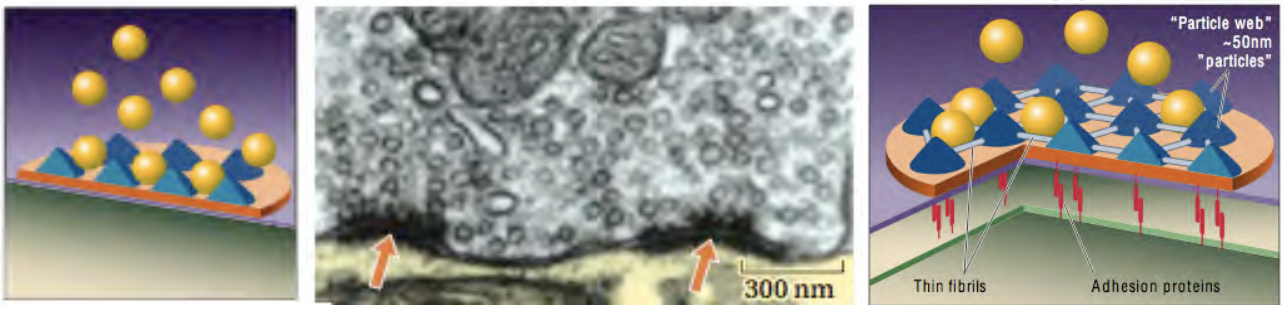


Fig. 16 Saccular hair cell in frog

Left: Schematic view of axon terminal vesicles approaching the releasing site. (yellow: vesicles), (blue: ¨web particles¨), (orange: releasing site), (purple: presynaptic membrane).
Middle: Two releasing sites (red arrows: synapses with active zone facing the postsynaptic partner).
Right: In addition to left scheme, the adhesion molecules (red), thin fibrils (grey) and the postsynaptic membrane(green)
Adapted from (Zhai and Bellen, 2004).

4.2.2. Ultrastructure of the dendritic spine (postsynaptic element)

4.2.2.1. The different types of dendritic protrusions

- S**tubby** spines have no constricted neck. The ratio width over length is higher than 1.
- T**hin spines** have a small head.
- The **mushroom spines** have a large head.
- The **filopodia** are thin and elongated protrusions without clear head (< 2x the width of the protrusion).

As PSD can be observed at **EM level**, classification is different for protrusion which shows a head devoid of PSD. These particular protrusions are then classified as **spine-like filopodia.**

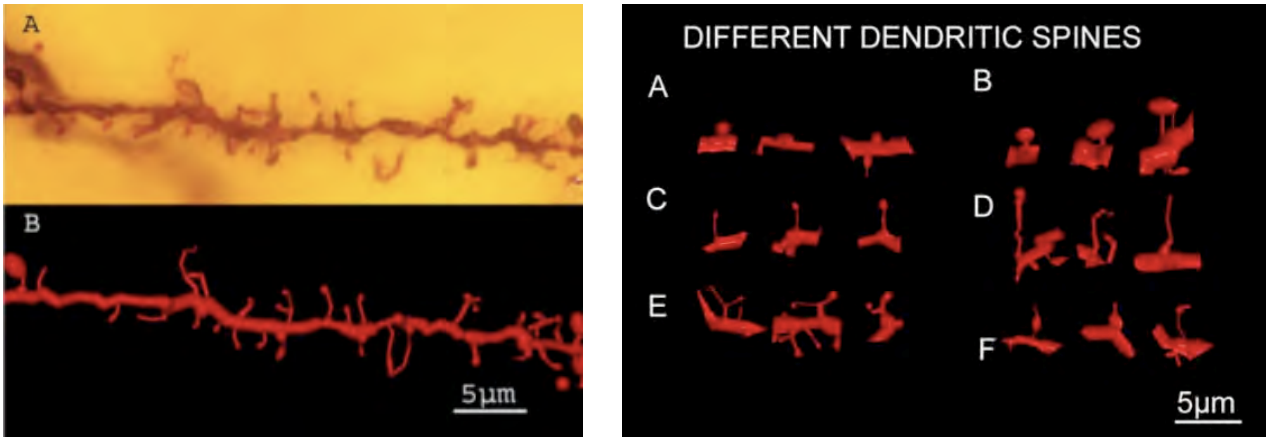


Fig. 17 3D Reconstructions from Cajal's fixed preparations

Left: Pyramidal cell (above, Golgi's method) prepared by Cajal and used hundred years later by Garcia-Lopez et al. for digital analysis. Fragment of an oblique apical branch with thin spines, mushroom spines, filopodia and branched spines.
Right: Different types of dendritic spines codified. A, Sessile spines. B, Mushroom spines. C, Thin spines. D, Filopodia. E, Branched spines. F, Spines with spinules.
Adapted from (Garcia-Lopez et al., 2006).

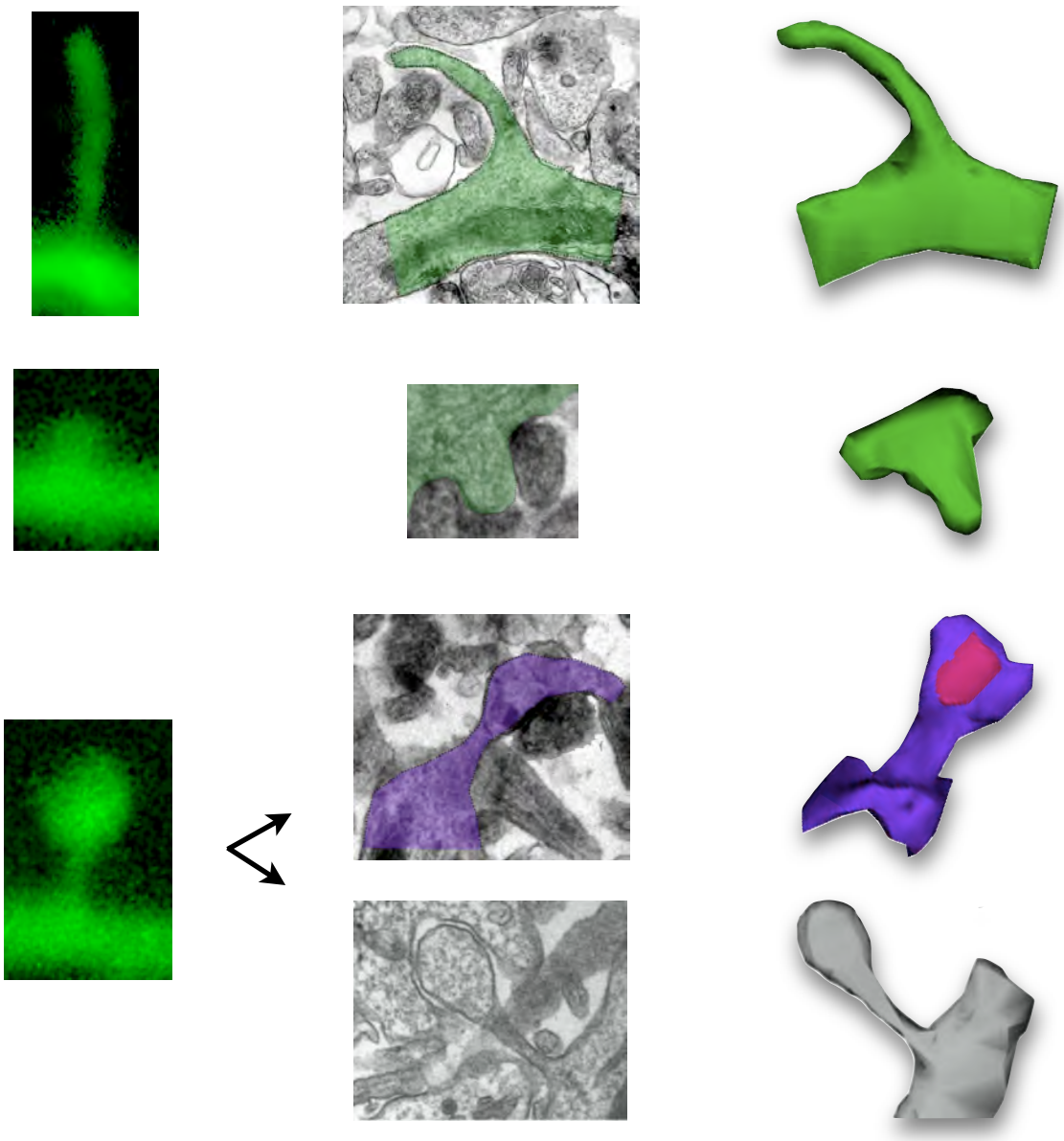


Fig. 18 Confocal & EM representation of the different types of protrusion

Left: Confocal images (left, courtesy of P.Klauser), EM images (center) and 3D reconstructions (right) of respectively:
Middle: ssEM images
Right: 3D reconstructions from the ssEM images
Top-> down:
- Filopodium, typically an elongated protrusion devoid of PSD.
- Stubby spine, a small protrusion without a salient spine neck or head.
- Mushroom spine, a protrusion with a head and making a synapse with a presynaptic partner.
- Spine-like filopodia which can be confused with a spine in confocal microscopy. However, the EM shows that it is devoid of PSD.

4.2.3. Structural and molecular organization of dendritic spines

4.2.3.1. Ultrastructure of the postsynaptic density

The PSD has been mainly investigated thanks to electron microscopy as light microscopy is blurred at that level of resolution. At ultrastructural level, the PSD is a dark line usually on the tip of the spine head. On EM images, the PSD is visible because its dense and complex meshwork traps electrodense material

(osmium, lead, acetate uranyl) during the embedding and contrasting procedures. The PSD is a versatile element that undergoes continuous changes (Luscher et al., 2000) with morphologies that exhibit a large variability.

As dendritic spines are highly dynamic structures, EM images can be considered as morphological snapshots in the chain of structural changes occurring at the synapse. Nevertheless, EM observations bring precious informations on the nature of synapses. Indeed, physiological changes induced by experimental conditions may lead to dramatical ultrastructural changes visible at high resolution level.

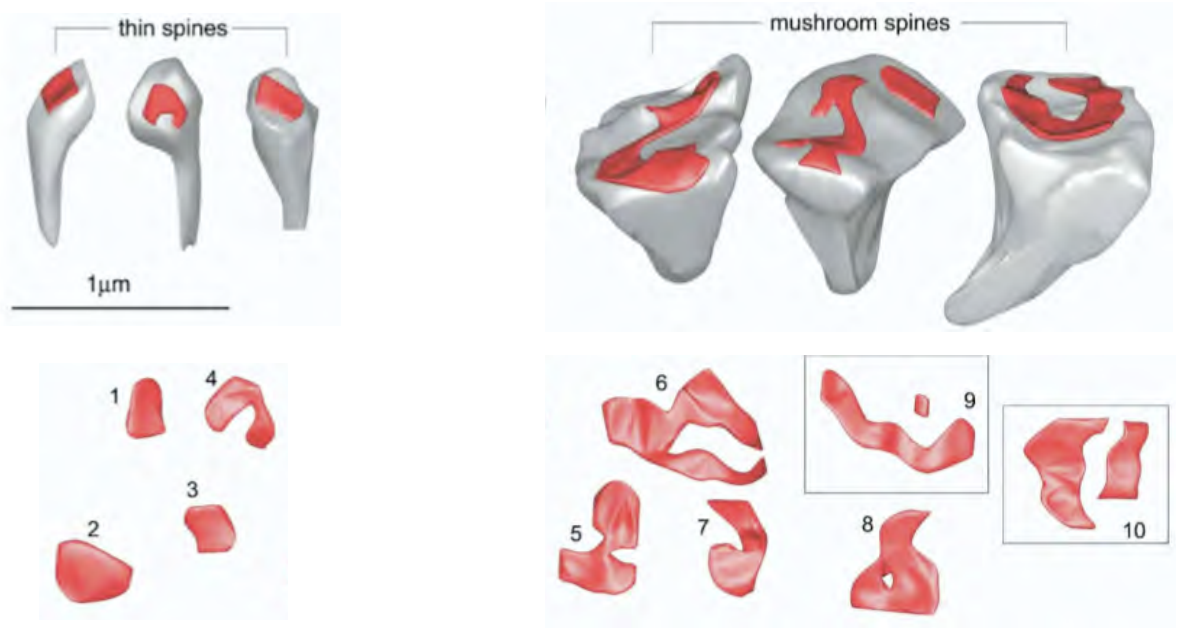


Fig. 19 3D Reconstructions of PSDs

Above: The size of the PSDs is correlated with the spine head volume. Moreover PSDs often show more complex features, especially in mushroom spines. Big spine head is often correlated with mostly exhibit complex-shaped PSD.
Below: PSDs are classified as: macular (1-4), usually on thin spines. Complex PSDs which comprises perforated or C-shaped (5-8) and segmented (9-10) PSDs on mushroom spines.
Adapted from (Donohue et al., 2006)

PSDs have been classified in many subtypes depending on the technique of analysis (2D or 3D) and their shape. Globally, PSDs can be divided in two major categories: macular, which are homogenous flakes found on small spines, and complex PSDs which comprise horseshoe (also called C-shaped) PSDs and segmented PSDs, formed by two or more distinct parts (fig.19). An other critical point besides the qualitative appreciation of PSDs is the quantitative approach used to define PSDs. Several studies focused on the size of the different elements of a dendritic spine (spine head volume, spine neck characteristics and PSDs) and reported potential links between spine morphology and synaptic functions (Freire, 1978, J Anat, 126, 193-201) (Spacek and Hartmann, 1983) (Harris and Stevens, 1989). The PSD area is itself proportional to the number of presynaptic vesicles (Nusser et al., 1998) and in a study of LTP in CA1 pyramidal neurons, a shift towards the formation of complex PSDs has been observed (Buchs et al., 1996)(Toni et al., 1999).

4.2.3.2. Molecular composition of the PSD

The PSD surfaces range from small discs to large irregular-shaped structures that can be intercepted with electron-lucent regions (Harris and Stevens, 1988; Harris and Stevens, 1989). The postsynaptic density can be regarded as a big membrane-associated protein complex (fig.20). This organelle is specialized for postsynaptic signaling and plasticity (Kasai et al., 2003). The PSD not only contains ionotropic glutamate receptors (NMDA & AMPA receptors) (fig.21), but also a variety of receptor tyrosine kinases, G protein–coupled receptors, ion channels, and cell adhesion molecules. Theses molecules mediate physical linkage and/or functional communication with the presynaptic specialization. In addition, they play a central function in postsynaptic signaling. These membrane proteins are assembled with cytoplasmic scaffold proteins, signaling enzymes, and cytoskeletal elements into a disk-like proteinaceous structure, ~200–800 nm (mean 300–400 nm) wide and ~30–50 nm thick, known as the PSD (Carlin et al., 1980). The peri-synaptic membrane within 100 nm of the PSD is likely different in molecular content and function than the extra synaptic membrane at greater distances from the PSD. The MAGUK family-member PSD-95, which is the best-studied scaffold protein of the PSD and which binds to NR2 subunits of NMDA receptors, is highly abundant in the adult forebrain PSD (1.7 pmol/20 μg of PSD protein; ~1% by mass). It appears to be much more abundant than its closest relatives PSD-93/chapsyn-110 (0.3 pmol/20 μg) and SAP102 (0.2 pmol/20 μg). PSD-95 binds to GKAP/SAPAP (guanylate kinase-associated protein/synapse associated protein-90--PSD-95-associated protein), which interacts with Shank, which in turn binds to Homer (Kim and Sheng, 2004). These scaffold and adaptor proteins also are abundant in the PSD. GKAP/SAPAP family proteins is almost equimolar with Shank family proteins, but only about 30% to 40% as abundant as PSD-95 family proteins, and two time as abundant as Homer family proteins (Cheng et al., 2006). 300 PSD-95 molecules can be counted in an average PSD (Chen et al., 2005). A pillar of scaffold proteins pervade through the PSD with a stoichiometry of ~400 molecules for PSD-95 family members; ~150 for GKAP/SAPAP family members; ~150 for Shank family members and ~60 for Homer family members (Cheng et al., 2006). Probably, these numbers are higher in large PSDs and lower in small PSDs (fig.21). The major scaffold proteins exercise strong guidance on the structure and function of the PSD (Sala et al., 2001; Kim and Sheng, 2004; Funke et al., 2005; Sala et al., 2008). In conclusion, these facts denote that the PSD, a well organized aggregate of hundred of different molecules, reveals peculiar features at the EM level, related to the different physiological states.

One of the questions raised by these data is how does the synapse “know” how many of these proteins to bring in the PSD? A second question would be the potential existence of a “master” organizing scaffold protein of the PSD.

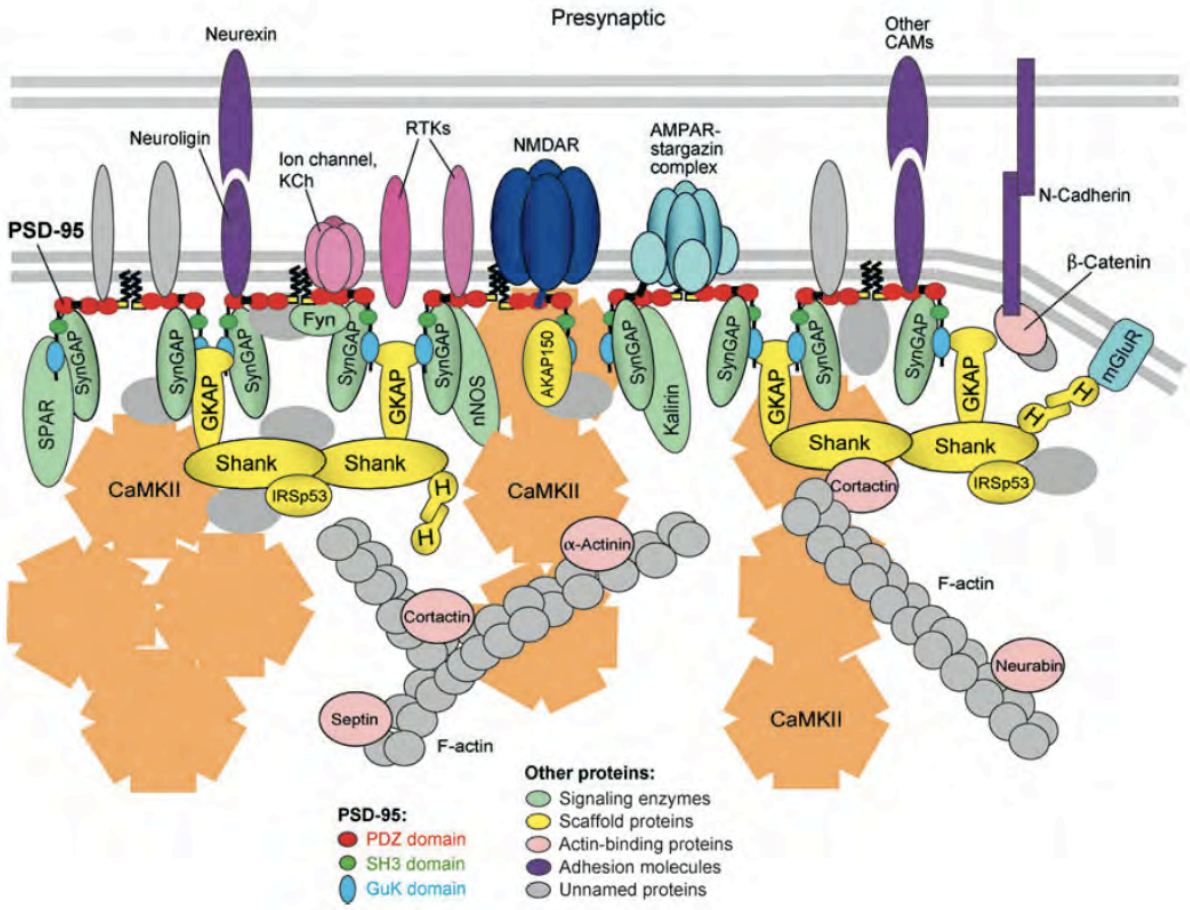


Fig. 20 Organization of proteins and protein-protein interactions in the postsynaptic density

Schematic diagram of the network of proteins in the PSD. Contacts between proteins indicate an established interaction between them. The postsynaptic density contain a multitude of proteins from different families that interact with each other.
Adapted from (Sheng and Hoogenraad, 2006).

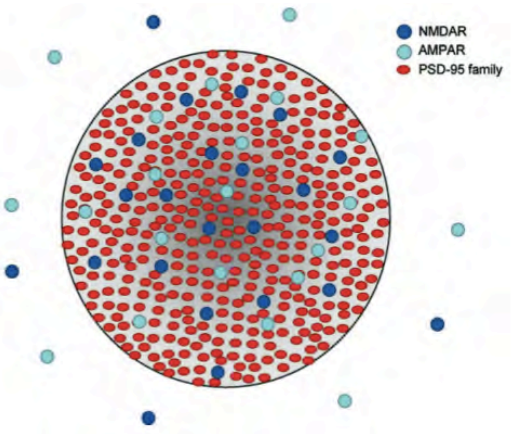


Fig. 21 Distribution of NMDARs, AMPARs and PSD-95 in the PSD

Schematic diagram of an average PSD (360 nm diameter) and surrounding extrasynaptic membrane viewed “en face” from the cleft side. Within the PSD (shaded gray), AMPARs, NMDARs, and PSD-95 family molecules are shown roughly at scale and with a calculated stoichiometry (Cheng et al). AMPARs and NMDARs are also present in the extrasynaptic membrane.
Adapted from (Sheng and Hoogenraad, 2006).

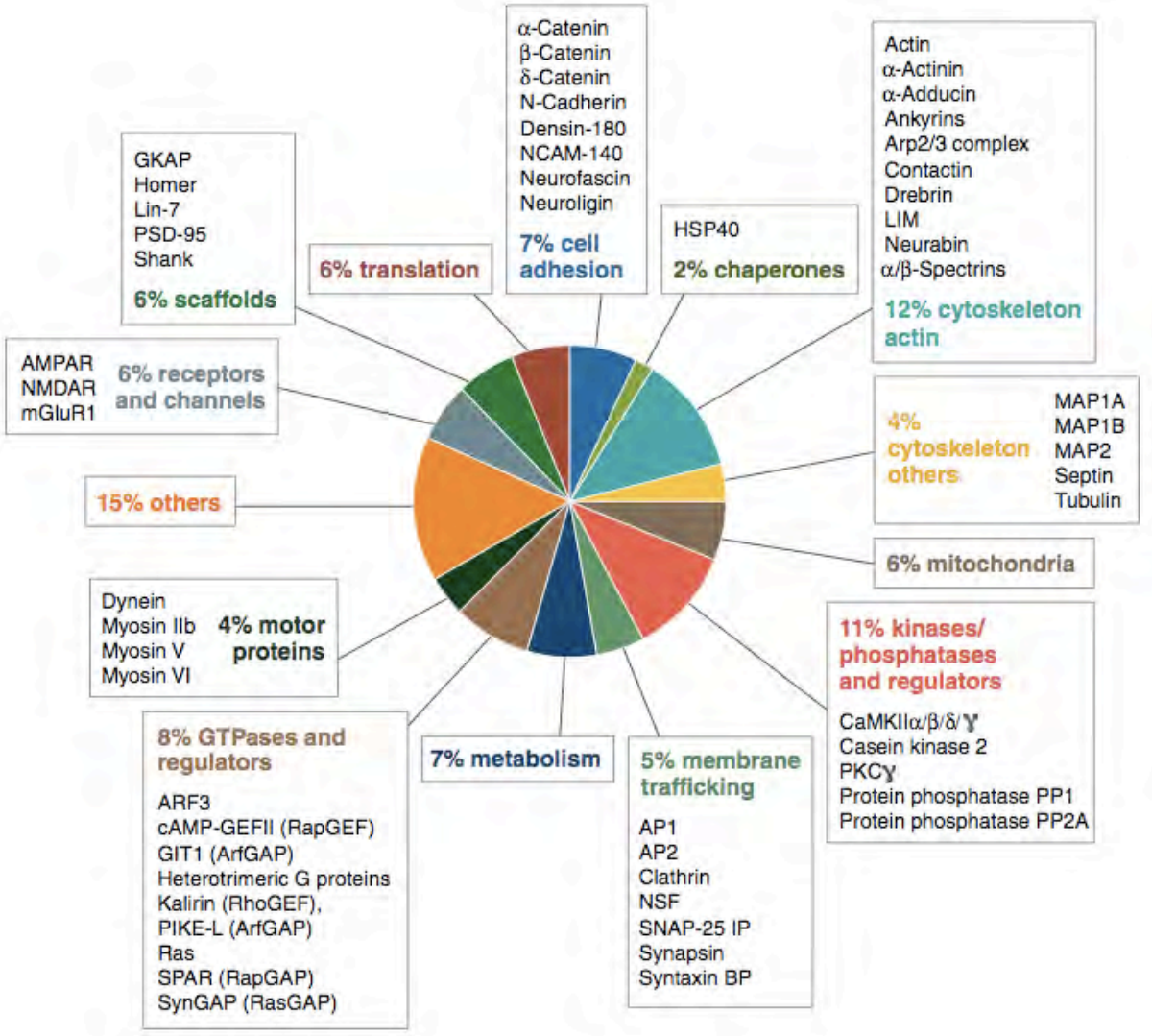


Fig. 22 Protein diversity in the PSD fraction

These proteins of the PSD fraction of the forebrain are categorized according to cellular function.
Proteins can be classified as following:
Cellular communication and signal transduction: Adhesion, kinase/ phosphates, receptors and channels.
Cellular organization: Cytoskeleton, membrane traffic, motors and scaffolds.
Energy: Mitochondria and metabolism.
Protein synthesis and processing: Translation and chaperones
percentage of PSD proteins per category was obtained from the mass spectrometry data by Peng and colleagues (Peng et al., 2004).
Adapted from (Sheng and Hoogenraad, 2006).

4.2.3.3. Dendritic spines organelles

Dendritic spines host multiple **membrane-bound organelles**. While rough **endoplasmic reticulum** is noticeable in the cell body and proximal dendrites of neurons, smooth endoplasmic reticulum (SER) predominates in distal dendrites and dendritic spines (Spacek and Harris, 1997). Newly synthesized membrane proteins allotted to the postsynaptic membrane are produced in the rough endoplasmic reticulum of the neuronal cell body. Proteins are then transported through the Golgi apparatus from which they are finally sorted to dendrites (Malinow and Malenka, 2002; Horton and Ehlers, 2004).

Translation machinery components, like **polyribosomes**, have been detected in dendritic shafts and dendritic spines. This evidence implies a capacity for local postsynaptic protein synthesis which may play a role in synapse plasticity (Gardiol et al., 1999; Pierce et al., 2000; Ostroff et al., 2002). ER exit sites and Golgi membranes, elements of the secretory pathway, are observed in hippocampal dendrites (Horton and Ehlers, 2004). The organelles are not equally shared among spines, the large spine bearing more of them. The endoplasmic reticulum (ER) forms a dynamic, continuous network in the dendritic shaft, from which it extends into a subset of dendritic spines, sometimes closely approaching the PSD (Spacek and Harris, 1997). The storage and release of calcium, as well as the transport of lipids and proteins of the plasma membrane and synapses are probably the most critical functions of the SER in dendrites and spines (Svoboda and Reenstra, 2002; Verkhratsky, 2005). The spine apparatus is a specialized compartment consisting of stacks of SER separated by electron-dense plates which is found in ~20% of spines. It is mainly located in the neck of large mushroom-shaped spines (Spacek and Harris, 1997). The spine apparatus is considered as a calcium store that may take part in calcium-dependent mechanisms of synaptic function and plasticity (Svoboda and Reenstra, 2002).

Mitochondria are often observed in dendritic shafts from which they diffuse the ATP into the spines. They are only occasionally observed in spines. Furthermore, they usually are restricted to big and complex spines. Mitochondria can be observed as tubules of variable sizes that fuse, divide, and branch in a dynamic reticular network. Beside of the provided ATP, mitochondria acts as buffer for the intracellular calcium. The mechanisms of apoptosis also involve mitochondria (Chan, 2006).

4.2.3.4. Actin cytoskeleton and its regulation in dendritic spines

Actin is highly concentrated in the spines compared to the dendritic shaft, and is highly enriched in the PSD, where it anchors receptors by interacting with a cohort of scaffolding proteins (Cohen et al., 1985; Kaech et al., 1997; Kuriu et al., 2006). Moreover, spine formation and morphology are regulated by actin filaments (Matus 2000, Zito et al. 2004). Spines contain actin in soluble pool of monomeric G-actin and as polymerized or filamentous F-actin that maintains the characteristic spine morphology (Halpain, 2000). F-actin forms organized bundles in the spine neck. Head shape modifications are related to the actin polymerization-depolymerization states (Star et al. 2002). The permanent turnover of actin filaments in dendritic spines most likely involves a tread-milling of existing filaments, with polymerization occurring at the fast growing ¨ barbed ¨ ends which are mostly oriented towards the surface of the spine. In contrast, depolymerization occurs at the ¨ pointed ¨ end (Carlier and Pantaloni, 1997; Pollard and Borisy, 2003). Induction of LTP briefly depolymerizes actin filaments (Ouyang et al., 2005). Thus maintenance of LTP and sustained spine enlargement require polymerization of F-actin (Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003; Lin et al., 2005). In opposite, LTD results in the depolymerization of actin as well as spine elongation or shrinkage of spine head (Chen et al., 2004; Nagerl et al., 2004; Zhou et al., 2004). Actin cytoskeleton is regulated by actin-binding proteins, like small guanosine triphosphatases (GTPases) of the Rho family, and changes in intracellular Ca^{++} concentration (Ethell and Pasquale, 2005; Tada and Sheng, 2006).

The Rho family of small GTPases is represented by a large number of proteins which comprises Rho, Rac, and Cdc42 (fig.23). These proteins act as binary switches cycling between GDP-bound inactive and a GTP-bound active state. In neurons, like in other cells, this switch is turned on or off by regulatory proteins, which include guanine nucleotide exchange factors (**GEFs**), GTPase activating proteins, and guanine nucleotide dissociation inhibitors. GEFs catalyze the exchange of GDP for GTP causing activation of Rho GTPases. Conversely, GTPase activating proteins (**GAPs**) and guanine nucleotide dissociation inhibitors **(GDI)** inactivate Rho GTPases. GEF-H1 has recently been reported as a mediator of AMPARs activity-dependent regulation of spine development (Kang et al., 2009).

Cdc42, a member of the Rho GTPases family, promotes actin nucleation and branching in the dendritic spine head by binding to **N-WASP** which is a member of the Wiskott-Aldrich syndrome family of proteins (Miki et al., 1996; Irie and Yamaguchi, 2002). N-WASP, by recruitment of G-actin, can form a super-complex with **Arp2/3 complex**. Once made active, the Arp2/3 complex nucleates actin polymerization and branching, a mechanism suggested to be involved in spine head enlargement (Higgs and Pollard, 2001). Cdc42 also promotes actin polymerization via the binding to the adaptor insulin receptor substrate p53 (IRSp53). IRSp53 is located in spines and it is known to mediate the actin cytoskeleton in non-neuronal cells (Abbott et al., 1999; Miki et al., 2000).

Rac1, another member of the Rho GTPases family, regulates actin dynamics in dendritic spines by activating the ARP2/3 complex via WASP family verprolin-homologous protein (WAVE)/Scar family proteins. IRSp53 seems to be the adaptor between Rac1 and the (WAVE)/Scar family proteins as the latter lack a binding domain to Rho family GTPases (Miki et al., 1998). **Cortactin**, a scaffold protein that regulate actin dynamics in dendritic spines, is translocated to the cell periphery under Rac1 influence (Head et al., 2003; Hering and Sheng, 2003). Both Rac1 and Cdc42 activate **Pak1** which is a serine-threonine kinase that phosphorylates and activates LIM kinases1 and 2 (Yang et al., 1998a; Yang et al., 1998b; Edwards and Gill, 1999; Edwards et al., 1999). The LIM kinases also are serine–threonine kinases. They can phosphorylate and inhibit the actin depolymerizing proteins ADF and cofilin in such a way that they are able to reduce actin filament turnover and cell motility.

RhoA, a third member of the Rho GTPases family, boosts activation of **LIM kinases** via another serine–threonine kinase called **ROCK** (Maekawa et al., 1999; Sumi et al., 2001). Both Pak1 and LIM kinase 1 have been shown to regulate dendritic spine shape, but their effects are not similar (Meng et al., 2002; Penzes et al., 2003). Pak1 activates actomyosin contractility by directly phosphorylating myosin regulatory light chain (Kiosses et al., 1999; Sanders et al., 1999; Bokoch, 2003). ROCK appears to stand as major RhoA effector in neurons (Li et al., 2002). In adjunction to LIM kinases, it activates myosin regulatory light chain by phosphorylation and inactivates myosin light chain phosphatase. These combined effects altering myosin regulation are essential for the biological activities of RhoA. Therefore this regulation may lead to dendritic spine increased contractility and spine shortening. ROCK seems to play a role in the regulation of the dendritic spine downstream of RhoA as ROCK inhibitor blocks the effects of activated RhoA on dendritic spines (Nakayama et al., 2000; Govek et al., 2004). Rho family GTPases also promote the synthesis of the acidic phospholipid PIP2, which can be linked to N-WASP as well as many actin modulation proteins, and regulates their activities (Chong et al., 1994; Yamamoto et al., 2001; Yamazaki et al., 2002; Weernink et al., 2004).

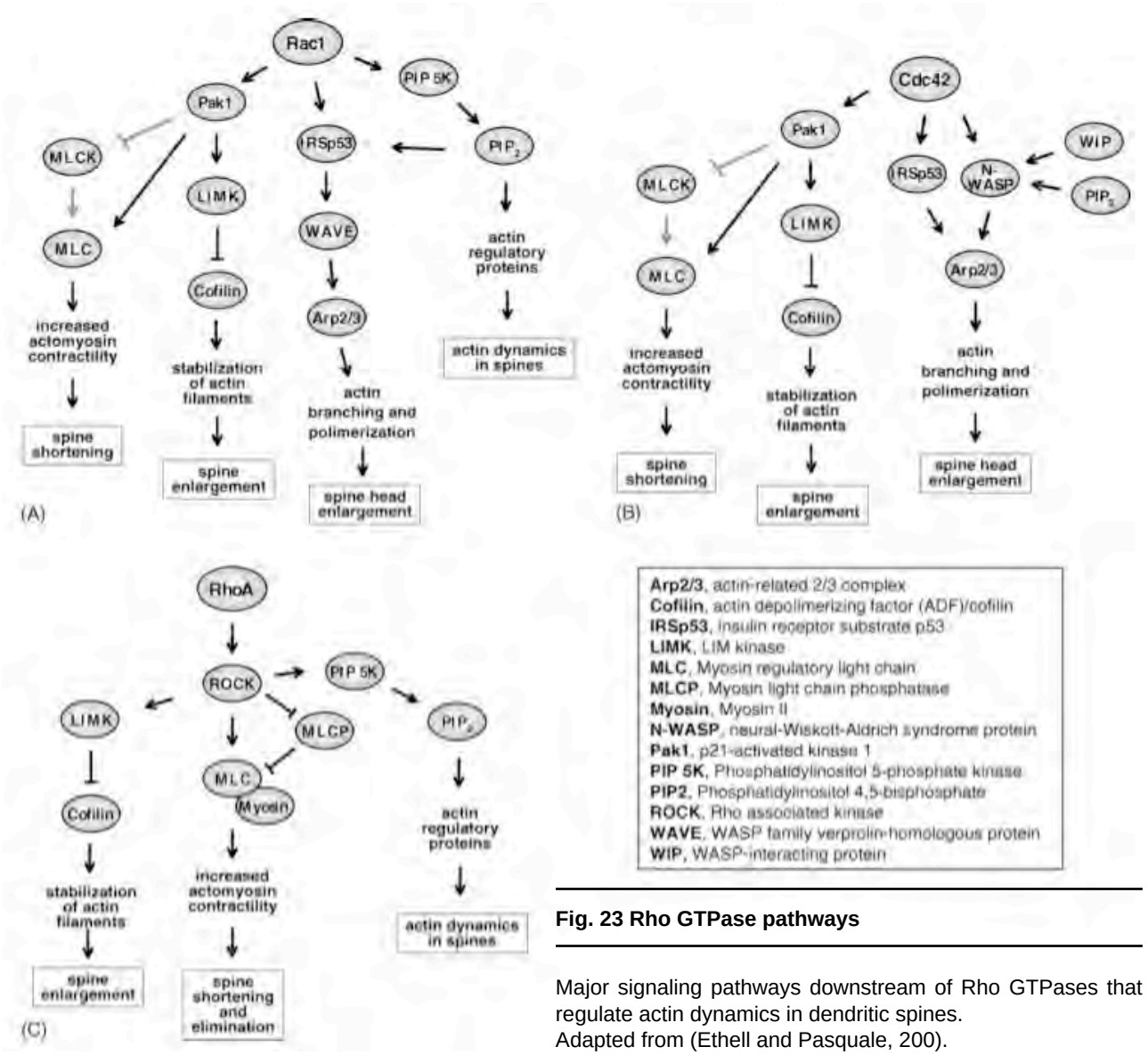


Fig. 23 Rho GTPase pathways

Major signaling pathways downstream of Rho GTPases that regulate actin dynamics in dendritic spines.
Adapted from (Ethell and Pasquale, 200).

Profilin is a promoter of actin polymerization that could facilitate LTP-induced actin assembly and spine enlargement (Ackermann and Matus, 2003). **Cofilin** is an actin binding protein that causes actin depolymerization. LTP or exposure to enriched environment cause phosphorylation-mediated inhibition of cofilin which promotes spine enlargement (Chen and Firestein, 2007; Fedulov et al., 2007).

Rap1 is an actin-binding protein that brings AF-6 to the synaptic membrane. AF-6 induces rearrangement of actin filaments and promotes removal of AMPA receptors (Xie et al., 2005) and spine elongation (Zhu et al., 2002). However, inactivation of Rap1 releases AF-6 from the synaptic membrane, thus regulating a different pool of actin filaments that promote recruitment of AMPA receptors to the synapse; spine enlargement with LTP is also influenced by AF-6 (Xie et al., 2005). Myosins IIb and VI are motor proteins enriched in the PSD. They control contractility of actin filaments and spine shape (Osterweil et al., 2005; Ryu et al., 2006). Myosin VI–deficient spines have disrupted clathrin-mediated endocytosis of AMPA receptors, suggesting a role in LTD (Osterweil et al., 2005).

4.2.3.5. Other scaffolding proteins

Two major families of scaffold proteins located at the synapse are believed to regulate spine morphogenesis. One is the **MAGUKs superfamily** and the second is the Shank family.

4.2.3.5.i. MAGUKs Superfamily

MAGUKs are defined by the following set of features (fig.24). They have a ~300 amino acid region with homology to yeast kinase (GK) which catalyzes the ATP-dependent phosphorylation of GMP to GDP. Though the GK domain in MAGUKs is catalytically inactive, it is always flanked by either a preceding SH3 (Src-homology-3) domain or followed closely by a WW (two conserved TrP residues) motif. Finally, MAGUKs always bear PDZ (PSD-95/Dlg/ZO-1) domains. All these modular motifs in MAGUKs are involved in protein-protein interactions (Funke et al., 2005).

The most important and abundant member of MAGUK family is PSD-95 protein highly enriched in the PSD. PSD-95 related molecules are encoded by four genes which give raise to PSD-95/SAP90 (synapse-associated protein 90), PSD-93/chapsyn110, SAP102 and SAP97. These proteins contain three PDZ domains (PDZ is the abbreviation for the first letter of: Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1)), a SRC homology (SH3) domain and guanylate kinase-like (GK) domain. Besides this common architecture, SAP97 bears a LIN2/LIN7 (L27) domain at the N-terminal. PSD-95 family members appear to play major roles in synapse organization and function (Funke et al., 2005). PSD-95 is the most studied of its family as it is the major constituent of the excitatory PSDs (Chen et al., 2005). It is also greatly involved in synaptic plasticity (Migaud et al., 1998) as it can occlude or mimic LTP in an expression-dependent manner (Stein et al., 2003). PSD-95 is able to directly bind NMDA receptors by their NR2A subunit, and it can bind AMPA receptors by the associated protein stargazin (Schnell et al., 2002). Therefore it plays a role in AMPA receptors targeting at the synapse and their regulation during synaptic plasticity (Chen et al., 2000; Schnell et al., 2002; Ehrlich et al., 2007). PSD-95 regulates the balance between the number of inhibitory and excitatory synapses (Prange et al., 2004). Besides synaptic function, some evidence lets imagine that PSD-95 affect synapse maturation (El-Husseini et al., 2000; Ehrlich et al., 2007) (De Roo et al., 2008b) and synapse number. Severe knockdown of PSD-95 impairs the development of synaptic structures (Ehrlich et al., 2007). In addition, PSD-95 mutant mice have variable spine densities in several brain regions (Vickers et al., 2006). PSD-95 regulation is a continuous and dynamic process between intracellular and synaptic pools (Bresler et al., 2001; Gray et al., 2006). Many synaptic proteins interact with PSD-95, such as synaptic GTPase-activating protein (SynGap), ErbB, fyn, neuroligins and syndecan (cell adhesion proteins), spine-associated Rap-guanosine-triphosphatase-activating protein (SPAR), kalirin-7, Ca2þ homeostasis (e.g. Ca2þ-ATPases 2a and 4b), nitric oxide synthase (NOS), thus participating in synapse formation or stabilization. Nitric oxide (NO) mediates trans-synaptic signaling (Kim and Sheng, 2004). Furthermore, PSD-95 overexpression gives rise to multi-innervated spines. This process is compromised when PDZ dependent interactions between NOS and PSD-95 are restricted (Nikonenko et al., 2008). SAP97 and PSD-95 have impact on the synaptic targeting and anchoring of AMPA receptors. SAP97 and PSD-95 interaction is mediated by binding of the N-terminal segment of SAP97 to the Src homology 3 domain of PSD-95 (Cai et al., 2006). Cai et al.

experiments in cultured hippocampal neurons showed that expression of green fluorescent protein-tagged PSD-95 triggered accumulation of SAP97 in synaptic spines, but this was totally inhibited by coexpression of PSD-95 (SH3 inefficient) constructs. Furthermore, overexpression of green fluorescent protein-tagged PSD-95 induced dendritic clustering of GluR1 subunit-containing AMPA receptors, which was mainly inhibited by cotransfection with SAP97 (N-terminal inefficient) and PSD-95 (SH3 inefficient) constructs (Cai et al., 2006).

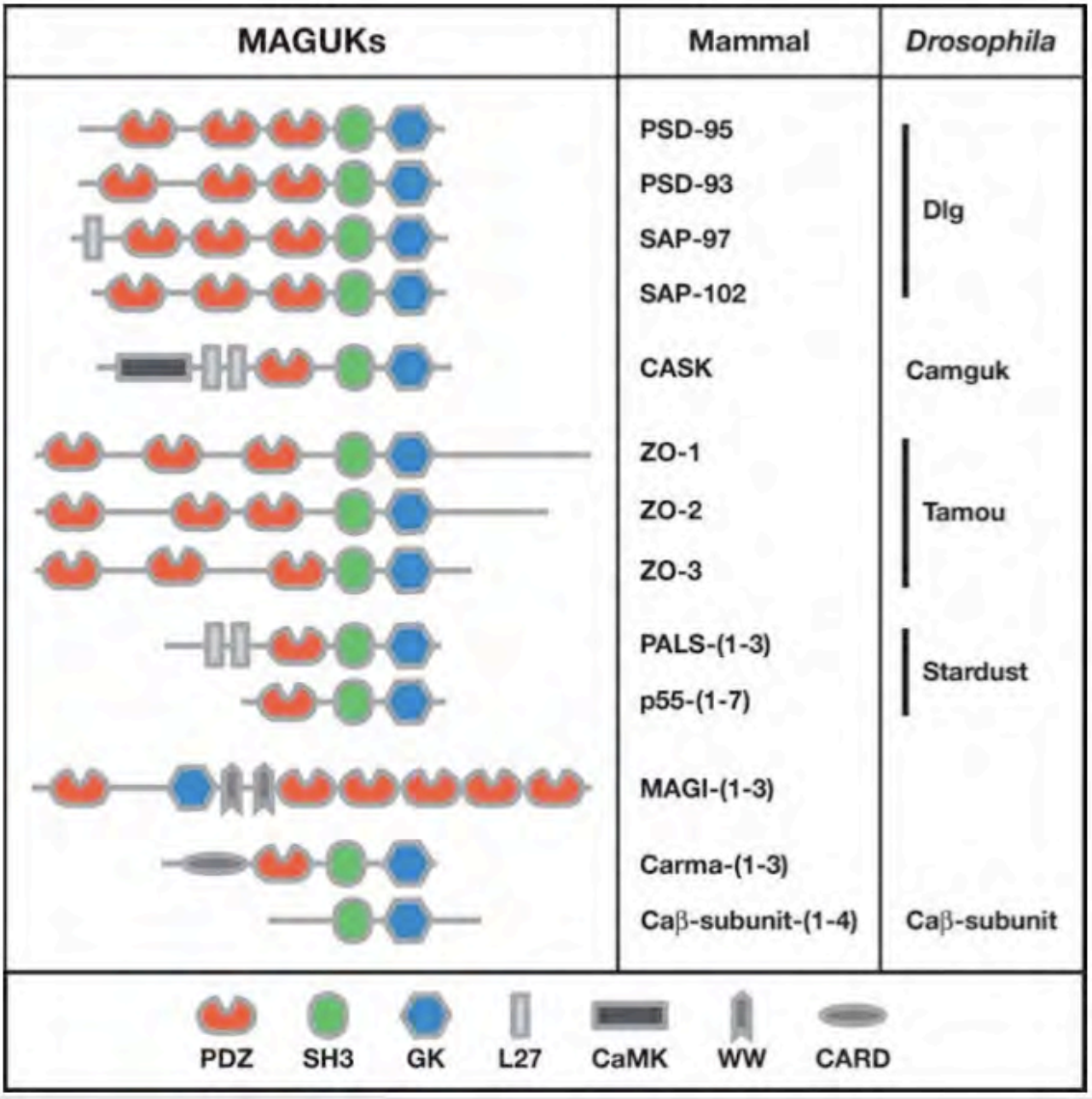


Fig. 24 The MAGUK family of proteins

Mammalian MAGUK structures are depicted, along with their Drosophila homologues. MAGUKs show three typical features, they have a PDZ domain, a SH3 domain and a GK domain. Beside that, they have supplementary or repeated domains that give them specific functions in the cell they are expressed.
Adapted from (Funke et al., 2005).

4.2.3.5.ii. SAP97

SAP97 or hDLG for human Homolog of Drosophila Discs large (Montgomery et al., 2004) is the next best-studied MAGUK protein after PSD-95. SAP97 is present at both pre- and postsynaptic compartments, where it is implicated in the processing of the GluR1 subunit of the AMPARs (Rumbaugh, G., Sia, G. M., Garner, C. C., and Huganir, R. L. (2003) J. Neurosci. 23, 4567– 4576). Interaction with NR2-type NMDA subunits and SAP97 have also been suggested (Bassand, P., Bernard, A., Rafiki, A., Gayet, D., and Khrestchatisky, M. (1999) Eur. J. Neurosci. 11, 2031–2043)(Gardoni et al., 2003). SAP97 interactions with GluR1 have been shown early in the biosynthetic pathway of GluR1-containing AMPA receptors (Leonard, A. S., Davare, M. A., Horne, M. C., Garner, C. C., and Hell, J. W. (1998) J. Biol. Chem. 273, 19518 –19524), (Sans, N., Racca, C., Petralia, R. S., Wang, Y. X., McCallum, J., and Wenthold, R. J. (2001) J. Neurosci. 21, 7506 –7516). This evidence confirms that PDZ protein interactions at the level of the endoplasmic reticulum-cis-Golgi play an important role in the sorting and surface expression of ion channels in neurons. SAP97 has different phosphorylation sites, among them ser39 in the L-27 domain and ser232 in the PDZ1 domain (Mauceri et al., 2004). SAP97 is involved in GluR1 delivery to the membrane via SAP97 ser39 phosphorylation by CaMKII (Mauceri et al., 2004). A ser39 CaMKII phosphorylation is also involved in the release of SAP97/NR2A from the endoplasmic reticulum. Moreover, a phosphorylation of the SAP97 PDZ1 ser232 domain by CaMKII disrupts SAP97/NR2A complex and consequently is responsible for NR2A insertion in the postsynaptic membrane (Mauceri et al., 2007). Few synaptic AMPA receptors associate with SAP97 (Sans, N., Racca, C., Petralia, R. S., Wang, Y. X., McCallum, J., and Wenthold, R. J. (2001) J. Neurosci. 21, 7506 –7516). SAP97 can influence synaptic AMPARs when overexpressed postsynaptically (Rumbaugh et al., 2003; Nakagawa et al., 2004b) and seems also to affect presynaptic function (Rumbaugh et al., 2003). Postsynaptic SAP97 has the stronger presynaptic effect by dramatically increasing the levels of the active zone protein Bassoon and the vesicle proteins synapsin and synaptophysin (Regalado et al., 2006). Functional PDZ domains, intact S97N and GK domains are required for SAP97 transsynaptic effect on synapse structures and functions. Moreover, an increase in postsynaptic SAP97 also promotes a selective augmentation of several binding partners including the AMPAR subunit GluR1 and the scaffold proteins ProSAP2, Shank1a, and SPAR/SPAL (spine-associated RapGAP/SPA-1-like protein). SAP97 associated to 4.1N binds Glur1 (Valtschanoff et al., 2000; Sans et al., 2001) via actin, actinin and CaMKII (Lisman and Zhabotinsky, 2001). Transsynaptic effects of postsynaptic SAP97 require multiple cell adhesion and signaling molecules including cadherins, integrins, and EphB receptor/ephrinB. All together, these data suggest that SAP97 plays a key role in coordinating the growth and functional organization of the presynaptic and postsynaptic components of synapses during both development and synaptic plasticity (Regalado et al., 2006). SAP97 has also been reported to have an impact in disease such as Alzheimer disease, via its connection with ADAM10 (Marcello et al., 2007) and in schizophrenia, according to an association analysis (Sato et al., 2008).

4.2.3.5.iii. Shank

Shank is a large scaffold protein with a multidomain composed by an ankyrin repeat close to the N-terminal, followed by a SH3, a PDZ domain, a long proline-rich region, and a sterile alpha motif domain

(SAM) at the C-terminal (Tiffany et al., 2000). Shank proteins are codified by three genes ranging from Shank 1 to 3. Shank proteins are linked to two glutamate receptor subtypes which are the NMDA receptors and the type-I metabotropic GluRs (mGluRs). (also see below)

4.2.3.5.iv. SAPAP/GKAP

The Shank PDZ domain binds to the C-terminal of SAPAP/GKAP which is another PSD scaffold protein belonging to the SAP90/PSD-95-Synapse-associated protein/guanylate kinase-associated protein family. It is also able to associate with the GK domain of PSD-95.

4.2.3.5.v. Homer

Homer interaction at the proline-rich domain ensures the association of Shank with type-I mGluRs. Homer proteins are encoded by three genes from Homer 1 to 3. Homer usually consists of an N-terminal EVH1 domain followed by a coiled-coil domain that mediates dimerization with other Homer proteins. The Ena/VASP homology 1 (EVH1) domain of Homer1 binds to a PPXXF or a quite similar sequence pattern present in Shank, mGluR1/5, inositol-1,4,5-trisphosphate (IP3) receptor, ryanodine receptor, and to different members of the TRPC family of ion channels (Xiao et al., 2000; Yuan et al., 2003). Because of its ability to self-associate, Homer isoforms containing the coiled-coil domain can physically and functionally link the proteins and receptors that bind to the EVH1 domain. The overexpression of Shank1 and Homer1b (a constitutively expressed splice form of the immediate early gene product Homer) (Xiao et al., 2000) in hippocampal neurons accelerates the maturation of filopodia-like protrusions in mature spines. Moreover, it promotes the enlargement of mature spines without increasing the density. Shank and Homer work in association and contribute to the accumulation of PSD proteins in dendritic spines, such as GKAP and NR1. They also increase the F-actin content in spines (Sala et al., 2001; Sala et al., 2008). An evidence for a global role of Shank in synapse maturation is that its overexpression also induces the maturation of the presynaptic compartment (Sala et al., 2001; Roussignol et al., 2005). Shank also binds to a large panel of proteins. The list contains the cortactin, Abp1 (Qualmann et al., 2004), the Rac1 and Cdc42 exchange factor αPIX (Park et al., 2003), and Cdc42-binding protein IRSp53 (Soltau et al., 2002). Homer binds to Rho GTPase-activating protein oligophrenin-1 (Govek et al., 2004). The interaction of Shank1 with Homer appears to be essential for promoting spine maturation, as it is for the interaction between cortactin and Shank3 (Roussignol et al., 2005). The enlargement effect can also rely on Shank1 and Homer1b capacity for recruiting the entire ER compartment to dendritic spines (Roussignol et al., 2005). Overexpression of Homer1a destabilizes synapses and diminishes the amount and the size of dendritic spines. The overexpression also reduces the synapse number of both AMPA and NMDA receptors. Thus the effect of Homer1a may contribute to the global activity-dependent loss of spines. Furthermore it acts negatively on the regulation of unstimulated synapses (Sala et al., 2003). Shank1 appears to promote the maturation of smaller, more plastic spines into larger, more stable spines (Hung et al., 2008).

4.2.3.6. Cell adhesion molecules

Cell adhesion molecules are an important class of molecules that are present on both side of the synapse that play as dynamic regulators of synapse functions. Interactions between them can induce the formation of new synapses or simply modulate the function of existing synapses. Adhesion molecules effects occurs through signaling cascades or secondary protein-protein interactions. Moreover, several studies indicate that particular interactions between adhesion molecules can regulate synapse formation, modify dendritic spine morphology, modulate synaptic receptor function and finally alter synaptic plasticity. The major families of cell-adhesion molecules at the synapse comprise namely neurexins and neuroligins, EphBs and ephrinBs, immunoglobulin (Ig)-containing cell adhesion molecules and cadherin. The following chapter will focus on the description of the cadherin complex.

4.2.3.6.i. Cadherin

The cadherins are calcium-dependent homophilic cell–cell adhesion molecules (Yoshida and Takeichi, 1982; Gallin et al., 1983; Peyrieras et al., 1983). They are single-pass transmembrane proteins that bear repeated extracellular domains in their extracellular part. The intracellular region is highly conserved and contains a juxtamembrane domain and a catenin-binding domain (fig.25). The juxtamembrane domain holds the GGGEED sequence, by which p120 catenin family member proteins (p120 catenin: Ctnnd1; NPRAP/d-catenin: Ctnnd2, p0071: Pkp4; and ARVCF: Arvcf) interact with cadherins (Suzuki and Takeichi, 2008). These proteins are suggested to regulate the surface stability of cadherins as well as the activity of the RhO family GTPases which play a central role in actin organization (Chen et al., 2003; Davis et al., 2003; Kennedy et al., 2005) (Goodwin and Yap, 2004). N-Cadherins provide a strapping adhesion between cells by the mean of catenins. N-cadherin possess a catenin-binding domain interacting with β-catenin (Ctnnb1). In turn, N-Cadherin and β-catenin are tethered to the actin cytoskeleton via α-catenin (Hirano et al., 1992; Kintner, 1992; Fujimori and Takeichi, 1993; Roe et al., 1998; Goodwin and Yap, 2004). The Cadherin-catenin complex alters actin polymerization by recruiting cortactin and the Arp2/3 actin nucleator complex which are able to delineate specific sites for the assembly of F-actin. On the other hand, the Cadherin-catenin complex, via α-catenin that associates to formin-1 (a member of the Diaphanous/formin-homology protein family) induce actin cable formation (Goodwin and Yap, 2004). Cadherin interacts with tyrosine kinases and phosphatases, but also with adaptor proteins. Moreover, catenins like β-catenin and δ-catenin function as scaffolding molecules by interacting with proteins containing PDZ domains (PSD-95/Dlg/ZO1) domains. By sequestering β-catenin, p120 catenin, and δ-catenin at the membrane, Cadherin influences and inhibits their transcriptional mediated activities (Goodwin and Yap, 2004). N-cadherin and β-catenin are randomly distributed along the length of free dendritic filopodia until they reach an axon. Then they accumulate at the filopodia-axon contact point (Togashi et al., 2002; Jontes et al., 2004). The N-Cadherin and β-catenin

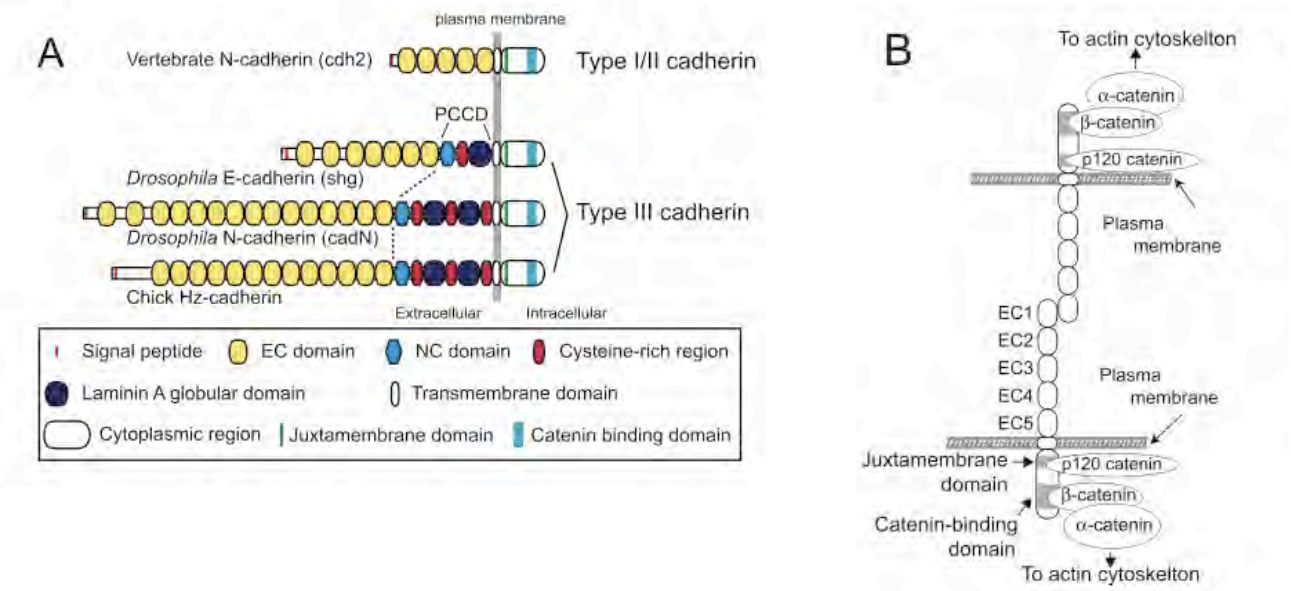


Fig. 25 Cadherins

A: Organization of cadherin-mediated cell-cell adhesion. Extracellular domain is versatile and conversely the intracellular domain is conserved in the three cadherins.
B: In situ scheme of two interacting N-Cadherin. Signaling and action of cadherin pass through the Cadherin-catenin complex.(Cadherin / beta and alpha catenin)
Adapted from (Suzuki and Takeichi, 2008, Dev Growth Differ, 50 Suppl 1, S119-30).

complex appears to mediate contact stabilization and further maturation and differentiation. Inhibition of cadherin contacts prevented spine maturation, and dendritic protrusions keep their long, immature, filopodial morphology. Neurons lacking α N-catenin also show immature filopodial morphologies (Salinas and Price, 2005). All together, these studies suggest that stabilization, mediated by the formation of cadherin-catenin-actin complexes, is required for the proper spine maturation. Another capacity of cadherins is to regulate the activity and localization of signaling molecules like cortactin, Arp2/3, formin-1, and the Rho family of GTPases. These associations with a large panel of molecules suggest that Cadherins are not restricted to structural regulations of the actin cyotskeleton. Cortactin, the Arp2/3 complex (Hering and Sheng, 2003), and Rho GTPases (Carlisle and Kennedy, 2005) are involved in the promotion of spine formation and stability. Studies using PSD-95 marker and kainate receptor subunit 6 (GluR6) show that cadherin-based contacts are important for the adequate localization of these postsynaptic density components (Honjo et al., 2000; Togashi et al., 2002)(Coussen et al., 2002). The δ-catenin, a neuron-specific catenin which is usually not contained in the classic cadherin-cytoskeleton complex, is able to form stable complexes with PSD-95. Cadherins are also involved in presynaptic active zones organization. Indeed, presynaptic active zones can assemble rapidly from pre-assembled clusters of presynaptic proteins that translocate along the axon in transport vesicles and are “trapped” at points of cell-cell contact (Ziv and Garner, 2004). Two main classes of transport vesicles have been observed so far. Firstly, the large dense-core vesicles called Piccolo-Bassoon transport vesicles (PTVs); they transport N-cadherin and several proteins associated with the active zone; secondly, the small pleomorphic vesicles that translocate along the axon in packets. They convey many of the distinctive molecules of synaptic vesicles (Ziv and Garner, 2004). Cadherin impact on vesicle localization is mainly mediated by β-catenin. It appears that β-catenin may interact with and localize PDZ domain-containing proteins that reversely interfere with the actin cytoskeleton, among which the PDZ domain-

containing Rho guanine nucleotide exchange factors (GEFs) (Fukuhara et al., 1999). Though cadherins and F-actin keep a significant role at mature synapses, more and more evidence indicates that neuronal activity mediates the distribution of cadherins, catenins (Salinas and Price, 2005), and F-actin (Colicos et al., 2001), which reversely affect synapse function and remodeling.

4.3. From spinogenesis to synapse formation

4.3.1. Spinogenesis

There are three largely accepted models that describe spinogenesis.

1. From filopodia to spine: a filopodia or a spine-like filopodia reaches an axon bouton, forms a synapse and then it is transformed into a spine.
2. From shaft synapse to spine: a bouton induces spine formation after contacting the dendritic shaft.
3. Spine growth preceding synapse formation: a spine emerges from the dendrite and contacts a bouton with which it form a synapse.

4.3.1.1. From filopodia to spine

The filopodia or spine-like filopodia probe the space surrounding their parental dendrite to find an appropriate presynaptic partner. It has been proposed that release of glutamate by axons may act as trigger for filopodia extension and guidance to presynaptic bouton (Portera-Cailliau et al., 2003). During early development, dendrites produce many highly motile filopodia (Dunaevsky et al., 1999; Lendvai et al., 2000) that can express PSD-95 and contact axon terminals (Marrs et al., 2001). But filopodia can also be observed after specific conditions such as induction of plasticity following ischemia or during regeneration after neuron injury (Maletic-Savatic et al., 1999; Jourdain et al., 2003; Ruan et al., 2009). Filopodia make contact on axons but in a transient way, and only a selected subset of these contacts get stabilized, over a range of minutes, via the generation of calcium transients (Lohmann et al., 2005). Lohman et al. noticed that this process of stable contact formation is not sensitive to glutamate receptors antagonist; nevertheless filopodia are able to discriminate between partners, as they never make stabilized contacts with inhibitory axons (Lohmann et al., 2005). Therefore, filopodia may initiate contact with potential partners likely via the expression of recognition or signaling molecules. However, it is yet unclear if filopodia make such preliminary contact before to make proper axo-dendritic synapses. But, their motility is probably an important advantage for synaptogenesis during early development as indicated by the decrease in synapses if motility is impaired by acting on Ephrin receptor signaling. Altogether the recent results suggest that the spine fate of developmental filopodia is controversial as filopodia are barely seen undergoing maturation process leading to stable spine (Zuo et al., 2005; De Roo et al., 2008a)(Wong et al., 1992).

4.3.1.2. From shaft synapse to spine

During the early development, pyramidal neurons show mainly excitatory contacts on dendritic shafts and not on spines or filopodia (Fiala et al., 1998). Later on, with network maturation, the number of spine synapses continuously increases while shaft synapse number decreases (Miller and Peters, 1981; Harris et al., 1992). These observations suggested therefore that spine synapses could be formed from initial shaft synapses. Supporting this interpretation, emergence of mature spines from shaft synapses has been observed (Marrs et al., 2001) under culture conditions following over-expression of tagged PSD-95. To account for the formation of shaft synapses, it has been proposed that axonal filopodia might play a role by prospecting for postsynaptic partners and by establishing synaptic contacts with dendritic shafts (Fiala et al., 1998). However, this model would expect axons to have convoluted trajectories, at least during development. This however is not the case as axonal trajectories are straight and contacts are made en passant (Yuste and Bonhoeffer, 2004). Indeed, in mature hippocampal network, the meshwork of axons is tight enough for short partner-matching trajectories.

4.3.1.3. Spine growth precedes synapse formation

Sotelo model states that presynaptic partners are not necessarily required for spinogenesis during development (Sotelo, 1990). Spinogenesis has been reported to precede synaptogenesis in developing slices cultures (De Roo et al, 2008) and after activity-induced plasticity (Nagerl et al., 2007) and even in anesthetized adult mice following experience dependent plasticity (Knott et al., 2006). In mature neurons, new spines often start by contacting multisynapse boutons (MSBs) before showing a PSD in EM observations (Toni et al., 2007). These results thus suggest that in later development and particularly after LTP induction spine formation precedes synapse formation, conferring an important role to the post-synaptic part in regulating spinogenesis. More recently however, it has been found that newly formed spines may very quickly become functional and express AMPAR dependent responses within tens of minutes. EM evidence for PSDs apparition were observed after a period exceeding one day (Knott et al., 2006), these evidence contrast with previous studies where the enrichment of synaptic markers and vesicle recycling was used as a measure of synapse formation (Friedman et al., 2000; Okabe et al., 2001). The reasons for the variability in speed of synapse formation is currently unknown, but these new data at least show that mature synapses can be formed extremely rapidly under specific circumstances. The role of spine growth in synapse formation might however be different during early development. Due to the prevalence of filopodia and shaft synapses then, a model involving a precursor role for filopodia and shaft synapses has been proposed. In this model, a first synaptic contact would be promoted by a filopodia reaching a bouton, then the filopodia would retract and pull the terminal to the dendritic shaft, thus allowing a spine to emerge (Fiala et al., 1998) (Marrs et al., 2001).

4.3.2. Phases of synapse formation

Conceptually, synaptogenesis can be divided into five discrete steps (fig.26):

1. The establishment of an initial contact during which one neuronal process (axon or dendrite) recognizes the other as a potential and appropriate target. This starting step is thought to be primarily ruled by several classes of cell adhesion molecules CAMs, a class of molecule that is involved in extracellular matrix- or cell-cell binding. It includes integrins, members of the cadherin and protocadherin family of calcium-dependent adhesion molecules, and members of the Ig superfamily of CAMs.
2. The inductive step triggering pre- and postsynaptic differentiation. It seems to involve bidirectional signaling events that occur when subclasses of CAMs or ligand/receptor complexes are formed at the initial contact site (Waites et al., 2005; Craig et al., 2006).
3. The Induction is followed by a relatively prolonged step that lasts 1–2 h, during which vesicles and synaptic proteins accumulate within the presynaptic bouton and at the cytoplasmic face opposing the postsynaptic membrane (Ahmari et al., 2000; Bresler et al., 2001; Marrs et al., 2001; Okabe et al., 2001; Niell et al., 2004). These nascent synapses are able to achieve both release and detection of neurotransmitter by postsynaptic receptors within the first 30 minutes of initial contact (Buchanan et al., 1989; Ziv and Garner, 2001).
4. This initial phase of synapse differentiation is then followed by a much more prolonged phase, lasting for hours to days, during which structural and functional maturation occurs. The synaptic maturation is accompanied by molecular changes in the composition of the active zone and the PSD. Mainly, synaptic maturation is also associated with an increased stability of the junction and a resistance to disassembly. For instance, nascent-immature synapses may only stay for hours or even less (Alsina et al., 2001; Niell et al., 2004; Meyer and Smith, 2006). Furthermore, they are sensitive to actin depolymerizing drugs such as latrunculin A (Zhang and Benson, 2001). They also depend on the presence of “generic” cell adhesion molecules such as N-cadherin. In contrast, more mature synapses are insensitive to these drugs (Zhang and Benson, 2001). Interestingly, in neurons older than 10 days, cadherins seem superfluous for joining and aligning presynaptic vesicle clusters with molecular markers of the PSD (Bozdagi et al., 2004), they seem to be much more stable (Ruthazer et al., 2006) as they last for days, for weeks, and even for months (Trachtenberg et al., 2002; Meyer et al., 2003; Holtmaat et al., 2005; De Roo et al., 2008b; De Roo et al., 2008c).
5. The maintenance of a mature synapse for days to years. The mechanisms that regulate this stability are currently essentially unknown.

4.3.3. Mechanisms of synapse assembly

To generate a new synapse, the components and proteins required for its function must be brought at proximity of the site where the new synapse will be formed. It has been proposed that this could occur in two ways:

1. Proteins made in the cell soma appear stochastically and independently at new sites of axodendritic contact. Once on site, they are assembled into the appropriate synaptic structures.

2. Proteins already form complexes within the cell soma. Subsequently, they are grouped and transported to contact sites making assembly of synapses more rapid and efficient.

Neuron appear to integrate both scenarios (Ziv and Garner, 2001; Waites et al., 2005; Craig et al., 2006). Presynaptic assembly appears to depend on vesicular intermediates that deliver pre-assembled protein complexes. These include synaptic vesicles precursors, which deliver most of the synaptic vesicle proteins to nascent synapses (Bauerfeind and Huttner, 1993). Piccolo is transported to nascent synapses in an approximately 80 nm dense core granulated vesicle together with other constituents of the active zone, including Bassoon, Syntaxin, SNAP-25, and N-cadherin, as well as chromogranin B (Zhai et al., 2001; Shapira et al., 2003). Many more elements should be added to form the entire picture (Ahmari et al., 2000). Postsynaptic assembly may depend more on the gradual recruitment of individual proteins, as the evidence for vesicular delivery of prefabricated postsynaptic protein complexes is still missing. Like in axons, in dendrites a rich repertoire of small and tubular vesicles has been observed in dendrites. These include endoplasmic reticulum (ER) and Golgi membranes as well as vesicle pools carrying either AMPA or NMDA receptor subunits (Kennedy and Ehlers, 2006). Together with the former, the presence of dendritic ribosomes and mRNAs (Steward and Schuman, 2001; Sutton and Schuman, 2005) let imagine that both cytosolic and integral membrane proteins can be locally synthesized, processed and inserted into nascent and mature synapses (Ju et al., 2004) (Miyata et al., 2005). Some studies demonstrate prefabricated protein complexes like NMDA receptors vesicles also containing Veli/MALS, CASK and the microtubule-dependent KIF17. The other studies show (Setou et al., 2000) postsynaptic proteins like PSD-95, GKAP and Shank moving in an actin-dependent manner in non-synaptic clusters. These proteins are then incorporated at sites close to functional presynaptic active zones (Gerrow and El-Husseini, 2006). Neuroligin1 is contained in a slow moving non-synaptic cluster which seems to induce the presynaptic clustering of synaptic vesicles.

All together these data raise the possibility that clusters of postsynaptic scaffold proteins, not only help the gathering of cell-surface CAMs, but may also trigger the formation of presynaptic boutons. Therefore, an imaginable scenario would begin with a cluster of PSD proteins which aggregate proteins such as neuroligin/SynCAM that are capable of inducing presynaptic formation. These additional units of postsynaptic material would then be added to the site following the synapse growth and maturation (Marrs et al., 2001; Prange and Murphy, 2001; Washbourne et al., 2002; Bresler et al., 2004; Gerrow and El-Husseini, 2006). Time-lapse imaging studies performed in dissociated hippocampal cultures indicate that PSD proteins accumulate gradually at nascent synapses with kinetics that have time constants in the range of 12 to 30 min (Bresler et al., 2001; Bresler et al., 2004).

4.3.3.1. Structural and functional maturation of synaptic contacts

The process following the initial formation of mostly transient synaptic connections last for a longer time. It is during this period that these connections are refined and stabilized. This transition from labile to stable involves numerous structural and functional changes. Morphologically, the number of clustered synaptic vesicles increases on presynaptic side; pronounced postsynaptic densities appear; and one can observe a shift from unstable nascent spines to mushroom and stubby mature spines (Yuste and Bonhoeffer, 2004). Finally there is a drastic decrease in spine mobility. These events are dependent on cytoskeleton dynamics (Zhang and Benson, 2001; Abe et al., 2004; Bozdagi et al., 2004)((Takeichi and Abe, 2005).

Related with these structural changes is an array of functional modifications that alter the firing properties of neurons concomitant with maturation of the nervous system (Scholz and Miller, 1995; Pravettoni et al., 2000). PSDs which contain NR2B subunits of the NMDA receptors in immature excitatory synapses switch to NR2A subunits during the second and third weeks of postnatal life as these synapses mature (van Zundert et al., 2004; Petralia et al., 2005).

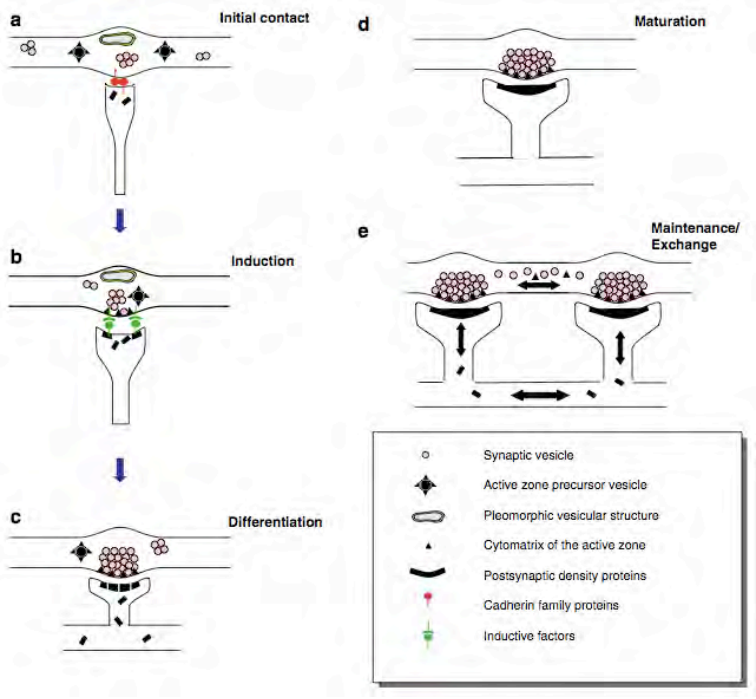


Fig. 26 Five steps of excitatory synapse formation

A: Inductive factors at these contact sites induce the formation of presynaptic active zones and PSDs by recruiting the proper protein components.
B: Synapse stabilization by adhesion molecules.
C: Dendritic spines are not yet fully formed, and both pre- and postsynaptic elements are highly sensitive to cytoskeletal perturbations.
D: Synapses attain their final protein composition after maturation.
E: The replacement and exchange of pre- and postsynaptic proteins enables synapses to be maintained over long periods of time.
Adapted from (Garner et al., 2006).

Activity appears to play an essential role in synapse formation and network maturation. This can be observed in many regions of the brain, including the visual, olfactory, and somatosensory systems, which requires synaptic NMDA-dependent neurotransmission to set up proper wiring (Constantine-Paton et al., 1990; Constantine-Paton, 1990; Sullivan et al., 1995; Foeller and Feldman, 2004; Ruthazer and Cline, 2004). However, the role of synaptic activity in influencing the formation and maturation of synapses is less clear as the brain develops normally without proteins essential for synaptic vesicles exocytosis (Verhage et al., 2000; Rosenmund et al., 2002). Activity appears to be important for synapse formation per se, but not for the gross architecture of the nervous system (Harms and Craig, 2005).

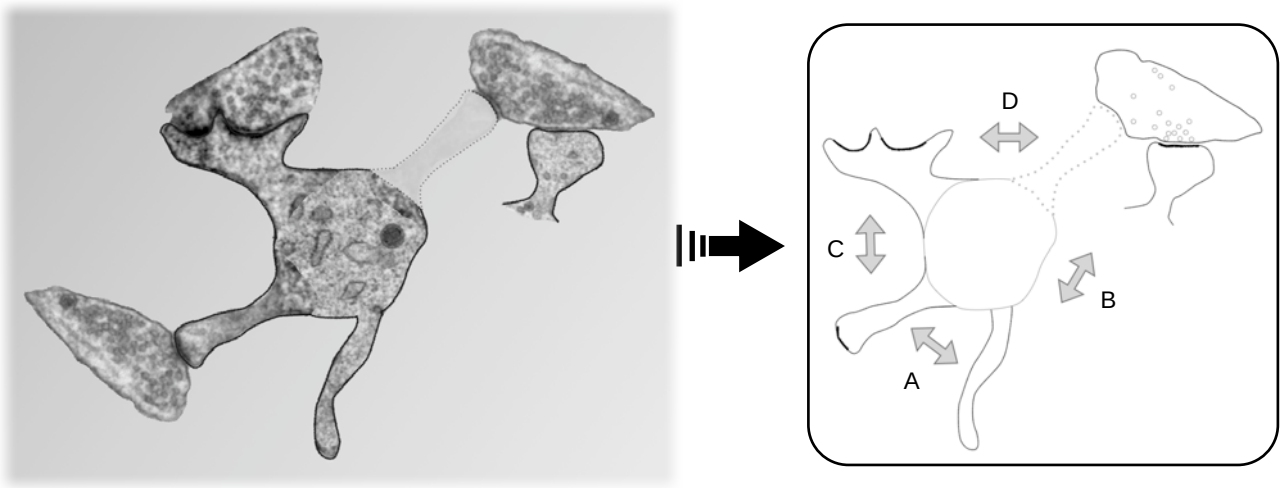


Fig. 27 Representation of different dendritic protrusions stages observed with serial section EM

Left: Photomontage of different protrusions stages observed in serial section Electron Microscopy **(**ssEM). ssEM is a special technique used to reconstruct volume of tissue thanks to serial images made from following sections that are cut with an ultra-microtome. Starting from the left clockwise: A small spine with almost no spine head but making a synapse with a presynaptic axon terminal (immature-like). Then a large and mature spine with perforated PSD making synapse with a unique presynaptic partner. Right, a sketch showing a disappearing spine which is, in some cases, in selective competition with an appearing spine. Below, a filopodia characterized by no PSD and no spine head and without active presynaptic partner.
Right: Schematic diagram resuming the macro changes occurring to dendritic protrusions.
A: Filopodial protrusions may switch to nascent spines after acquiring a PSD and making a synapse with a facing presynaptic active zone.
B: Filopodia are versatile protrusions that appear quickly, but only a small percentage of new filopodia turn into stable protrusions (De Roo et al., 2008).
C: Many works showed that spines changes their size depending on the stimulation they experience. Small spines are often enlarging after specific stimulation like LTP. Contrarily, large spines may also change their size under certain conditions like LTD for instance, thus diminishing their volume.
D: Neuronal remodeling is a dynamic process that shows appearing and disappearing spines or protrusions. Homeostatic and/or hebbian mechanisms change the spines' parameters, thus remodeling the neuronal network in such order to fit to the new environmental stimuli.

4.3.3.2. Maintenance of synaptic structure

In vivo imaging studies of neurons in mature somatosensory, visual cortex and organotypic hippocampal slice cultures have shown that, whereas mature synapses do turnover, many remain stable over days, weeks, and even months (fig.27) (Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005a; De Roo et al., 2008b; De Roo et al., 2008c). Ehlers pulse-chase studies showed that the average turnover rate of synaptic proteins in a synaptosome preparation is ~5 h, with individual components ranging from 4 to 15 h (Ehlers, 2003). Remarkably, chronic changes in neuronal activity dramatically influence the turnover of some dendritic spines, so that an increase in activity causes a faster turnover (t=2–10 h) of NMDA receptor subunits, SAP102, protein phosphatase1, and A-kinase-associated protein 75, whereas a decrease in the activity slows down their turnover (t=20–30 h). Studies at central synapses indicate that both receptor and structural protein dynamics are markedly faster in their turnover and exchange rates than at peripheral synapses. This is an argument for the more plastic nature of central synapses. The global activity state of a neuronal network influences the protein turnover that has been estimated to be between 3 to 20

hours (Ehlers, 2003). Still, imaging studies investigating the dynamics of individual postsynaptic proteins reveal a great volume and rate of exchange occurring at presumable stable structural junctions. As observed with FRAP experiments, many postsynaptic molecules like for instance SAP90/ PSD- 95, SAP97, PSD- Zi p45/ Homer1c, α-actinin; neurabin-l, actin, brain-enriched GK domain-associated protein, ProSAP1/Shank2, ProSAP2/Shank 3, AMPA receptor subunit GluR1, NMDA receptor subunit NR1, CaMKII, N-cadherin undergo an important rate of removal and reintegration into individual synapses. This phenomenon is partially activity dependent (Okabe et al., 2001; Yao et al., 2003; Bresler et al., 2004; Nakagawa et al., 2004a; Nakagawa et al., 2004b; Iki et al., 2005; Sharma et al., 2006).

All together, these studies provide the evidence that the multimolecular complexes located at synapses are highly dynamic structures. In addition, individual molecules are continuously exchanged with the molecules in extra-synaptic pools (Inoue and Okabe, 2003; Triller and Choquet, 2005).

4.4. Functional characteristics of synapses

4.4.1. The two types of ligand-gated channels on postsynaptic cells

When the receptors are directly coupled with ions channels, such receptors are called ionotropic. When the receptor is indirectly coupled through a G-protein pathway to an ion channel,it is called metabotropic. Most synapses in the CNS express several types of receptors.

4.4.2. The different steps of the chemical transmission

Pioneering work at the squid giant synapse showed how action potentials stimulate the release of synaptic vesicles by triggering the opening of voltage-dependent Ca^{++} channels in the presynaptic terminal (Llinas et al., 1981; Augustine et al., 1985). In the case of giant squid axons we are confronted with a two-stage process. In the first stage, Ca^{++} channels open rapidly (0.1 ms) during the peak of the action potential (Wu et al., 1999). However, the Ca^{++} current is small because the voltage is so positive that the electrochemical driving force is low. The electric driving force can be explained as the difference between Nernst equilibrium potential for an ion and the membrane voltage. The current through a channel is the product of the channel conductance and the electrochemical driving force. The second stage occurs during the descending phase of the action potential; the lowered voltage augments the driving force and thereby strongly increases the Ca^{++} influx. This two-stage process accounts for a substantial part (0.4 ms) of the synaptic delay at giant synapses.

4.4.3. The polarity of the synapse

A synapse is defined as excitatory when an excitatory postsynaptic potential (EPSP) occurs. As seen above, an EPSP is provoked by a depolarizing cation influx through the membrane following the binding of neurotransmitters to the postsynaptic receptors. Conversely, a synapse is called inhibitory when the binding of the neurotransmitter to the postsynaptic receptors induces an influx of anions through the membrane. This anion influx hyperpolarizes the postsynaptic zone and produces an inhibitory post synaptic potential (IPSP).

The most common neurotransmitters in the central nervous system are the two amino acids glutamate and gamma-aminobutyric acid (GABA). Glutamate acts as an excitatory neurotransmitter, and GABA plays a role of inhibitory neurotransmitter.

4.4.4. Postsynaptic currents

4.4.4.1. EPSPs

In the brain, EPSPs occur by the mean of ionotropic glutamate receptors which major subtypes are N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. Receptors' nomenclature is defined by their respective synthetic agonist. Non-NMDA receptors are responsible for the early phase of the EPSP and NMDA receptors generate the late phase due to their relatively slow opening and closing rate. Glutamate receptors show the following characteristics:

-AMPA receptors: cation channel conduction is high for Ca^{++}, Na^{+} and K^{+} ions. The GluR2 subunit of AMPA modulate permeability of cations, especially for calcium. AMPARs lacking GluR2 subunits are susceptible to be blocked in a voltage-dependent manner by a class of molecules called polyamines.

- NMDA receptors: cation channel conductance is high for Ca^{++}, Na^{+} and K^{+} ions; the permeability is voltage-dependent due to its voltage-dependent block by Mg^{++}. Accordingly an important level of activity is required to induce the opening of NMDA receptors. Not all synapses express both types of receptors. It is generally believed that immature synapses lack AMPA receptors and only express NMDA receptors. They are called "silent synapses" because of the activation of NMDA receptors by the released glutamate under basal conditions results in no currents due to the Mg^{++} block. (Isaac et al., 1995; Liao et al., 1995).

4.4.4.2. IPSPs

IPSPs are mainly GABA receptors (GABAR) dependent in the brain. There are three classes of GABA receptors: $GABA_A$, $GABA_B$, and $GABA_C$ (Barnard et al., 1998; Chebib and Johnston, 2000). $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels permeable to Cl^{-}, whereas $GABA_B$ receptors are G-protein-coupled receptors that usually activate K^{+} channels or inhibit voltage-gated Ca^{++} channels.

4.4.4.3. Miniature EPSC (mEPSC)

A mEPSC is a spontaneously occurring synaptic event caused by the spontaneous release of one vesicle. It is generally measured after blocking action potential with tetrodotoxin to insure that there is no release due to spontaneous action potential. The amplitude of mEPSC is determined by the number of AMPA channels open by the transmitter and is used as a parameter to asses postsynaptic processes in traditional quantal analyses. This is however not always valid as mEPSC amplitude can also be affected by vesicle glutamate concentration and a multi-vesicular release mode. The frequency of mEPSC is due to the capacity of the presynaptic vesicles to release glutamate into the cleft, known as probability of release. Therefore mEPSC frequency is a parameter for presynaptic activity.

4.4.5. Plasticity of dendritic spines

Dendritic spines are dynamic structures the stability of which depends upon activity, Hebbian type of plasticity and homeostatic processes (Sjostrom et al., 2008). Dendrites bear protrusions that appear and disappear depending on their intra- and extra-cellular molecular environment and depending on the level of activity of the neuronal network (Harris, 1999; Luscher et al., 2000; Bonhoeffer and Yuste, 2002; Holtmaat et al., 2005; De Roo et al., 2008c; Holtmaat et al., 2008). Dendritic spine plasticity is often sorted in structural and functional plasticity. Structural plasticity involves the morphological changes affecting the neck and the head of the spine and mechanisms of synaptogenesis. This includes fast movements and oscillations of spines, taking place in the range of seconds, but also changes in size of spine heads, which may occur within minutes, or even formation of new synapses over periods of minutes to hours. On the other hand, functional plasticity is characterized by all changes in strength of synapses that may depend upon pre- as well as postsynaptic changes, including processes of receptor recycling. These two forms of plasticity are likely to be linked to each other: It has indeed been shown that the efficacy of the synapse is closely correlated to the spine size (Harris and Stevens, 1989; Knott et al., 2006). Therefore, morphological changes of the spine also inform to a certain degree on the synaptic contatc and vice versa.

4.4.5.1. Functional plasticity

Functional plasticity aims at fine tuning of the efficacy of transmission at the synapse. Pre- and postsynaptic compartments undergo a dynamic change of their constitutive elements. Changes occur at the nanometer scale: receptors as well as their subunit composition and their associated partners are modified in the number and nature. Endo- and or exocytosis mechanisms and lateral diffusion from the center to the periphery of the synapse alter receptors location. Furthermore, cascade of molecular phosphorylations or dephosphorylations finely set the biophysical properties of the synapse throughout its life.

4.4.5.1.i. LTP

Long term potentiation ((LTP) (and subsequently long term depression (LTD)) is the classical example of activity-dependent process occurring in the brain. LTP is often considered as the basic mechanism underlying memory as it fits well with the famous postulate of Hebb (fig.30). Indeed, the conceptual idea underlying the protocol causing LTP is fathered by the Hebb's postulate predicitng that learning and memory would involve synaptic strengthening elicited by the coordination of pre- and postsynaptic cells firing.

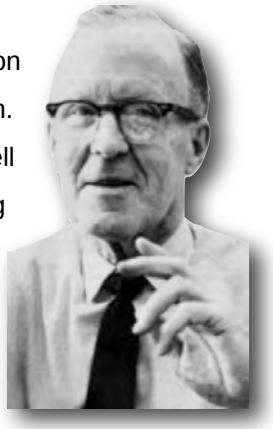


Fig. 30 Hebb

His theory exerted an important influence in the understanding of selective neuronal plasticity.

"When an axon of Cell A is near enough to excite a Cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."

Ref. Hebb, D. O. The organization of behavior. New York: Wiley; 1949

Timothy Bliss and Terje Lømo (fig.31) while working in the lab of Per

Andersen published the famous 1973's paper: ¨ Long-lasting *potentiation of synaptic transmission in the dentate area of the anesthetized rabbit following stimulation of the perforant path* ¨. (Bliss and Lomo, 1973) Photo: J. Lisman. They demonstrated in anesthetized rabbit hippocampus that brief, high frequency stimulation of the perforant-pathway input to the dentate gyrus produced a long-lasting increase of the extracellular recorded field potential. This phenomenon has been shown in subsequent studies to last for weeks or months.

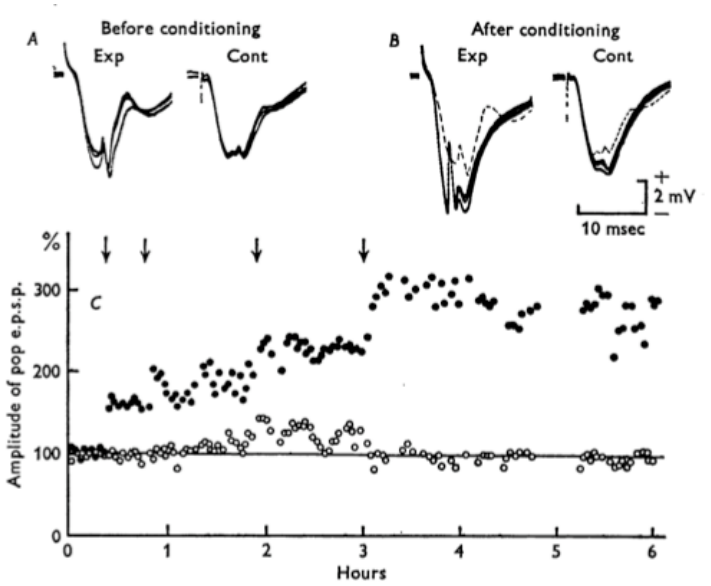


Fig. 4. An experiment in which all three standard parameters of the evoked response were potentiated. Three superimposed responses obtained in the synaptic layer for both the experimental and control pathways are shown in *A* (before conditioning) and in *B* (2·5 hr after the fourth conditioning train). *C*, graph showing the amplitude of the population e.p.s.p. for the experimental pathway (filled circles) and the ipsilateral control pathway (open circles) as a function of time. Each point was obtained from the computed average of thirty responses by measuring the amplitude of the negative wave 1 msec after its onset. The values are plotted as percentages of the mean pre-conditioning value. Conditioning trains (15/sec for 10 sec) were given through a medially placed conditioning electrode at the times indicated by the arrows.

Fig. 31 The Bliss & Lomo LTP

Left: ¨Historic¨ sample figure with figure legend of the famous (Bliss and Lomo, 1973).
Right: Bliss, Andersen and Lomo in Stockholm

Long-term depression (LTD) is a lasting decrease in synaptic efficacy that follows specific types of electrical stimulation in the hippocampus (Bear and Abraham, 1996). It results from persistent weak synaptic stimulations. LTD seems to produce spine shrinkage and requires NMDARs and calcineurin activation (Zhou 2004), but as single spine undergoing LTD have been less studied, LTD will be mainly kept apart from the following description. It is certain that in an activity-dependent network system, increase of synaptic strength in specific spines is followed by a decrease in the others to sustain a hierarchy in the process relying information storage mechanisms, and so LTD plays an important role in counterbalancing LTP-like neuronal network activity.

The NMDAR-dependent form of LTP occurring at CA1 pyramidal neurons is triggered by the depolarization or NMDARs. Thus, Mg^{++} is removed from NMDAR channels which makes them active. Activation of NMDARs leads to an influx of Ca^{++} through the NMDARs channels (Lynch et al., 1983) (Malenka et al., 1988); then the calcium rise induces activation of the Ca2+/calmodulin-kinase II (CaM-KII), indispensable for LTP induction (Malenka and Nicoll, 1999; Lisman et al., 2002). And an increase in Ca^{++}

intracellular concentration, like for instance by a calcium influx, has been observed to induce a rapid switch from a diffusive AMPARs state to a stationary one, their accumulation at neuronal surface (Borgdorff and Choquet, 2002). Besides internalization at synaptic and extrasynaptic sites (Ashby et al., 2004), regulation of AMPARs also relies on lateral trafficking from extra-synaptic pools. Moreover the proportion of juxtasynaptic AMPARs inversely fluctuates with the degree of synaptic stabilization (Tardin et al., 2003). Despite the uncertain provenance of the GluR1/2 subunits, they replace GluR2/3 subunits in an activity and CaMKII dependent manner (Hayashi et al., 2000). For instance, GluR1 subunits need Ser831 site phosphorylation by CaMKII during LTP (Barria et al., 1997; Derkach et al., 1999). An exception for CaMKII in LTP induction is observed in young animals (Kirkwood et al., 1997), that may be explained by the type of subunit expressed at the synapse. Zhu et al. showed that the GluR4 subunit mediates activity-dependent AMPAR delivery in immature hippocampus, and this delivery was NMDAR activity dependent (blocked by APV) but not CaMKII activity-dependent (Zhu et al., 2000). Interestingly, impairing Ser831 phosphorylation by CaMKII do not prevent GluR1 trafficking, but blocking PKA (a cAMP-dependent protein kinase) Ser845 phosphorylation site of GluR1 does prevent its trafficking (Hayashi et al., 2000). Thus, phosphorylation of GluR1 by PKA is necessary for trafficking (Ehlers, 2000) but is not sufficient for AMPARs externalization (Shi et al., 2001). However, CaMKII activity may relieve Glur1 interactions with proteins that restrict hetero-oligomeric GluR1/ GluR2 receptors from synaptic delivery (Shi et al., 2001). Studies of Tavalin and colleagues show that the neuronal A-kinase anchoring protein AKAP79/150, a molecule linked to GluR1 via SAP97, interacts with PKA promoting basal phosphorylation of Ser845. In addition, AKAP79 brings PKC activity near the receptor, therefore accelerating Ser-831 phosphorylation and selectively shifting the dose-dependence for PKC modulation of GluR1 receptor currents by approximately 20-fold. As an effect, low concentrations of PKC are as effective as much higher CaMKII concentrations. Moreover, AKAP79 also interacts with PP2B (a Ca^{++}/ CaM activated ser/thr phosphatase) thus conferring a calcium- and PP2B-mediated downregulation to GluR1 receptor currents supposed to occur during LTD (Tavalin et al., 2002).

The **MAGUKs proteins PSD-95 and SAP97** contain alternative N-termini expressing either double cysteines that normally are palmitoylated (alpha-isoforms) or an L27, for Lin-2 and Lin-7 protein, domain (beta-isoforms). Present in many family of scaffold proteins, the L27 domain is an interaction module that can function as an organization center of large protein assemblies required for establishment and maintenance of cell polarity. Alpha-isoforms of PSD-95 and SAP97 affect AMPAR-mediated synaptic strength independent of activity, while the effects of beta-isoforms are regulated by activity in a CaMKII-dependent fashion. The N-termini feature of the predominant endogenous forms of PSD-95 (alpha-isoform) and SAP97 (beta-isoform) rules their respective roles in regulating synaptic function, which means for PSD-95 that the synaptic effects of the beta-isoforms are masked by the endogenous alpha-isoform of PSD-95. This mainly confers an activity-independent influence of PSD-95 on AMPARs, whereas SAP97 mainly confers an activity-dependent influence on AMPARs (Schluter et al., 2006). In addition, spine levels of PSD-95 are tightly related to AMPARs levels through binding to stargazin (Bayer et al., 2001; Bredt and Nicoll, 2003; Kim and Sheng, 2004).

N-ethylmaleimide-sensitive factor (**NSF**) participates to membrane fusion events such as synaptic vesicle exocytosis (Rothman, 1994), moreover its specific interaction with the subunit GluR2 is required for synaptic expression of AMPARs (Shi et al., 2001). In addition, association between NSF and GluR2 appears

to play an important role for maintaining AMPARs at the synapse: the replacement of NSF by AP233 seems to initiate a clathrin-dependent internalization of AMPARs (Lee et al., 2002). All together, the data suggests that NSF-GluR2 interaction is required for the externalization and stabilization of AMPARs at the synapse (Malinow and Malenka, 2002). PICK1 (a C kinase-1 interacting protein) interacts with protein kinase C (PKC) and GluR2/3 to modulate AMPARs trafficking during bidirectional plasticity (Terashima et al., 2008). In opposition, **GRIP/ABP** (glutamate receptor interacting protein/AMPAR binding protein) stabilizes at the synapse or into the spine the AMPARs containing GluR2/3 subunits to which it is bound (Collingridge and Isaac, 2003). As seen above, stargazin is concentrated at the synapse via its association with PSD-95 and is tightly correlated with AMPARs anchoring at the postsynaptic density (Nicoll et al., 2006). Stargazin plays a critical role in the expression of AMPAR during plasticity. Additionally numerous other components of the synapse appear to participate to the regulation of LTP mechanisms. This includes small as Ras and Rap that relays the CaMKII and NMDARs signaling which promotes AMPARs trafficking. In addition, small GTPases disrupt actin function and therefore affect LTP expression (Krucker et al., 2000).

4.4.5.2. Structural plasticity activity-dependent

4.4.5.2.i. Membrane oscillations

Protrusions show spontaneous and continuous morphological change sof their shape. This occur within periods of seconds in hippocampal dissociated cultures (Fischer et al., 1998) or in slice cultures (Dunaevsky et al., 1999) (Tashiro and Yuste, 2004). Protrusions' volume is not believed to be altered by these small modifications which seem to be elicited by basal intrinsic motility. These oscillation-like movements depend on actin constant rearrangement at the periphery of spines and they are activity-independent. Such movements have also been described in vivo, although the data are slightly divergent as they concern the spine length and as they are reported over minutes (Lendvai et al., 2000) (Majewska and Sur, 2003) (Majewska et al., 2006).

4.4.5.2.ii. Size modifications

Remodeling of spine shape that implies volume changes is observed in a time scale of minutes to hours. It is thought to allow spine morphology including head and neck size to adapt to a new environment. While filopodia protrusions vary mainly in length, spines usually modify their width (Majewska et al., 2006). EM and confocal studies show a continuum in spine morphologies. The shape of a spine is defined by its maturation and is fashioned by the alterations occurring in an activity-related manner. New protrusions appear as filopodia or as thin spines with a tiny head (Knott et al., 2006) that usually grows as they become stable. The neck, initially long and thin, then becomes wide and short as maturation goes. In declining spines, head mainly shrinks before spines disappear (Holtmaat et al., 2006).

4.4.5.2.iii. Spine enlargement induced by LTP

Several studies have shown that spines tend to enlarge upon induction of LTP. The first evidence for this was provided by Fifkova shortly after the description of LTP. This was based however on a global EM analysis likely to be affected by multiple possible caveats. The first demonstration of a selective enlargement

of potentiated synapses was provided by Buchs et al. (Buchs and Muller, 1996), by an EM staining approach that revealed activated synapses in the tissue. More recently, direct evidence for spine enlargement as a result of LTP induction was made possible through the development of 2-photon imaging techniques and uncaging of glutamate (Matsuzaki, 2004). This effect has now been reproduced in several studies, although to variable extent depending upon the stimulation protocol used. The structural modifications of the spine are believed to result from intracellular Ca^{++} accumulation triggered by glutamate activation of Ca^{++} permeable channels-associated receptors, NMDARs (Matsuzaki, 2007). Ca^{++} via calmoduline activates CaMKII (Hayashi et al., 2000; Matsuzaki et al., 2004). NMDARs activity is able to induce both stargazin phosphorylation, via activation of CaMKII and PKC, and stargazin dephosphorylation, by activation of PP1 downstream of PP2B, the latter supposed to be involved in LTD (Tomita et al., 2005b). Overall, spine enlargement may be linked to two types of regulations. First it can reflect the accumulation of material at the synapse that results from the increased receptor and other associated protein trafficking. At the EM level, spine enlargement has usually been associated with an increased complexity of PSDs and namely the formation of segmented PSDs. Second, enlargement probably also reflects a reorganization of the cytoskeleton in the potentiated spine. Consistent with this, LTP is dependent upon actin polymerization. Actin reorganization is regulated by several signaling systems, including the small G-proteins of the Rho family and Rap (Okamoto et al., 2004; Kennedy et al., 2005) (Xie et al., 2005). GTPases are themselves regulated positively or negatively by guanine nucleotide exchange factors (GEF) or GTPase-activating proteins (GAP). A recent study suggests that adhesion molecules, through signaling to Rho GTPases and PAK proteins could result in spine enlargement (Xie et al., 2008). Additionally, f-actin reorganization could also control AMPARs accumulation and the molecular trafficking and anchoring that occurs within stimulated PSDs (Kim and Lisman, 1999; Krucker et al., 2000; Zhu and Malinow, 2002; Fukazawa et al., 2003; Tomita et al., 2005a).

Small spines display a weak neck conductance due to the long, thin shape of their necks, which prevents Ca^{++} from diffusing from the spine head into related dendrite, leading to a large increase in Ca^{++} concentration (Matsuzaki, 2007). In contrast to small spines, large spines display a large neck conductance through their relatively short and thick neck. Thus, the Ca^{++} entering via the NMDARs leaks into the related dendrite (Noguchi et al., 2005), which may prevent Ca^{++} concentration within the spine head from rising to levels sufficient to cause spine enlargement and LTP. LTP induces spine head enlargement merely on small spines (Matsuzaki et al., 2004), thus increasing spine neck conductance (Noguchi et al., 2005) which is consistent with the relationship between volume and neck conductance under resting conditions. Spine head volume is closely correlated with the number of functional AMPA receptors (Matsuzaki et al., 2001), whereas expression of NMDARs is also correlated but does not tend to zero, even in small spines (Matsuzaki, 2007). Nevertheless, spine increase in volume before accumulation of AMPARs is detected, indicating that distinct mechanisms might underlie early changes in morphology and receptor content (Kopec et al., 2006). Mitochondria and polyribosomes may benefit from neck conductance by entering the spine head (Ostroff et al., 2002). Subsequently, CaMKII and activity-regulated cytoskeletal protein (ARC) may be supplied by polyribosomes (Sutton and Schuman, 2005).

4.4.5.3. Protrusions turnover

Dendritic spines undergo a turnover and experience processes of continuous growth and elimination of spines (Grutzendler et al., 2002; Trachtenberg et al., 2002). Mechanisms of spinogenesis or pruning occur over hours to months during development or adulthood and represent the most consequent changes that are observed at spine level. Spinogenesis is high during early postnatal life with an overproduction of protrusions that are subsequently refined through activity (Zuo et al., 2005a; Zuo et al., 2005b). Spine density decreases in the young brain until it reaches the level that will persist throughout adulthood. In the mature brain, fine-tuning mechanisms are likely to be important for synaptic plasticity. Spine loss makes possible to remove inappropriate connections while the apparition of new protrusions contribute to the establishment of new contacts. New protrusions can be formed following LTP induction, whereas protrusions disappear following application of LTD protocols (Nagerl et al., 2004). Therefore, protrusion growth and spine elimination are the Janus feature accompanying bidirectional plasticity of excitatory transmission.

In vivo, the overall stability of protrusions augments with age but differs throughout cortical areas and experimental conditions (Xu et al., 2007). Filopodia are protrusions with the faster turnover resulting in survival of only few hours or less (Zuo et al., 2005b). Conversely, the percentage of spines which are stable feature for one month varies between 55-66% in the somatosensory cortex (Trachtenberg et al., 2002; Holtmaat et al., 2005) to 73% in the visual cortex of young animals (Grutzendler et al., 2002). In adults, a similar category of spines represent 73-93% in the somatosensory cortex (Holtmaat et al., 2005; Xu et al., 2007) and 96% in the visual cortex (Grutzendler et al., 2002). LTP promotes a selective long-term stabilization over a period of several days for the spines that have been stimulated, an effect that contrast with the increased tendency to elimination of the spines that have not underwent activation by LTP (De Roo et al., 2008c). Moreover, newly formed protrusions do not appear just anywhere, but tend to cluster around activated spines (De Roo et al., 2008c). These new spines also become functional, and while functional, tend to remain stable. Together with the evidence that LTP induction is facilitated between spines located close to each other (Harvey and Svoboda, 2007), this provides therefore a means to promote spatiotemporal clustering of synaptic signals, a property recently shown to be critical for determining the characteristics of plasticity and processing at synapses on small or remote dendrites (Sjostrom et al., 2008).

5. Thesis purposes

The central objective of my PhD work was to analyze the role of specific synaptic proteins in defining the morphological characteristics of excitatory synapses. The work summarized in the introduction indicates that synapses are dynamic structures that are continuously formed and eliminated, that they can change shape and organization as a function of development or activity and therefore that molecular mechanisms are involved in the regulation of these morphological features. To address this question, I used hippocampal organotypic slice cultures and tested different approaches to label CA1 neurons with fluorescent markers and express specific mutant or wild type synaptic proteins and then analyze their ultrastructural properties. Among the multitude of molecules expressed at the synapse, I studied two proteins member of different families and implicated in the regulation of either spine or PSD morphology or spine formation mechanisms:

-The MAGUKs-family related SAP97.

-The cadherin-family related N-Cadherin.

To examine the functions of these two molecules on determining synaptic morphologies, I have over expressed their mutant and wild type genes in pyramidal CA1 neurons using a biolistic technique. Then I have observed the effects of these two molecules on spine and synapse morphology in serial section electron microscopy.

5.1. Aims of this work

1. Confocal - EM correlations of spine types and morphologies

The first aim was to analyze the correlation of spine type occurrence observed with confocal images and serial section electron microscopy (ssEM). Confocal microscopy allows to visualize most if not all spines of a neuron, but usually not their PSDs, leaving unclear which spines are synapses. In contrast, EM analyzes show clearly the ultrastructural details of a few spine synapses, but not their distribution in terms of type or properties on a neuron. It was important therefore to use these two complementary approaches to define the general characteristics of spine synapses in organotypic cultures.

2. Characterization of the functional role of SAP97 in spine and synapse remodeling.

The second part of the work investigated the role of the MAGUK protein SAP97 in determining the morphological characteristics of PSDs and synapses. Work by Irina Nikonenko showed that overexpression of another member of this family, PSD-95, profoundly affected the structural organization of excitatory synapses. It was therefore of interest to understand whether this was also the case with SAP97 and whether there would be differences. For this I overexpressed wild type SAP97 using the biolistic system, in pyramidal neurons of the hippocampus. Pyramidal neurons were observed using confocal microscopy and ssEM. The objective was to assess a number of morphological parameters of reconstructed spine synapses of SAP97 transfected neurons and compare them to control non transfected or eGFP transfected cells. The parameters analyzed included: spine density, spine volume, spine shape, PSD area, PSD organization, number of terminals contacting one spine.

3. Characterization of the functional role of N-Cadherin in synapse and spine formation.

The last part of my work was to define the morphological changes induced by the overexpression of the wild type (WT-NCad) and the mutant form (Δ390-NCad) of N-Cadherin. N-Cadherin is an important adhesion molecule which has been shown to play a role in the mechanisms of spine morphogenesis and also to be required for the induction of synaptic plasticity. Work in the lab by Pablo Mendes further suggest that N-Cadherin could be important for the stabilization of spine synapses. We were therefore interesting to understand whether and how expression of N-cadherin at a synapse could affect its morphology. I therefore used the same approach as for SAP97 and compared the effects of overexpressing N-cadherin or a mutant form of N-cadherin that acts as a dominant negative molecule to determine how it affected spine morphology. Again I analyzed the same parameters as for SAP97.

METHODS

1. Hippocampal slice cultures

Organotypic hippocampal slice cultures are prepared from newborn 6 to 8 days old rats. As the cytoarchitecture has already taken form, cells survive explantation more readily. Cross sections of 450-500 µm in thickness are performed perpendicular to the longest side of the previously dissected hippocampus and can be incubated for weeks at interface. Following the Stoppini model, (Stoppini et al., 1991) slices are sustained at the surface of medium by a semi porous membrane allowing gas or molecular exchanges either from the top or from the bottom. The 10 days slice culturing induces shrinkage of the culture to an approximate thickness of 100-150 µm. Nevertheless the three-dimensional hippocampal cytoarchitecture is conserved to a large extend. Afferent fibers are cut during dissection and slice preparation. But, only few synaptic rearrangement occur. The lack of extra-hippocampal afferents results in vacant synaptic sites at the distal part of dendrites (Buchs et al., 1993) and in the establishment of aberrant mossy fibers collaterals (Zimmer and Gahwiler, 1984). Biochemically, lactate dehydrogenase remains at an immature stage, suggesting an incomplete shift from anaerobic to aerobic glycolysis that maybe confers additional resistance to hypoxic conditions (Schousboe et al., 1993). The aberrant development of recurrent connectivity that appears into in vitro aging cultures can promote epileptic activity; though the maturity of functional activity in in vitro organotypic hippocampal slice cultures approaches what is observed in real conditions (Muller et al., 1993b).

This in vitro model is a unique way to observe hippocampus with confocal as it is too deep for in vivo investigations. It also suits long lasting recording and observations as cultures are stable and the environment can be strictly followed. The developmental aspect also participates in the interest for this model. Indeed, intense spinogenesis occurs at the end of the first postnatal week, which matches slice preparation time. However, the critical period for neuronal network establishment and spines formation happens before dissection (Buchs et al., 1993; Muller et al., 1993a). Moreover hippocampal CA1 synapses are believed to be representative of many types of central synapses. Therefore, mechanisms described for hippocampal studies seems applicable to many other synapses in the brain.

2. Transfection/constructs

Organotypic hippocampal cultures were transfected or cotransfected at 11 DIV with the different constructs using the hand-held Gene Gun biolistic technique (DNA-coated gold microcarriers; 1.6 µm; Helios Bio-Rad Laboratories) according to the instructions of the manufacturer. Usually, one out of four transfected slice cultures (with 1-10 transfected pyramidal cells per slice) showed efficient transfection of pyramidal cells. Nevertheless transfection occurrence strongly depend of the constructs used; eGFP was easy to transfect, the NCad construct either mutant or wild type were relatively easy to transfect; SAP97 for unknown reasons has been very difficult to transfect, which means that only few cells with sufficient labeling and an acceptable quality could be kept. The transfection of other cell types is also observed, but without affecting pyramidal neurons observations. Transfected cells were used for morphological experiments 2 days after transfection. For eGFP control, slice cultures were transfected with the pc-DNA3.1-EGFP plasmid as previously described (De Roo et al., 2008) under cytomegalovirus (CMV) promotor. For N-Cadherin mutant (D390-NCad) and WT form of N-cadherin transfections, myc-tagged wild type and mutated forms of mouse N-Cadherin were kindly provided by Dr R.L. Huganir of the Johns Hopkins School of Medicine, Baltimore, Maryland, USA. SAP97-GFP transfection were performed using rat SAP97-GFP fusionned protein cloned into GW1 vector and under CMV promotor, it was kindly provided by the Bredt lab of the University of California, San-Francisco, USA.

3. Confocal Imaging

Short imaging sessions (10–15 min) of transfected slices are carried out with an Olympus Fluoview 300 system coupled to a single laser 488 nm. Laser intensity in all these experiments is kept at the minimum to avoid photo damage. Observations of morphology with confocal are performed to ensure proper fitness of transfected cells before fixation and embedding protocol. At low magnification, I looked at the intensity of the eGFP expression, because small protrusions might not be visible if the fluorescence is not sufficient, therefore biasing the observations. Then, I observed the overall aspects of the neuron. I checked the specific characters related to a pyramidal neuron like its triangular-shaped cell body, the presence of large apical dendrites and several smaller dendrites at the base. I also controlled the quality of the neuron, like the absence of bubbling or dying dendrites, which are often also almost devoid of spines.

3.1. Immunohistochemistry for confocal microscopy

To reveal PSD-95 and nNOS molecules, a proteolytic treatment known to unmask hidden antigenic epitopes (modified from Burette et al., 2002) has been applied. Immunostaining was performed using the following antibodies: 1:8,000 primary anti-nNOS rabbit polyclonal antibody (Millipore) applied overnight at 4° C followed by 1:1,000 secondary anti-rabbit antibodies (Alexa Fluor 555; Invitrogen) applied for 2 h at room temperature and 1:20,000 primary anti-PSD-95 mouse monoclonal antibody (Thermo Fisher Scientific) applied overnight at 4° C followed by 1:1,000 secondary anti-mouse antibodies (Alexa Fluor 647; Invitrogen) applied for 2 h at room temperature. The images were taken with a confocal microscope (LSM 510; Ar laser

458/488/514 nm, 25 mW; HeNe 543 nm, 1mW; HeNe 633 nm, 5 mW; Carl Zeiss, Inc.) using a Plan-Apochromat 63× NA 1.4 oil objective.

4. Tissue preparation for EM

4.1. Immunocytochemistry

Slice cultures were fixed and processed (pre-embedding immunoperoxidase protocol) as described (Nikonenko et al., 2008). The slice cultures were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, cryoprotected, freeze thawed in liquid nitrogen, and incubated overnight in 1:500 primary rabbit anti-GFP antibody (Millipore) at 4° C and then in 1:200 biotinylated secondary goat

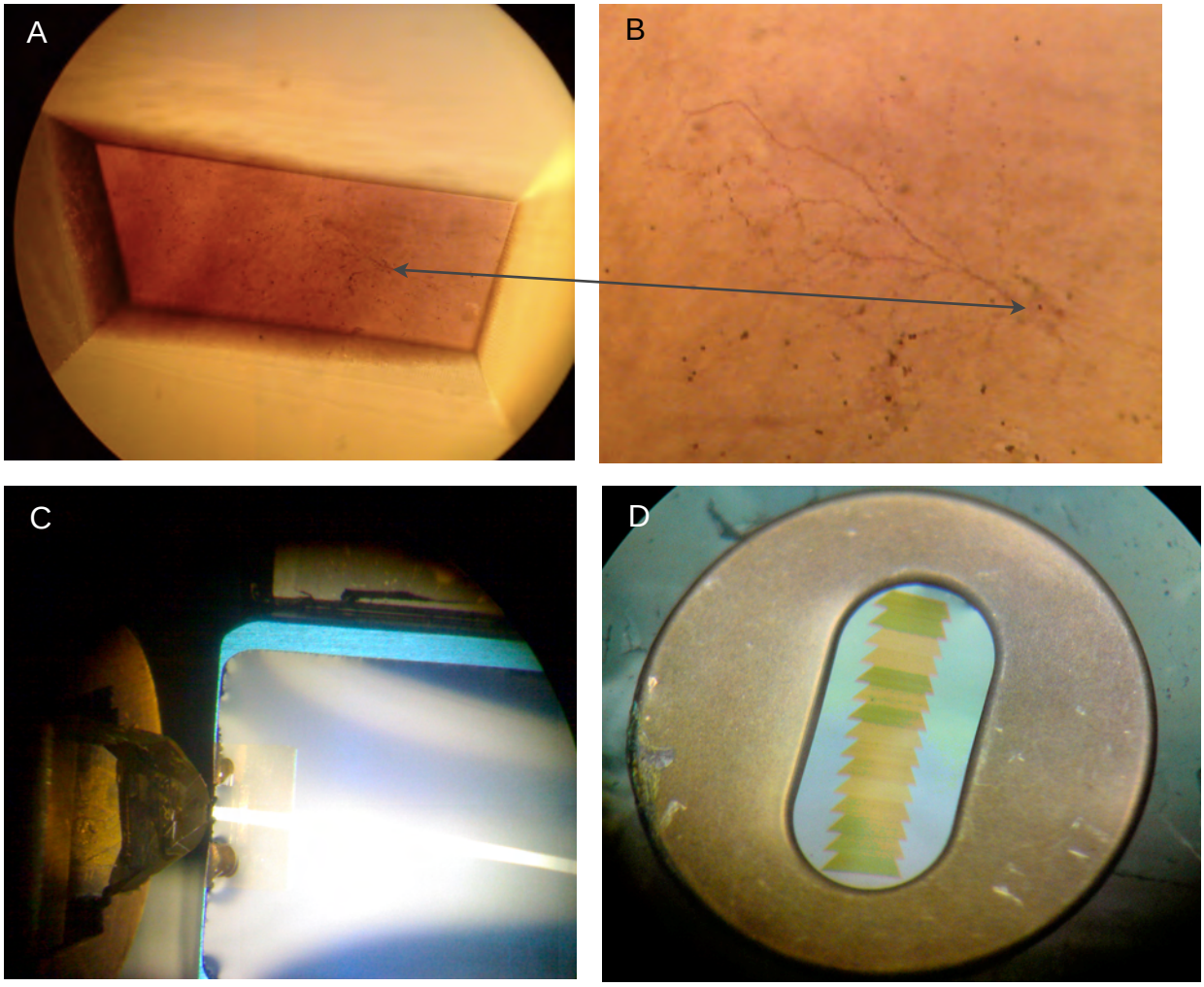


Fig.33 Details of EPON block ultra-cutting

A: A neuron embedded in EPON block. The neuron is visible thanks to DAB staining. The block is cut in a pyramid-shaped way for navigation reasons.
B: Zoom in on the neuron shown in A. Gold bids used for transfection are visible as black dots.
C: Top view of the ultra-microtome, with diamond knife on the right closing the blue recipient containing water. The block, left, making round circles is touching the diamond knife. Floating on the water, the slices are weakly stuck to each other forming a gold-colored tape.
D: A copper grid with formvar film on which are deposed a piece of slice tape. The colors of the slices vary in function of their thickness, from blue to light gold. (green is a reflection artifact)

anti-rabbit antibody (Jackson ImmunoResearch Laboratories) and avidin biotin peroxidase complex (Vector Laboratories) followed by 3,3'-diamino-benzidine tetrachloride and 0.015% hydrogen peroxide. The slice cultures are postfixed in a fresh solution of 1% OsO_4 for 45 mn and then contrasted in 1% uranil acetate for 10 mn. After a double 5 min rinse in distilled water, the samples are dehydrated in ethanol and propylene oxide and are embedded in Epon (Fluka, Buchs, Switzerland).

4.2 Ultrathin cutting

After embedding in EPON resin (Fluka), the cultures are stuck on the top of EPON blocks (fig.33). They then undergo glass knives trimming around labeled cells of interest with a Reichert Ultracut S ultramicrotome (Leica). Subsequently, trimmed blocks are cut with diamond in 65-nm serial ultrathin sections (Reichert Ultracut S Leica). Cutting is followed by plomb and uranyl acetate contrasting, respectively 15 mn for both.

5. Electron Microscopy

Images of labeled dendrites from the middle portion of CA1 stratum radiatum are taken at a magnification of 9,700-15,000 using transmission electron microscope (Tecnai 20; FEI Company) equipped with digital cameras (Mega View III; Soft Imaging Systems).

5.3. Tiles aligning

Aligning is a time consuming step that aims to position and distort images to re-create a three dimensional volume from individual juxtaposed micrographs taken from serial section electron microscopy. To reach this goal, Photoshop software (Adobe) is commonly used. To improve the comfort and the precision of the alignment process, we wrote a new aligning software called Mosaic Builder (Spaltenstein Natural Image). Helped by a simple interface and a relative surface area reference scale that instantly shows the change in size occurring during the matching of two images, the user can easily navigate in the operating field. Four anchor points located at the corners of each image are used to direct proper alignment. The images are then distorted in real time using a perspective transform based on these anchor points. The use of a perspective transform that can be applied in real time by modern graphics hardware eliminates the need to store both original images as well as distorted images, thus allowing more flexibility in file management as well as reducing storage needs. The multi colored layer interface gives salient labeling to each layer, facilitating high specificity of alignment. This software was written by Joel Spaltenstein of Spaltenstein Natural Image in collaboration with Lorenzo Poglia for the Electron Microscopy orientated interface and logo creation. Mosaic builder was written for Apple Mac OSX using the Cocoa programming interfaces.

5.4. 2D Counting (ssEM images)

Part of the counting of spine types has been done thanks to an Osirix plug-in made by Joël Spaltenstein that supports ROIs and dissociated counting (each parameter was sorted in a different counter). Osirix is a Mac OSX software created at the Hôpital Universitaire de Genève by Antoine Rosset, and originally used for medical image analysis. For the spine and PSD occurrence analysis, the counting was done by scrolling through serial EM images. Spine types were defined and sorted following the parameters exposed in the introduction:

- **Filopodium**, typically an elongated protrusion devoid of PSD.
- **Stubby spine**, a small protrusion without a salient spine neck or head.
- **Mushroom spine**, a protrusion with a head and making a synapse with a presynaptic partner.
- **Spine-like filopodia** which can be confused with a spine in confocal microscopy. However, the EM shows that it is devoid of PSD.

See below for 3D counting (Reconstruct)

5.5. 3D Reconstruction

Digital serial electron micrographs were aligned using Mosaic Builder. Surface, volume, and length measurements as well as 3D illustrations were performed using the software Reconstruct developed by J.C. Fiala and K.M. Harris (SynapseWeb: http://synapses.clm.utexas.edu/tools/index.stm; Fiala, 2005).

5.6. Analyzed parameters

Synapses are defined by the presence of a PSD facing at least two to three presynaptic vesicles. Complex or perforated PSDs are defined by the presence of a discontinuity on a single section. Terminals were identified by the presence of an enlargement of the axonal shaft containing vesicles and facing at least one PSD. Statistical analysis are done from data (length, surface, volume, type of object) collected using Reconstruct sofware (see above).

-Lengths were measured using the tracing and calculating tool of Reconstruct.

-Spine surfaces were measured using Reconstruct that calculated the surface from the spine manually traced outlines .

-Spine volumes were also measured using Reconstruct which calculated the volume from the same spine manually traced outlines.

-The PSD surfaces were not measured directly from a single trace, but from two traces that helped to diminish the errors as sketched in the figure 34:

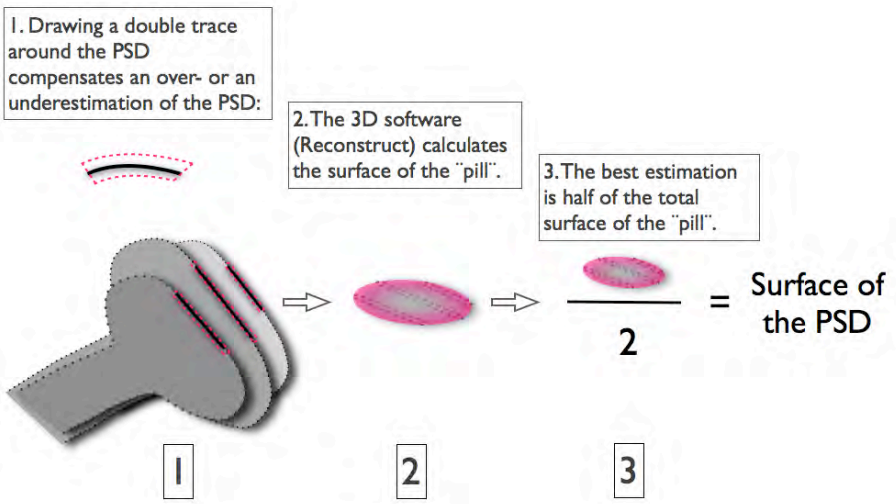


Figure 34 PSDs measurement

-The object type sorting was done manually. I have checked all the spines, PSDs and axons one by one using either the 3D reconstructions or the aligned serial section EM images in a dialectic manner.

Statistics were performed using Prism 5.0a for Mac OSX (GraphPad Software, Inc). Classification of spines and PSDs follow introduction characterization paragraph.

6. Technical developments

An important technical aspect of my PhD work has been the collaboration with J. Spaltenstein for the development of 3D-associated reconstruction software. Indeed, serial section electron microscopy (ssEM) images are taken in series, therefore they need to be aligned for 3D reconstructions. In preamble of my results, I will describe the steps that I have taken until I found the best manner to transfect CA1 neurons, and subsequently the more efficient way to reconstruct ssEM images in 3 dimensions.

6.1. Electroporation

As a first approach, I have tested the electroporation method to stain cells. Electroporation is a physico-chemical way to introduce molecules through the cell membrane. The idea is based on the relatively labile nature of the phospholipid bilayer's hydrophobic/hydrophilic interactions, and its ability to spontaneously reassemble after disturbance. Pores in the cell membrane are temporarily formed by quick voltage shocks, thus allowing polar molecules to pass through before the membrane get resealed. To assure the molecular flow through the membrane, a positive pressure is also applied to the pipette. Parameters have to be properly set to get the best setting allowing the penetration of the material in the pipette without altering the neuron integrity.

The goal of the project was to fill neurons of organotypic hippocampal slice cultures with a dye, and subsequently with plasmids. Many electrical simulation burst protocols have been tested, as well at different sizes of pipettes with different resistances and therefore the load of electrical driving force applied to the neuron. Tests were made with sulforhodamin and Alexa (488nm) fluorescent dyes. Stimulation parameters were set to: two hundred 1ms-long square pulses with an interpulse delay of 4ms and an amplitude of 10V were delivered to each neuron. Pipettes containing dyes or the plasmids were made such as to be set between 2 and 5 M. Voltage applied varied between 5 and 10 volts, nevertheless 10 volts were the more convenient. 6-7 mbar were applied to the micropipette. I could reproducibly label pyramidal neurons to visualize the entire dendritic tree and morphological details such as spines (would be good to have an image). Several problems were however encountered: pipettes had a tendency to clog depending upon the dye concentration. Also the fluorescence tended to dim with time making long-term analyses more difficult. As a second step, we tried to use the same protocol to then introduce plasmids into CA1 neurons that would allow to render cells fluorescent for several days. For these experiments, I used a pcDNA vector with a concentration of 1 to 2.3 % of plasmid expressing the fluorescent protein eGFP. These experiments were however not successful. In more than 20 trials with each time around 5 neurons electropored, we have only observed a few times very weak fluorescent neurons. However, fluorescence dim quickly, and was not observed on subsequent days. Results were negative although we could confirm that electroporation had been successful by adding a fluorescent dye in the pipette at the moment of current stimulation. Possible reasons for the failure to transfect cells with this technique are multiple: i) the electroporation protocol used might have damaged cells that did not survive on subsequent days; ii) the level of gene expression is too low to efficiently render cells fluorescent; iii) transfection is not efficient because the plasmid is introduced in the

cytoplasm and not in the nucleus of the cell. Whatever the explanation, the low efficacy of transfection obtained with this approach made its use inadequate for my experiments.

6.2. Lentivirus

As a second approach, we used lentiviruses to transfect pyramidal neurons in slice cultures. Lentiviruses are members of the Retroviridae. The common taxonomic denominator besides from structure and composition is their replicative strategy which includes reverse transcription of the virion genomic RNA into double stranded DNA and its subsequent integration into host genome. The 3 major common features shared by retroviruses are their coding domains which contain the information for the following virion proteins:

-**gag**, which directs the synthesis of internal virion proteins that form the matrix, the capsid, and the nucleoprotein structures.

-**pol**, which contains the information for the reverse transcriptase, integrase and protease enzymes.

-**env**, from which the surface and transmembrane components of the viral envelope protein are derived.

Due to extensive deletions made on different viral sequences (fig.34), lentiviral vectors are unable to replicate once integrated within host genome. However, one of their major advantages over other members of the retroviral family is their ability to infect non-dividing cells such as neurons, hematopoietic stem cells or hepatocytes.

For these experiments, I used the lentiviral construction FUGW containing an eGFP sequence under the control of strong ubiquitous promoter (Gift from Dr. Marc-Olivier Sauvain from the Prof. D. Trono Lab, university of Geneva). One critical parameter for successful transduction is the titer of lentiviral particles. The batch used had an MOI of $2x10^7$ TU/ml. The major problems that arose during our experiments were on one hand the level of expression that was not regular and of low intensity, and on the other hand the relatively large number of transfected neurons. We performed infections in a way that the lentiviral vectors were leaking out of the pipette freely diffusing in the slice culture in the figure 35b (A). The precise level of the site of transfection (z-axis) was difficult to evaluate and to reproduce. Thus, neurons that were transfected were surrounding the point of pipette contact, creating a group of eGFP expressing neurons. This high concentration of eGFP positive cells in a small region was a major problem for imaging spines and for the follow-up of spine behavior, due to the increased background level of fluorescence, figure 35b (B). Furthermore, it was quite difficult to relocate a dendritic segment on the following observation time points as the tangled neuron arborizations were forming a dense maze, figure 35b (C). For all these different reasons we decided to stop this project.

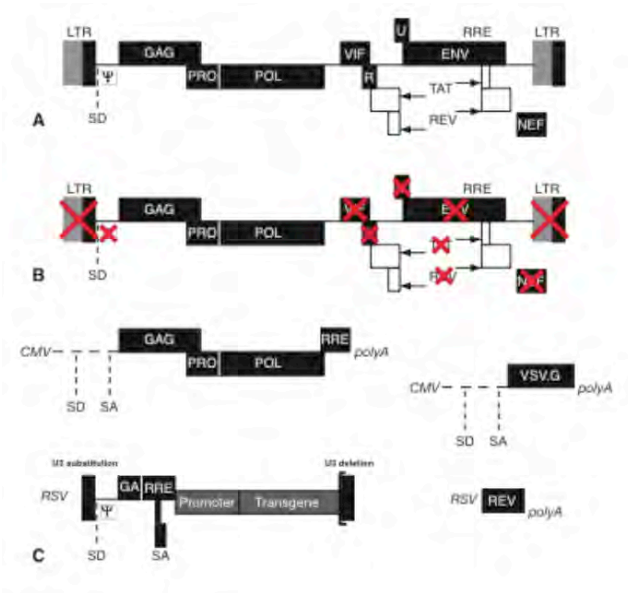


Fig.35a From HIV-1 virus to lentiviral vector

A: Schematic drawing of the wild type HIV provirus.
B: The different deletions leading to the production of the third generation HIV1-derived packaging construct.
C: The 4-plasmid system required to produce the third-generation lentiviral vector: the split gag-pol/rev packaging constructs, the heterologous envelope (in this case VSV-G) plasmid and the SIN transfer vector.
Adapted from Lentiviral Vectors, Ed. D. Trono, 2002, Springer-Verlag.

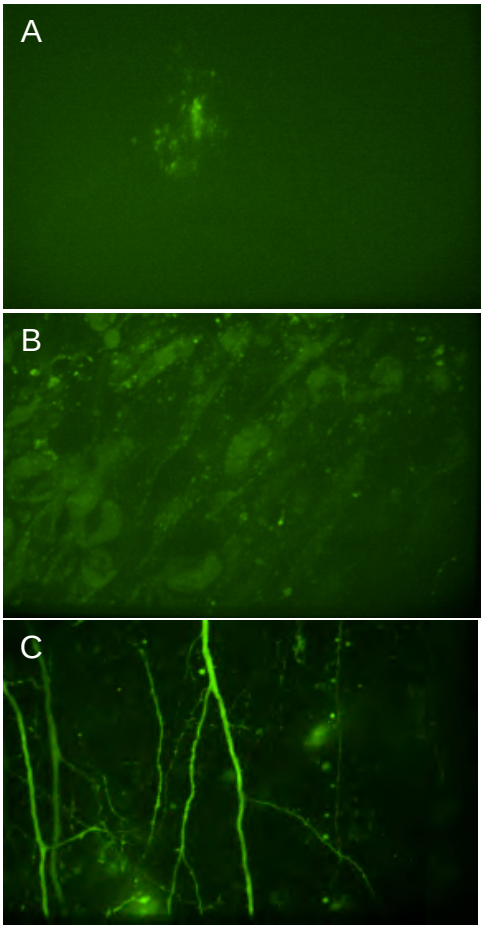


Fig.35b Expression of eGFP via lentiviral vectors in hippocampal slice cultures

A: Spot of neurons expressing eGFP via lentiviral vectors. Expression is higher around site of infection by the micropipette.
B: Same neurons showing a weak eGFP expression.
C: Dendritic segments from neurons expressing eGFP via lentiviral vectors.

6.3. Softwares developed for Electron Microscopy (EM) analysis

New electron microscopists take a long haul apprenticeship before to become experts. Critical points are:

- The tissue embedding. Immunocytochemistry, from the immuno to the DAB staining need to be finely tuned as too much of staining blur cell ultrastructure, and weak staining will reduce the chance to find the cell during ultra-cutting process.
- Ultra-cutting requests an excellent dexterity (tissue and the grids bearing them can easily be damaged), patience, calm and a sense of 3D representation.
- SsEM observations and image shooting request 3D visualization abilities to take account of the entire object.
- Alignment has to be accurate to original dimensions despite the deformations that are applied to the images.
- 3D Reconstructions have to match with the original neuron. In addition, parameters of analysis need to be clearly set before getting started with analysis. Indeed, image interpretation is difficult when considering small elements such as PSDs.

Altogether, electron microscopy requests a rigorous spirit as each step has a dramatic consequence on the ultimate result, furthermore it is a time and energy consuming process that finally leads to 3D reconstruction.

6.4. OsiriX

A particular part of this process was the EM data analysis. In collaboration with Joël Spaltenstein, that coded the plug-in for the OsiriX Software used for confocal images analysis (De Roo et al., 2008a), I initiated the project of creation of an OsiriX plug-in adapted to EM image analysis (fig.37). This was done in close collaboration with Joël Spaltenstein who wrote the program. This plug-in allowed attribution of regions of interest (ROIs) on images. This was very helpful to measure lengths, but also to count synapse types. By navigating up or down the 2D serial section EM images, I could check every synapse entirely. The plug-in was implementing a database which was subsequently easily exported for statistics. OsiriX, a Mac OS related software, was well adapted to be customized to my special needs. Here under a snapshot of an OsiriX window: the red dots indicate the ROI's pins and the attributes of the analyzed spine.

Another time-consuming and important point occurring before the analysis consists in the alignment of EM images. At the time of argentic micrographs, the images were scanned one by one and then aligned with difficulty. Digital images have helped a lot and have offered the possibility to analyze bigger surfaces and volumes. Nevertheless, the problem of distorted EM images was still a major problem. Photoshop (Adobe) was the commonly used EM aligning software in our lab until I decided to optimize the process (fig. 36). Photoshop is not an alignment software, it is heavy, unadapted and not easy to use:

- Photoshop requires a huge computer resource as it takes into account every single pixel of a loaded image, and that even if the image is out of the scene.
- The blending tools are very slow as Photoshop uses its own code to compute the image modifications.
- Raw EM images display a panel of grayscale. The superposition of gray images containing a maze-like pattern generates a confusing display.

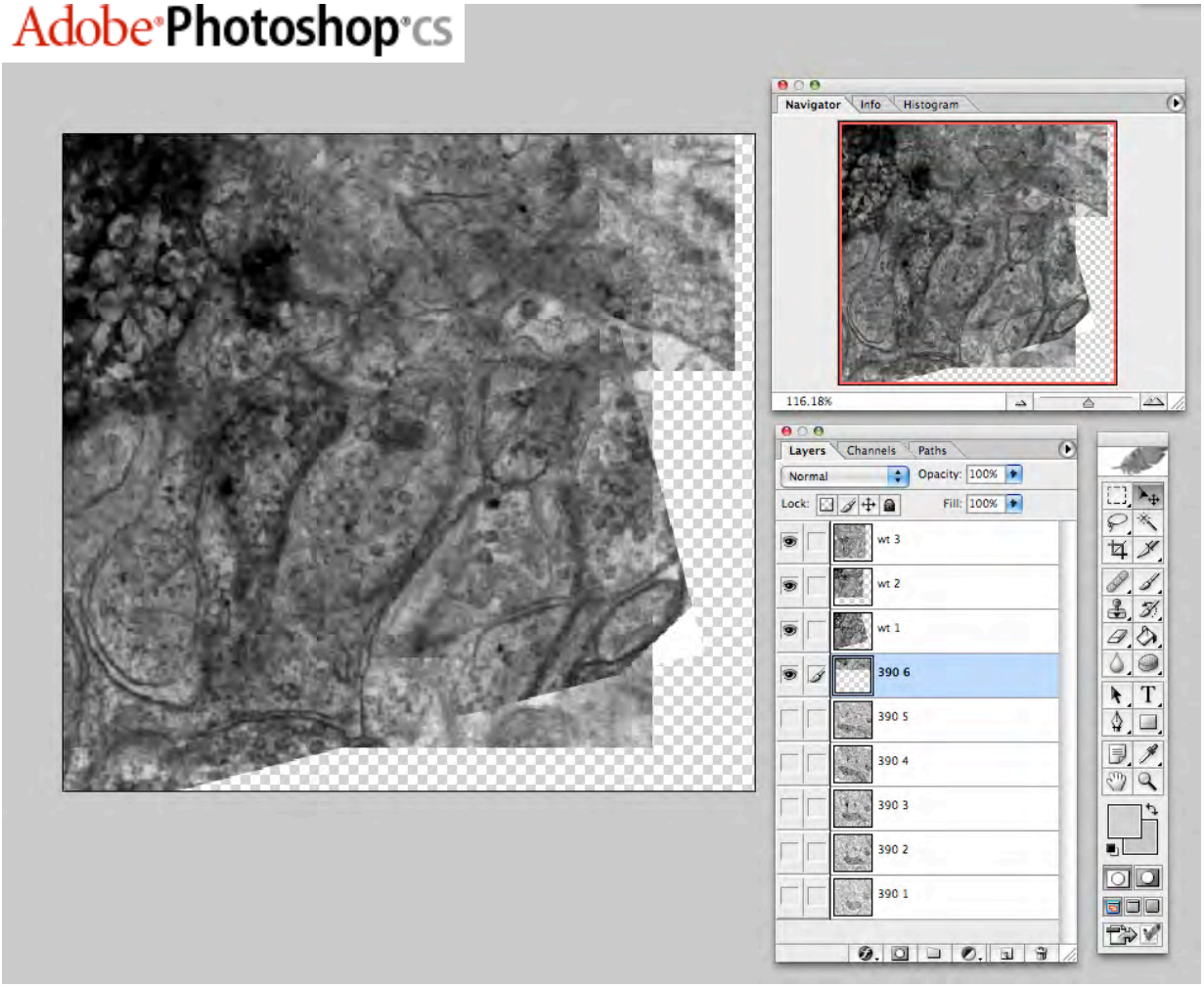


Fig.36 Adobe Photoshop ssEM images window layout

Superposition of layers in a Photoshop window snapshot. Navigation, handling and speed of execution are not comfortable.

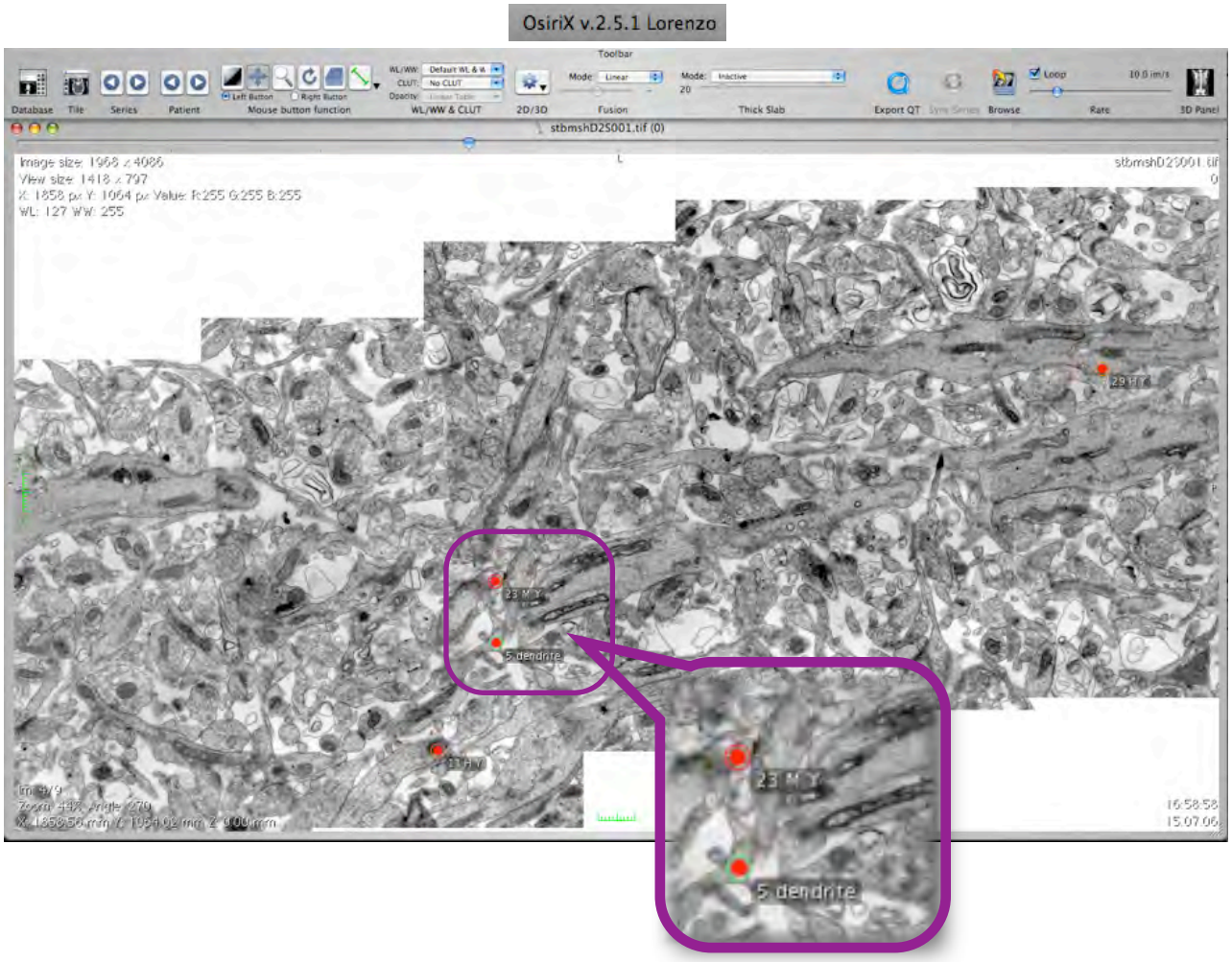


Fig.37 OsiriX ssEM images alignment working window

OsiriX window (Lorenzo's plug-in version). This working window shows an aligned level of ssEM images on which red dots represent counting attributes with the number on the left and the quality attribute on the right.

As Joël Spaltenstein (a Mac OS coding specialist) and I were already adapting OsiriX for EM analysis, we tried to modify and implement OsiriX for image alignment. The Mac OS X is an intuitive operating system that offers new possibilities and new solutions for image-related softwares. Among other interface solutions, we added artificial and contrasted colors to layers. This trick greatly facilitated the visual discrimination between layers, meanwhile enhancing layers superposition precision. This brought a faster handling and an enhanced comfort in comparison with Photoshop alignment (fig.36/38).

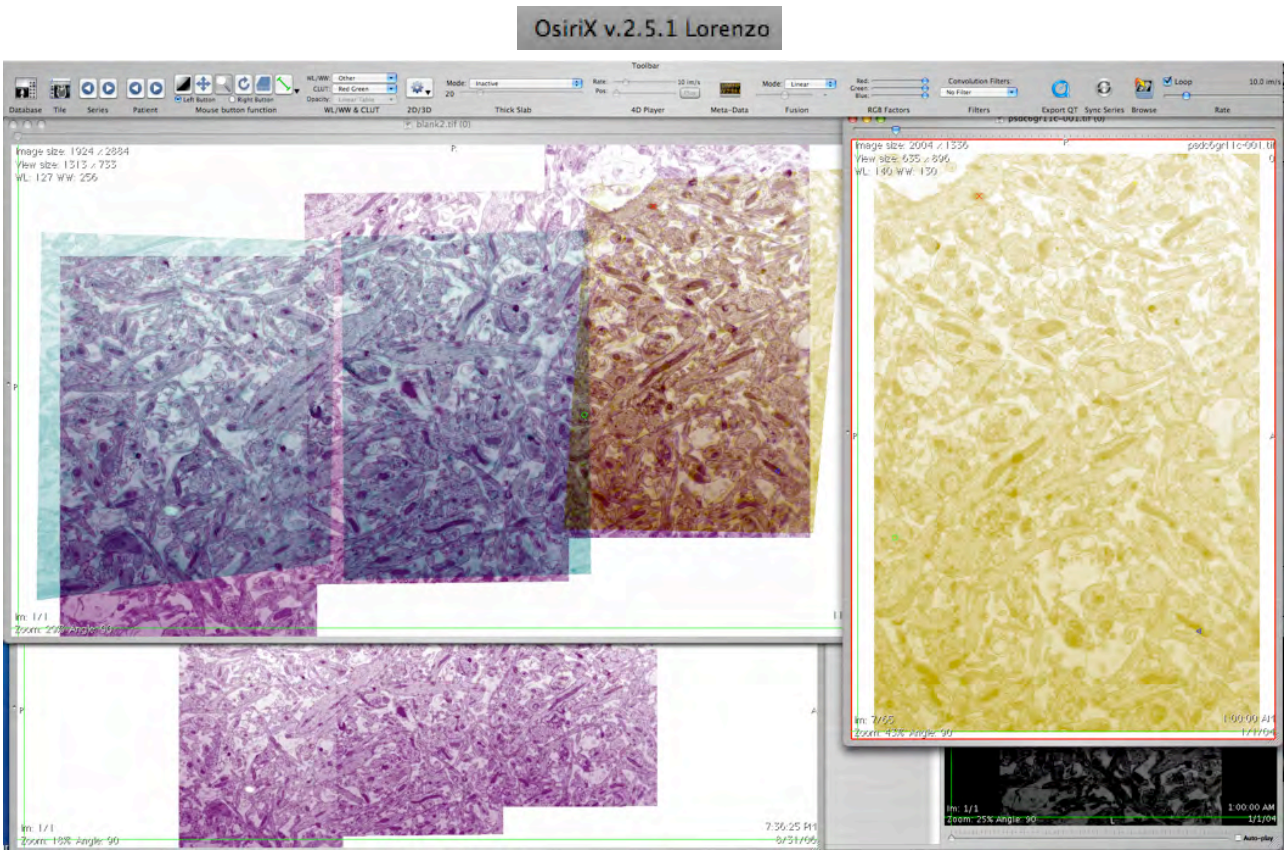


Fig.38 OsiriX ssEM images window layout

An OsiriX trial for image alignment. Layers of different levels are contrasted with colors (this interface solution was kept for Mosaic Builder). Nevertheless, OsiriX was still too slow and its architecture too constraining for the solutions we wanted to implement.

Despite its interest as software, OsiriX was written for medical image analysis and the coding architecture was already established. Therefore it was difficult to introduce dramatic coding changes like the one we thought would be useful for efficient EM alignment software. However, we made a project tryout in which we attempted to introduce several solutions we had imagined. The results were not satisfying, execution was too slow and the interface solutions were too difficult to insert in the OsiriX coding architecture.

Consequently, Joël Spaltenstein and myself were persuaded to generate a new program from the beginning with the objective to optimize EM alignment efficiency. Following my requests and interface suggestions as electron microscopist, J. Spaltenstein wrote a new software that we called Mosaic Builder.

6.5. Mosaic Builder (MB)

6.5.1. Why Mosaic Builder?

The name Mosaic Builder comes from the process of alignment. The ¨Mosaic¨ comes from the idea of ancient mosaics built by Romans, where colored pieces of hard material were assembled to form big pictures. A digital 3D reconstruction is made out of series of EM images. Each slice of the tissue provides a series of EM images. Therefore, to reconstruct the big picture, images are aligned, within a level, and then throughout the levels. As images suffer optic deformation, even the adjacent images coming from the same serial section level do not match with each other. Therefore, each image is distorted to fit with the preceding one.

The reason to start a new project was the possibility to choose each brick of the coding architecture, meanwhile offering a light coded software.

6.5.2. Coding

Mosaic Builder (MB) was written in Objective-C using Cocoa frameworks, an object-orientated language that allows us to take advantage of all native Mac OS X graphic handling capabilities, thus making the image alignment optimized for a low GraphicRAM consumption. Indeed, although giga-bytes of images can be loaded, only the image that appears on the screen requires Graphic RAM, allowing the user to blend and manipulate images in real-time.

6.5.3. Principle of interface

MB is an intuitive interface software. Most of the operations can be done directly on the images (see fig.39-40). Images are imported in a displayed database. Selected images belonging to this database are eligible for contrast and light adjustments or autolevel. The images are then drag-and-dropped on the working window. The right hand-side working window panel or navigation window contains the layer list. Every line is flanked by check-box or logical-box related to a special attribute: color, visibility, opacity. Images on the working window can be activated by a simple click. The interface design is intuition orientated, the user just manipulates images without having to go in the headbox to choose a specific tool. Activated image displays a double ¨tool¨ frame. The external blue frame has anchored points that allow to distort the image. Depending on keypad push and grasped point selection, the effect is either an homothetic image distortion or an simple image distortion. The inner red circle is a steering wheel with four grasping points. It allows to turn the image around its center point. Image motion is performed by clicking any point of the image. As transformation induces surface changes, an error scale bar on the left of the activated image indicates the

percentage of surface modification. This very useful read-out allows user to chose an acceptable error margin. The block of images can also be moved, moreover allowing an easy zoom-in and zoom-out navigation. One of the most important thing of MB is the export concept. Photoshop exports images without preserving the localization of the image into the aligned work. The trick in Photoshop to keep localization needs the freezing of each level into a mixed level image/grey-backgroud block. This process induces the addition of useless data to files. In contrast, MB considers a virtual origin around which images are mapped. This dramatically diminishes the size of files. Moreover, exporting process with MB is just a one click-process. This virtual mapping opened future development possibilities, especially for automatic alignment process.

6.5.4. Discussion

The final result was achieved after a number of trials and discussions about the best way to get the simplest interface and a smart coding architecture. The result improved the alignment speed process compared to Photoshop, while also enhancing the comfort for the users. Speed and comfort are central points as aligning images takes weeks of harsh work.

6.5.5. Future developments

The basic architecture of MB, by its simplicity, allows to implement the interface with solutions derived from the commonly used MAC OS X related softwares. Moreover, MB can easily be customized for specific user requests.

Development of Mosaic Builder for EM analysis, automatization and objects counting is currently being pursued at the Scientific Computing and Imaging Institute, University of Utah. (A computational Framework for Ultrastructural Mapping of Neuronal Circuitry, James R. Anderson et al., PLoS Biology 2009).

Altogether, this project was a great opportunity to enter the field of digital imagery. The interaction with a person coming from a different field, and working on such a specialized topic was an exciting challenge. I have been lead to new ways of thinking. Indeed, my needs had to fit the possibility offered by the informatics. Interesting discussions have arose from this interaction and allowed me to learn informatics specificities and requirements for image analysis.

The tile name window contains all the images imported to MB. Images can be adjusted thanks to the contrasting and brightness tools, or auto-level. Images are then drag-and-dropped in the working window.

The navigation window allows to select the level and the image. Image attributes like visibility, color or opacity are also selected thanks to this window.

The working window contains tile levels. The alignment process is performed in this window. See next figure for details.

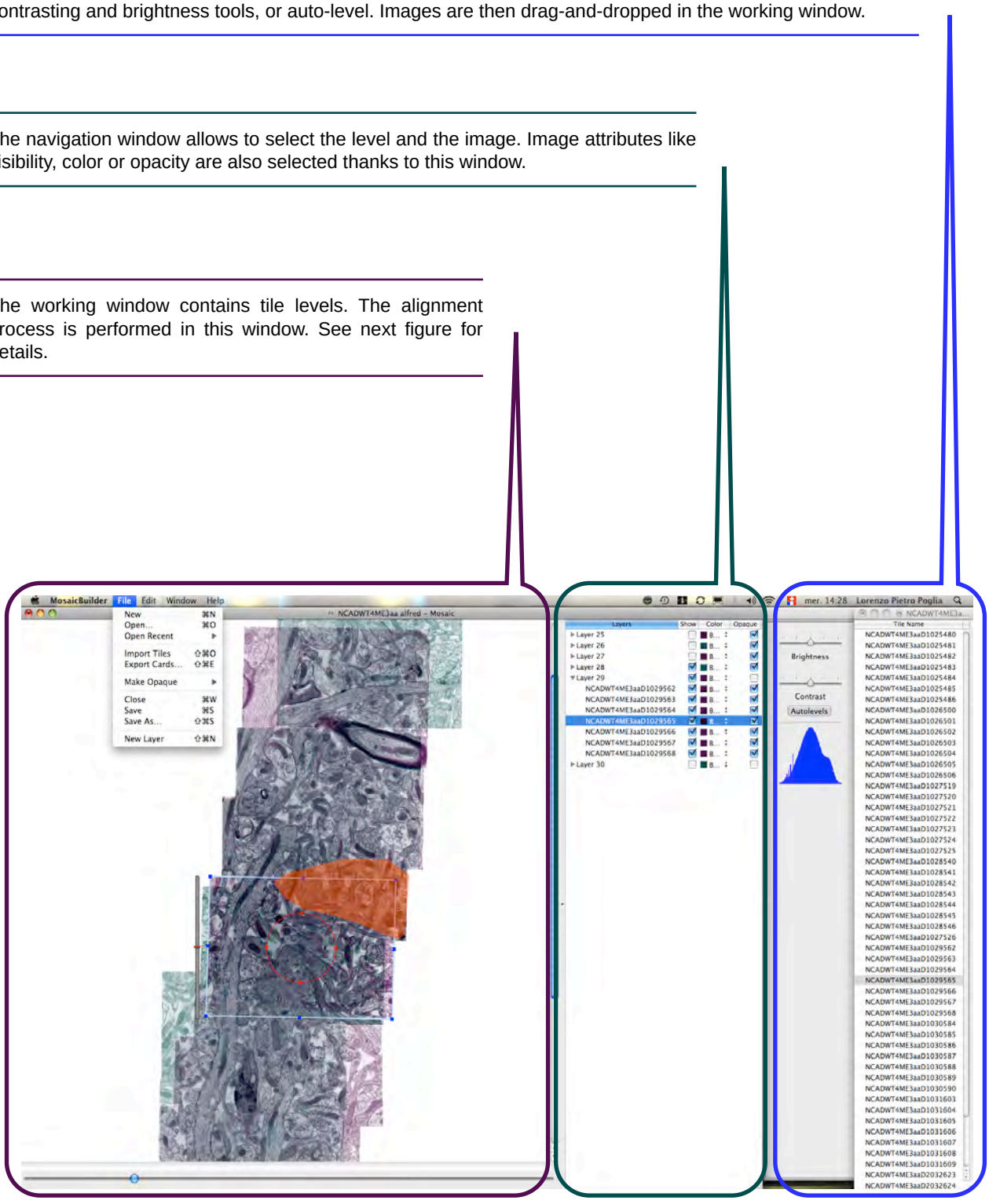
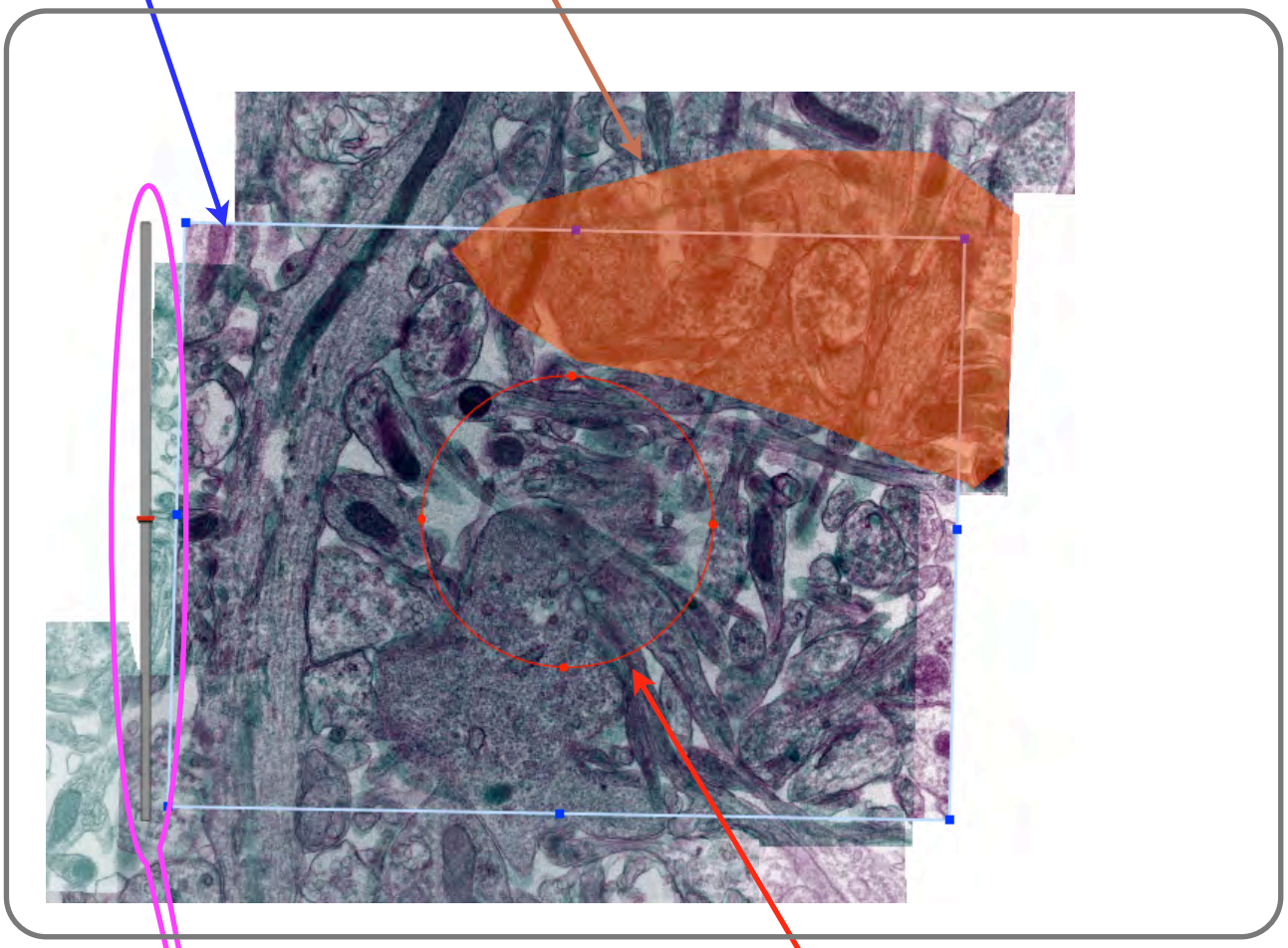


Fig.39 Tiles juxtaposed during alignment process. Tiles of the same level share the same colour. Activated frame is visible in the middle of the working window

The blue squared frame contains four anchor points at the edges that are used to distort the image, and four side points that are used for lateral stretching.

An arbitrary selected image zone can be removed. The zone is selected by simple mouse drawing around the zone to be cut. This tool allows to diminish wobbled overlapping.



The red circle frame contains four grasping points that are used to turn the image around its center point.

The error bar scale allows to constantly check the distortion effect on image size

Fig.40 MB alignment tools description. Zoom in on a multi layers ssEM alignment working window

RESULTS

1. Control eGFP CA1 pyramidal neurons

The transfection method that has been finally used is the hand-held Gene Gun biolistic technique. A convenient amount of neurons express transfected genes, therefore allowing individual observations. In figure 41A is represented a whole slice culture observed in light microscopy and confocal microscopy at 4x. Analysis of spine density and spine types were performed on higher magnification images of dendritic segments taken with a 40x objective such as shown on figure 41B and C(40x + 10x digital zoom are requested for dendritic spines observations). Similar observations have been performed on neurons, subsequently prepared by immunohistochemistry for EM.

2. Confocal - EM correlations of spine types and morphologies

Spine synapses in hippocampal organotypic slice cultures are extremely dynamic, with many of them (up to 20%) being formed new or eliminated every day. It was therefore important to determine under these conditions how spine types and morphologies observed at the confocal level corresponded to EM data. For this, dendritic segments from 3 organotypic hippocampal slice cultures of 22 DIV were analyzed from serial sections and compared to analyses made in eGFP transfected pyramidal neurons using confocal imaging. To more precisely define the characteristics of the protrusion population, the protrusions were classified into 3 main categories: filopodia, defined as long protrusions without head or widening at the tip, stubby spines, defined as protrusions without a neck, and mushroom spines, protrusions with a neck and a widening at the tip (fig.42A). Analysis led to the following results with confocal microscopy observations carried out on 720 protrusions from 10 CA1 pyramidal neurons in 11 DIV cultures, the proportion of the protrusions was as follows: filopodia (8 ± 2%); stubbies (19 ± 4%); mushrooms (72 ± 4%). At 25 DIV: filopodia (4 ± 1%); stubbies (20 ± 3%); mushrooms (77 ± 3%) (fig. 42B). Thus, the main difference between 11 DIV and 22 DIV is a diminution of filopodia that can be interpreted as a difference in developmental stage where young slice cultures exhibit more filopodia. At EM level, criteria for protrusions classification are similar to confocal but take PSDs into account; PSDs are observed as thin black densities on the postsynaptic protrusion tips, facing active zones. Therefore, protrusions without PSDs were considered as filopodia (fig. 42A,right)(see further for PSD type occurrence). The proportions of protrusions were the following in the 22 DIV cultures: n=3 cultures; 99 protrusions analyzed; filopodia (12%); stubby (18%); and mushroom spines (70%)(n=3 cultures; 99 protrusions analyzed). As illustrated in figure 42B, the ratios so obtained nicely correlated with the data obtained by confocal microscopy.

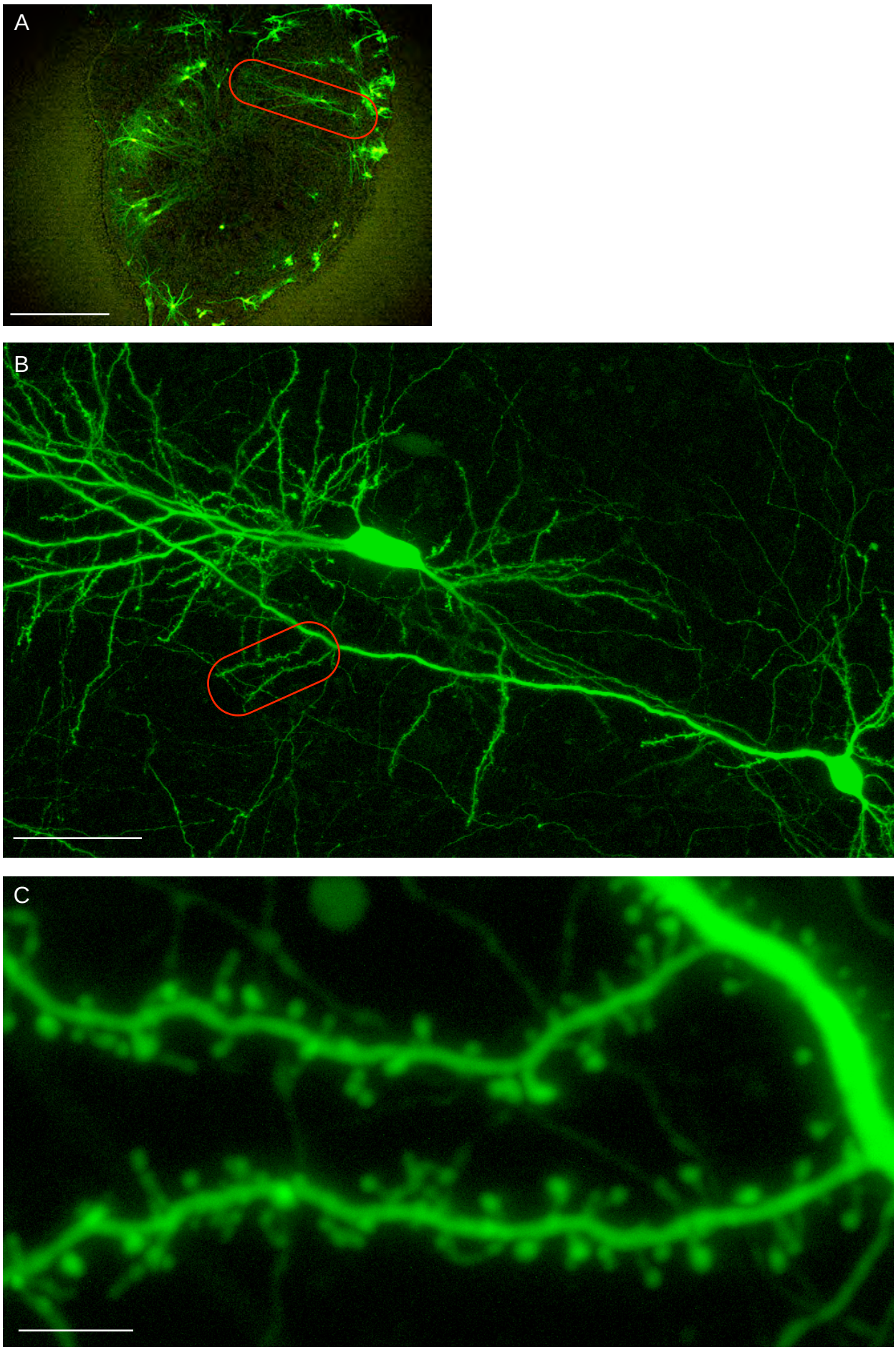

A
B
C

Fig.41 CA1 pyramidal neurons and dendrites observed with confocal microscopy

Organotypic hippocampal slice culture transfected with the biolistic system.
A: Slice culture observed with both transmitted light and confocal microscopy (4x). Red cartridge comprises neurons shown in B.
Scale bar: 100 µm
B: Same slice culture as A, observed with confocal microscopy (40x). CA1 pyramidal neurons were transfected with the biolistic technique. This system allows to transfect few neurons that can be observed individually. Cell-body and dendrites are devoid of bubbling.
Scale bar: 50 µm
C: Dendrite observed with confocal microscopy (red cartridge from (B), 40x+10x digital zoom). Dendritic spines show a regular eGFP labeling. Dendritic spines have normal shapes and a density of circa one spine per µm.
Scale bar: 5 µm

Continuing the characterization of the dendritic spine population, I focused my work on the PSD occurrence in the dendritic protrusion population. This EM analysis also supported the work of the colleagues on the presence of PSD on dendritic spines reported by the staining of PSD-95--DsRed2 transfection showing that 88% of the 109 mushroom spines analyzed exhibited a clear PSD staining, whereas the 7 filopodia exhibited none of the staining (pyramidal neurons from the CA1 region of organotypic hippocampal slice culture; 11DIV) see (fig.43).

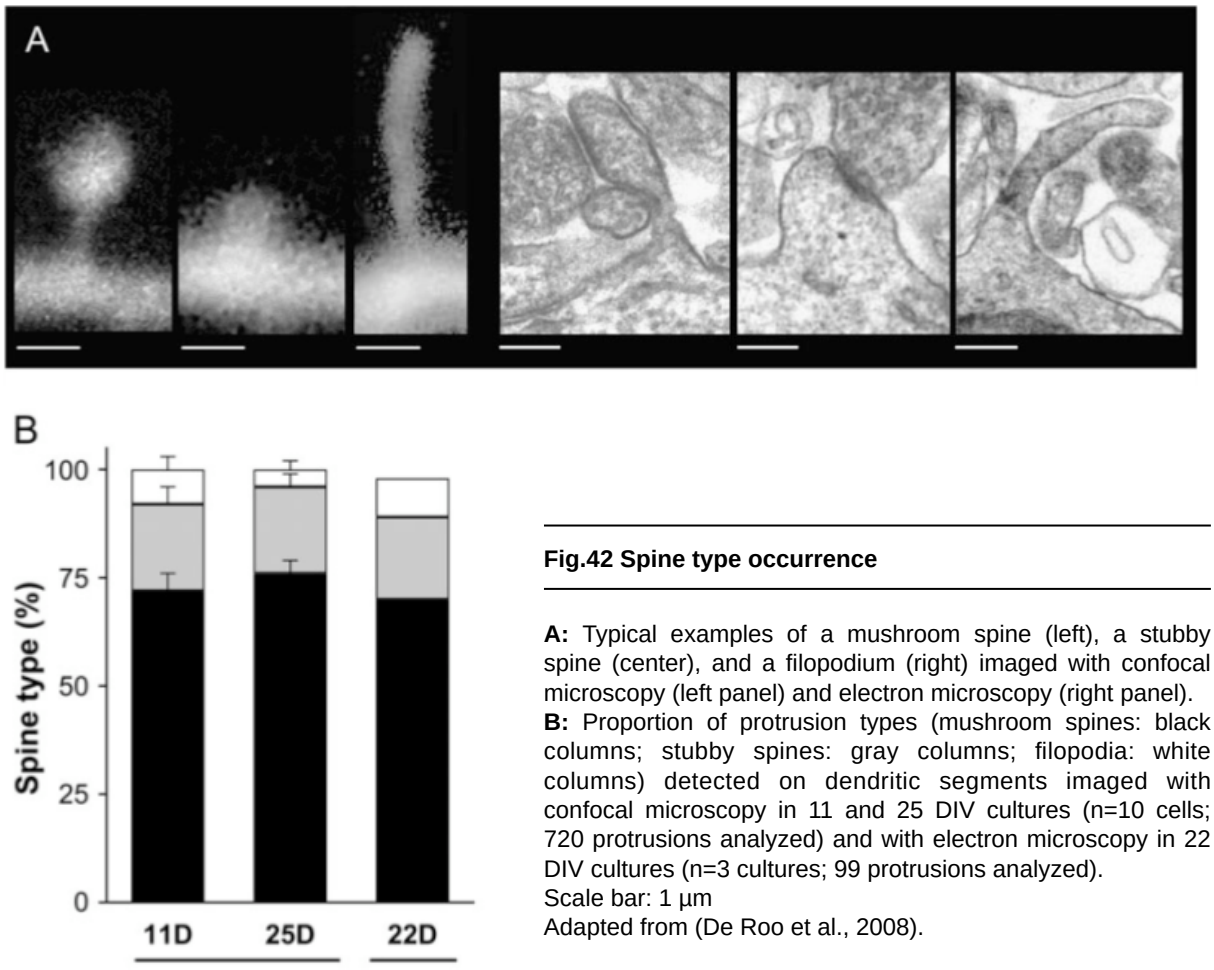


Fig.42 Spine type occurrence

A: Typical examples of a mushroom spine (left), a stubby spine (center), and a filopodium (right) imaged with confocal microscopy (left panel) and electron microscopy (right panel).
B: Proportion of protrusion types (mushroom spines: black columns; stubby spines: gray columns; filopodia: white columns) detected on dendritic segments imaged with confocal microscopy in 11 and 25 DIV cultures (n=10 cells; 720 protrusions analyzed) and with electron microscopy in 22 DIV cultures (n=3 cultures; 99 protrusions analyzed).
Scale bar: 1 µm
Adapted from (De Roo et al., 2008).

To carry out this analysis of the percentage of PSDs on dendritic protrusions, 68 protrusions from 3 different cells were analyzed from serial sections, which included macula (81 ± 8%), complex (13 ± 7%), no-PSD (6 ± 2%) as illustrated in (fig.43). The 4 cases of spine-like protrusions without PSDs that could be observed confirmed the existence in slice cultures of a small fraction of protrusions that are devoid of PSDs (see also Arellano et al. 2006). Note that the spine-like protrusion illustrated in figure 44 is also devoid of presynaptic partner. This shows an example where protrusion is formed before the apparition of a synapse.

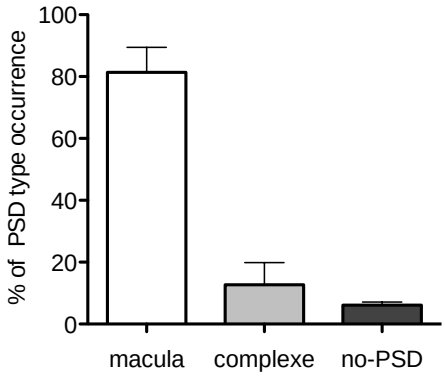


Fig.43 Occurrence of PSD type

Analyses were performed on ssEM images (n=68 protrusions out of 3 pyramidal neurons). Macular PSD type are the most abundant (81±8%),on the left, in white. Complex PSD (13±7%), (middle, light grey) and protrusion without PSD (6±2%), (right, dark grey) are less abundant in the segments analyzed. N.B. Variability is important throughout a similar set of pyramidal neurons. Nevertheless, these results are representative of the gross tendency in PSD type occurrence. See introduction for PSD type description.

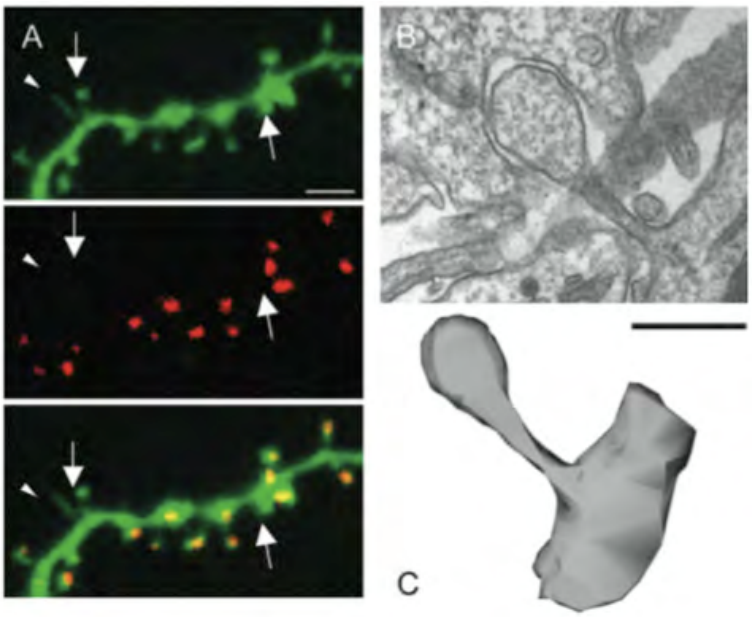


Fig.44 Absence of PSD-95--DsRed2 staining in a small fraction of dendritic spines

A: Illustration of a dendritic segment of a CA1 pyramidal cell cotransfected with EGFP and PSD-95--DsRed2 showing that most dendritic spines visualized with EGFP (upper panel) exhibit a PSD-95--DsRed2 (n=109;88% PSDs) staining (middle panel) that collocates with the spine head (merged; lower panel). This contrasts with the absence of PSD-95--DsRed2 staining in 2 spines (arrows)and one filopodium (arrowhead).
B: Electron microscopic illustration of the existence of a spine-like protrusion that is devoid of PSD and even lacks a presynaptic partner.
C: Three-dimensional reconstruction of the spine-like protrusion illustrated in (B) (scale bar: A 2 µm; B, C 0.5 µm).
Adapted from (De Roo et al., 2008).

3. SAP97

Based on analyses made at light microscopy on dissociated cultures, SAP97 has been proposed to be involved in transsynaptic signaling (Regalado et al., 2006). This result is to be considered in the context of a very recent study made by I. Nikonenko in the laboratory (Nikonenko et al., 2008). Through serial sectioning and 3D EM reconstruction of dendritic segments of pyramidal neurons transfected with PSD-95, she found that PSD-95 over-expression resulted in the formation of multiinnervated spines, with up to 7 presynaptic terminals contacting one single spine. Through genetic and pharmacological manipulations, she could show that this effect was mediated by the release of nitric oxide, which promoted terminal differentiation of axonal shafts into axonal boutons and contact formation. It was therefore very interesting to test whether SAP97 produced similar effects and how it might affect the morphology and synaptic organization of spine synapses.

Organotypic hippocampal slice cultures were transfected with a plasmid expressing wild-type SAP97 tagged with eGFP under the CMV promoter. Transfected cells were observed two days after transfections, analyzed with confocal microscopy, fixed, embedded and processed for EM serial sectioning and reconstruction. Confocal observations showed that SAP97-eGFP was present in the whole neuron, but with an increased expression in dendritic spines yielding a punctate aspect similar to that observed with PSD-95 (fig. 45A-B). Transfected neurons exhibited a normal architecture at low magnification. However, high magnification observations showed dendritic spines characterized by a fluffy appearance, poorly delimited borders and enlarged shapes (fig.45C-D-E).

SAP97 transfected neuron protrusions look bigger than control eGFP neuron protrusions. In addition dendrites showed protrusions making fluffy aggregates that made confocal observations and analysis poorly effective. After confocal observations, neurons fitting quality parameters (fluorescent intensity, cell quality) were then fixed for the EM Immunohistochemitstry. Ultra-thin sections of 65 nm were cut out of the tissue containing neurons of interest and contrasted with lead and uranyl acetate for 15 mn each. A simple 2D EM survey of SAP97-eGFP transfected neurons spines exhibited shapes that were easily noticeable as being unusual (fig.46-49). The protrusions were extending long and thin arms around presynaptic terminals forming deep engulfing protrusions (fig.46-49). These protrusions showed clear PSDs (fig.47D) that were facing presynaptic active zones with several synaptic vesicles. Analysis of the morphological parameters of reconstructed spines showed that the spine volume was increased (mean±sem: eGFP: n=4 cells; 98 spines analyzed, 0.053 ± 0.0045 μm^3; SAP97: n=4 cells; 45 spines analyzed; 0.281 ± 0.043 μm^3 comparing to PSD-95: 0.144 μm^3; (Nikonenko et al., 2008)), that the PSD size was increased (eGFP: n=4 cells; 80 spines analyzed, 0.042 ± 0.0042 μm^2; SAP97: n=4 cells; 41 spines analyzed; 0.333 ± 0.052 μm^2 comparing to PSD-95: 0.189 μm^2; (Nikonenko et al., 2008) that the number of spines with macular PSD was strongly decreased (eGFP: n=5 cells; 108 spines analyzed, 57.2 ± 4.7% ; SAP97: n=4; 42 spines analyzed, 20.25 ± 4.8%)(fig.53a). The proportion of filopodia was however not significantly different (eGFP: n=4; 108 protrusions analyzed, 7.8 ± 2.3%; SAP97: n=4; 45 protrusions analyzed 10.4 ± 3.2%). More interestingly, these analyses also revealed the presence of several spines contacted by multiple presynaptic boutons (fig. 53b), a situation similar to that found in the case of PSD-95 overexpression. The proportion of multi-innervated spines in SAP97-eGFP transfected neurons was (n=4; 43 spines analyzed 43 ± 8.7 %, thus significantly increased with regard to eGFP transfected cells (eGFP: n=5; 108 spines analyzed, 4.2 ± 1.9%

n=5; 108 spines analyzed), and even larger than in PSD-95 transfected neurons (29.1 ± 2.9% ; (Nikonenko et al., 2008)).

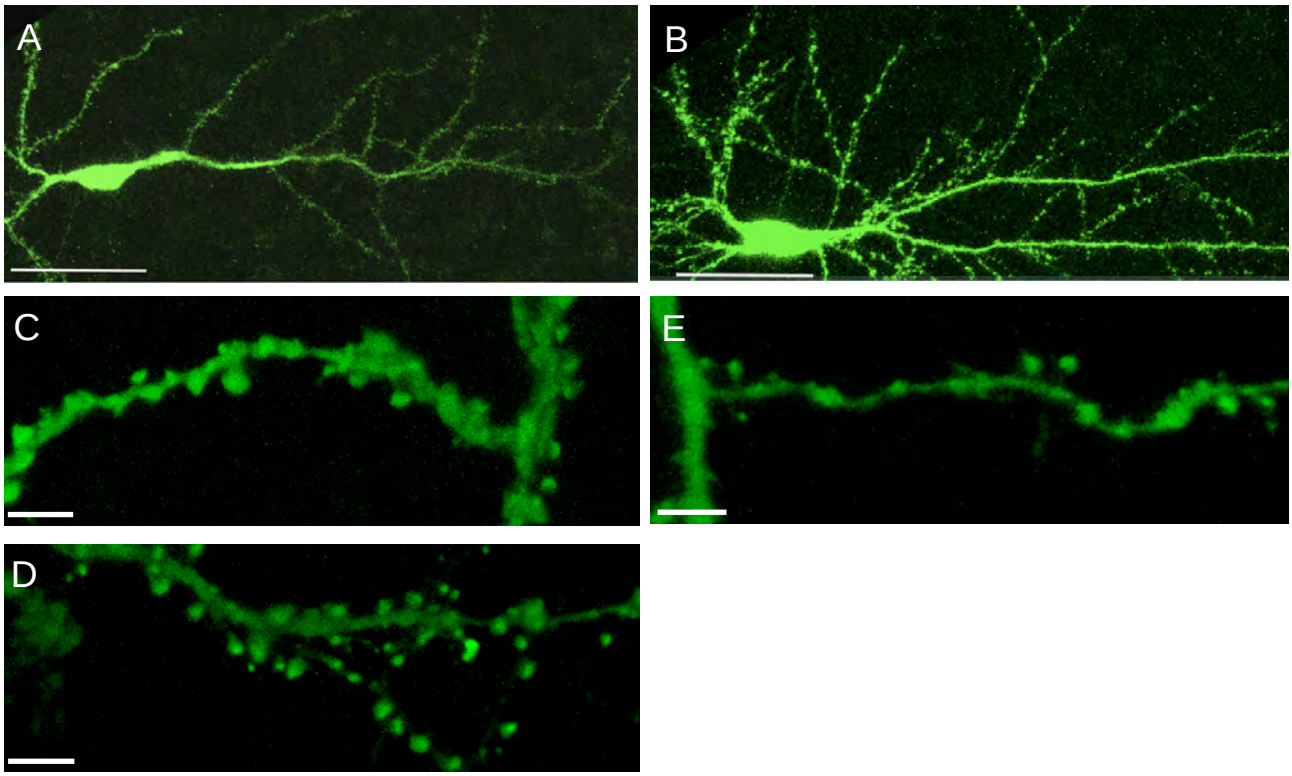
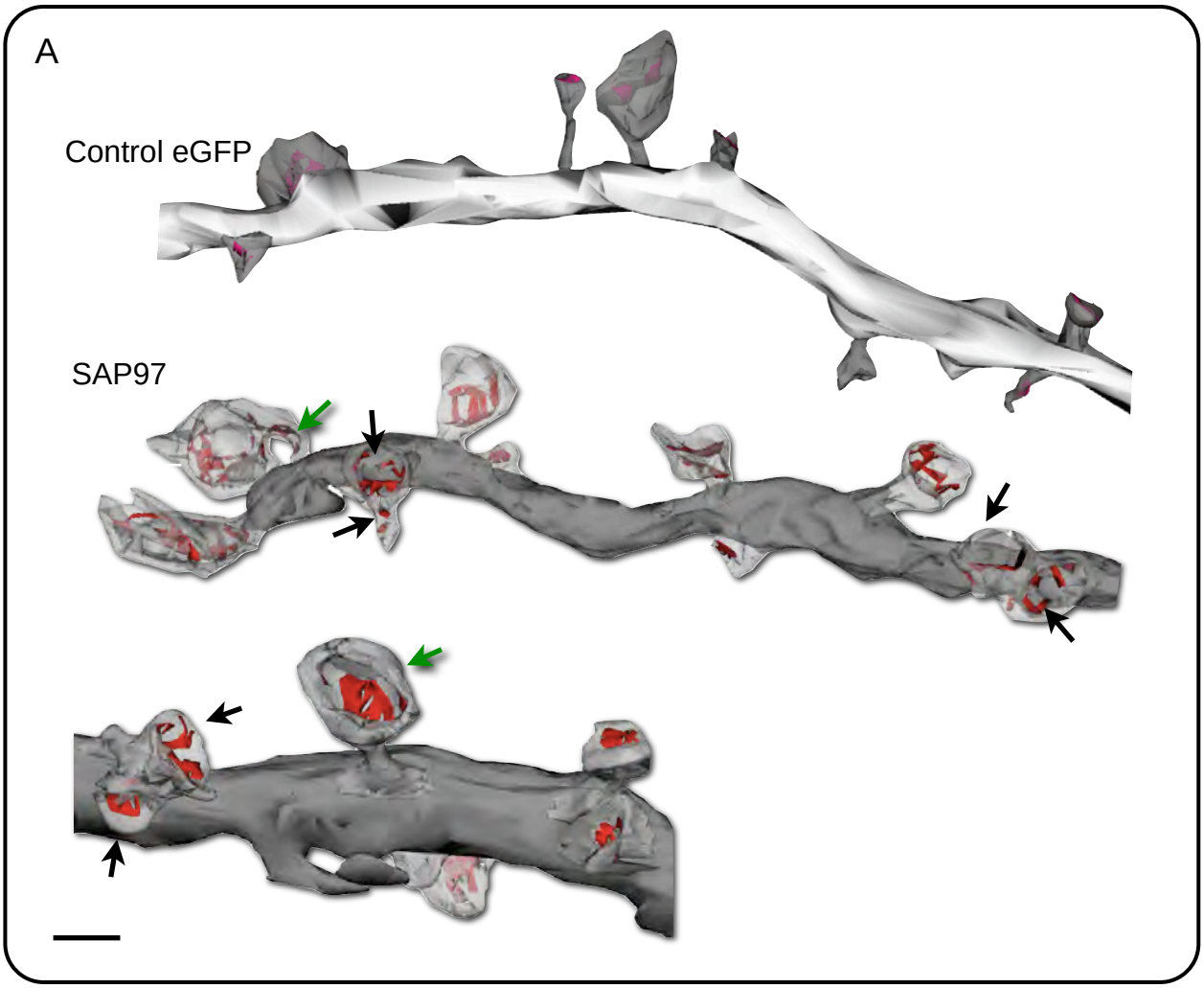


Fig.45 SAP97 & SAP97--L-NAME expressing neurons present particular features

A: SAP97 expressing neurons observed in confocal microscopy (40x). Dendritic segments bear spines, and the overall neuron looks healthy.
B: SAP97--L-NAME expressing neurons observed in confocal microscopy (40x). SAP97 expressing neurons often presented faded eGFP expression.
C, D: SAP97 dendritic segments exhibit typical big and fluffy protrusion aggregates. Notice that it is difficult to discern spines individually. (40x+10x digital zoom).
E: SAP97--L-NAME dendritic segments exhibit spines hardly differentiable from eGFP spines when observed with confocal microscopy (40x+10x digital zoom).
Scale bar (A, B: 50 µm; C,D,E: 4 µm)


A
Control eGFP
SAP97

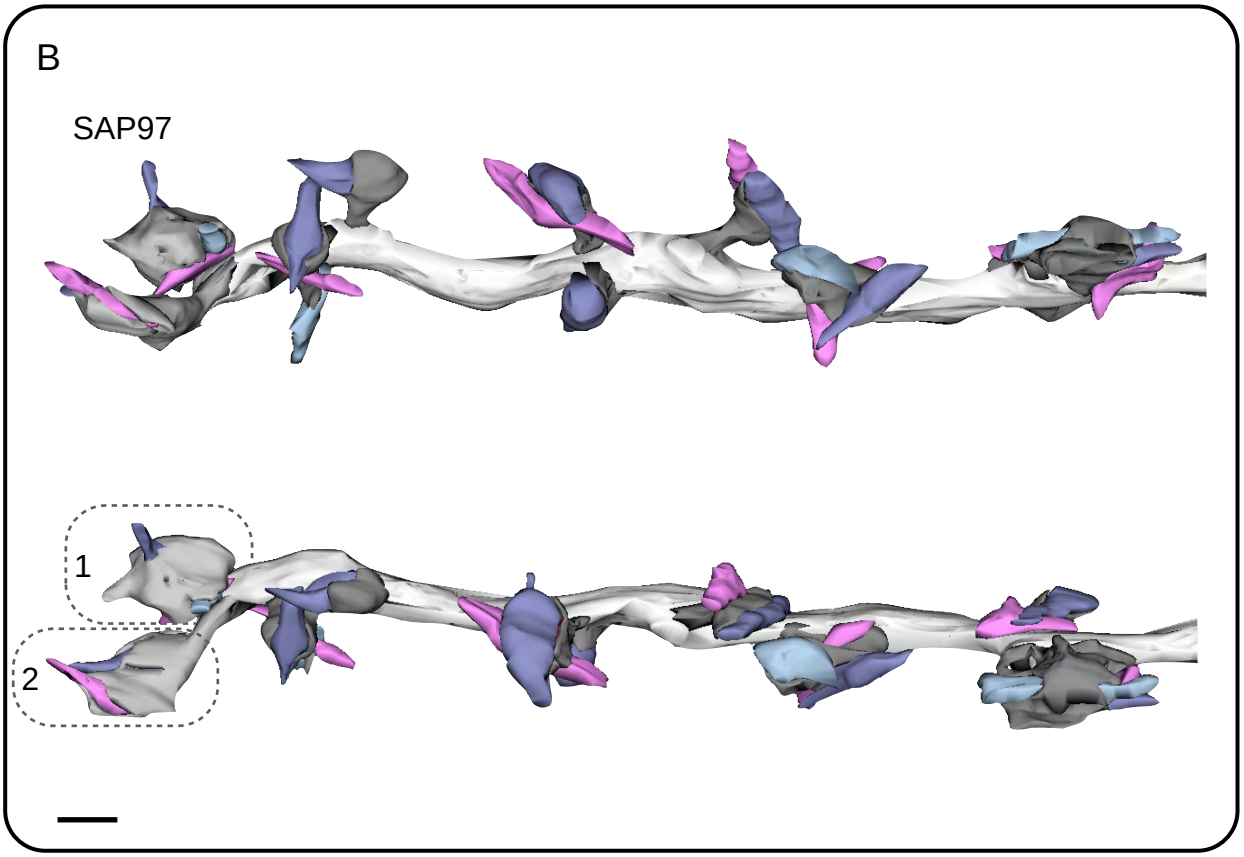

B
SAP97
1
2

Fig. 46 3D reconstruction of eGFP control and SAP97-eGFP transfected neurons from ssEM images

A: 3D ssEM reconstructions of eGFP control (above) and SAP97 expressing neurons (below, dendritic segment is shown as zoomed in the second SAP97 expressing neuron segment). Spines have very large heads that form a sheet around presynaptic axon terminals. In some cases, the spine head is almost completely engulfing the axons bouton (green arrows). Moreover, spine heads present multiple PSD's (red) locations (black arrows) that for most of them are connected with different presynaptic partners, thus giving to the spine its multiinnervated specificity.
B: Same SAP97 dendritic segments than in A viewed with two different angles. Spines are in grey, and each axon making contact is represented in one of the 3 code color (1st bouton in purple, 2nd in magenta, 3rd in light blue)
Scale bar: 1 µm

As nitric oxide was shown to mediate the formation of multiinnervated spines in the case of PSD-95 overexpression, we also checked whether this was the case with SAP97-eGFP transfected neurons. For this, slice cultures transfected with SAP97-eGFP were incubated for 2 days with L-NAME (200 µM), a blocker of the nitric oxide synthase (NOS). 3D analysis of these transfected cells showed that the proportion of multiinnervated spines was markedly reduced, almost to the control level (SAP97--L-NAME: 8.8± 3.2%, n=4, 53 spines analyzed, fig.53b-C). Thus L-NAME prevented multiinnervated spine formation without affecting the changes in spine morphology produced by SAP97 expression (fig.50-51) like spines enwrapping presynaptic boutons. As SP97 is not known to bedirectly bound to NOS, we wondered how increased SAP97 expression could promote multiinnervated spine formation through nitric oxide. We test therefore the possibility that increased SAP97 expression recruited additional PSD-95 which in turn could promote the expression of NOS. To examine this possibility we used immunohistochemistry to label PSD-95 in SAP97 expressing cells. Confocal microscopy analysis demonstrated an increase in the size of PSD-95 puncta (PSD-95 antibodies 1/20000/-Alexa blue anti-mouse secondary antibodies) co-localized with SAP97-eGFP overexpression (fig.52). All together, these results indicate that expression of SAP97 markedly affects the morphology of excitatory spine synapses. Part of the effect is related to the expression of SAP97 and leads to an enlargement of the PSD and an increased size of spine heads. However SAP97 also promotes the formation of multiinnervated spines through a mechanism that involves the recruitment at the synapse of PSD-95 and probably NOS.

Another peculiar feature observed at EM level was the way SAP97-eGFP overexpressing spines were en-sheathing the axonal boutons. Axonal boutons in some cases were surrounded by the spine making contact with them (fig.48-49). Interestingly, SAP97--L-NAME spines were also en-sheathing their presynaptic partner although they were barely observed as multiinnervated spines (fig.50-51).

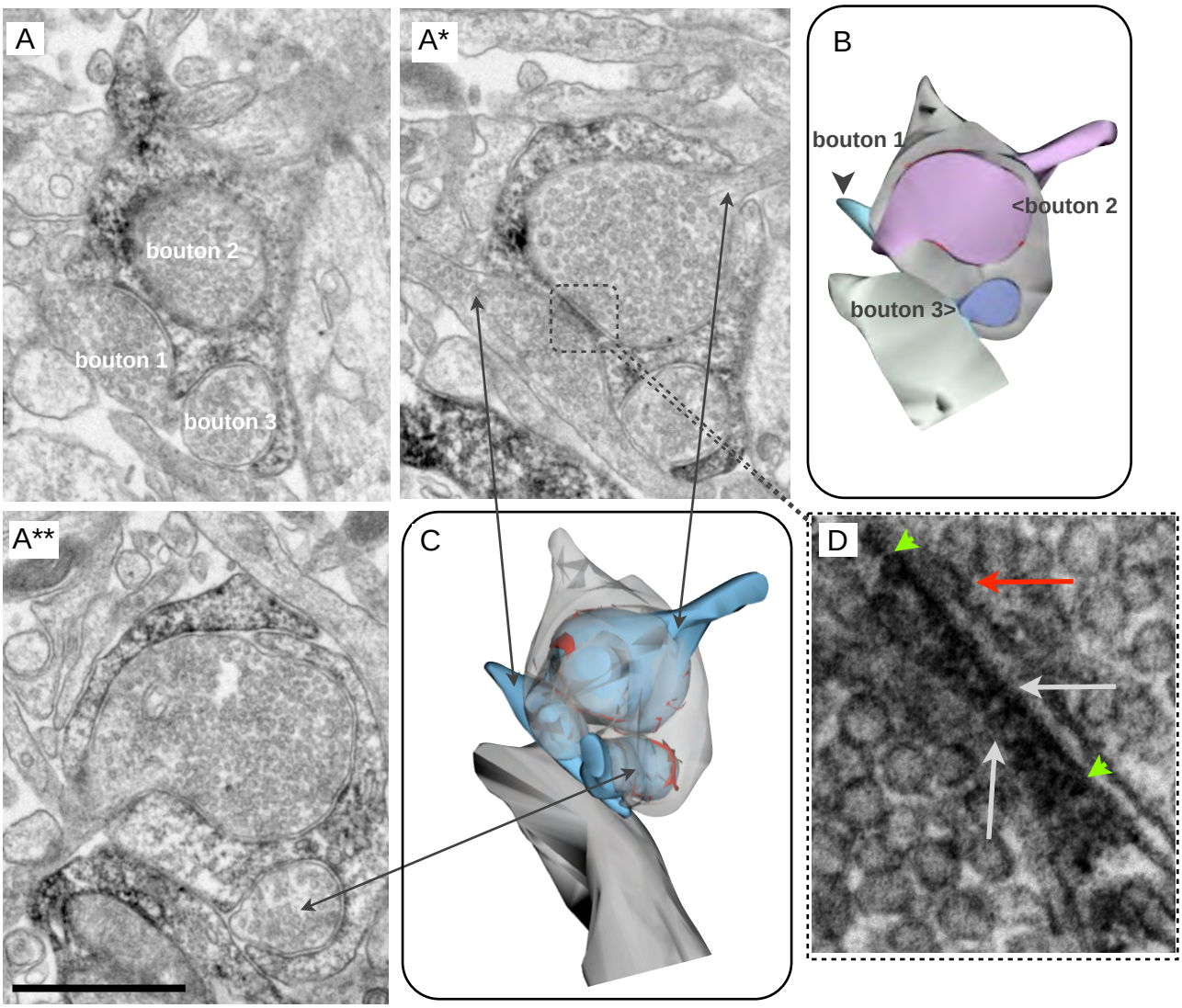


Fig.47 Details of the B1 spine of fig.46

A: ssEM images (15.000x) of 3 levels of the B1 spine of fig.46. 3 different axons are making contact with one spine. Synapses are found on both sides of the spine, which is facing two different boutons. NB the overlapping sheets of the spine around the bouton on A**.
B: Coronal cut of the same spine showing the detail of the spine en-sheating around axonal boutons (blue/purple/green).
C: 3D reconstruction of the previous spine (gray in transparency, dendrite in dark gray) connecting the three boutons (blue). The PSDs of the spine are visible as red spots disseminated around each boutons.
D: Zoom (150.000x) on a thin portion of the spine where two PSDs (gray arrows) are facing active zones of two different boutons. The axonal vesicles are touching and even fusing with the membrane (red arrow). The green arrow heads are delimiting one of the two measured PSDs.
Scale bar: 1 µm

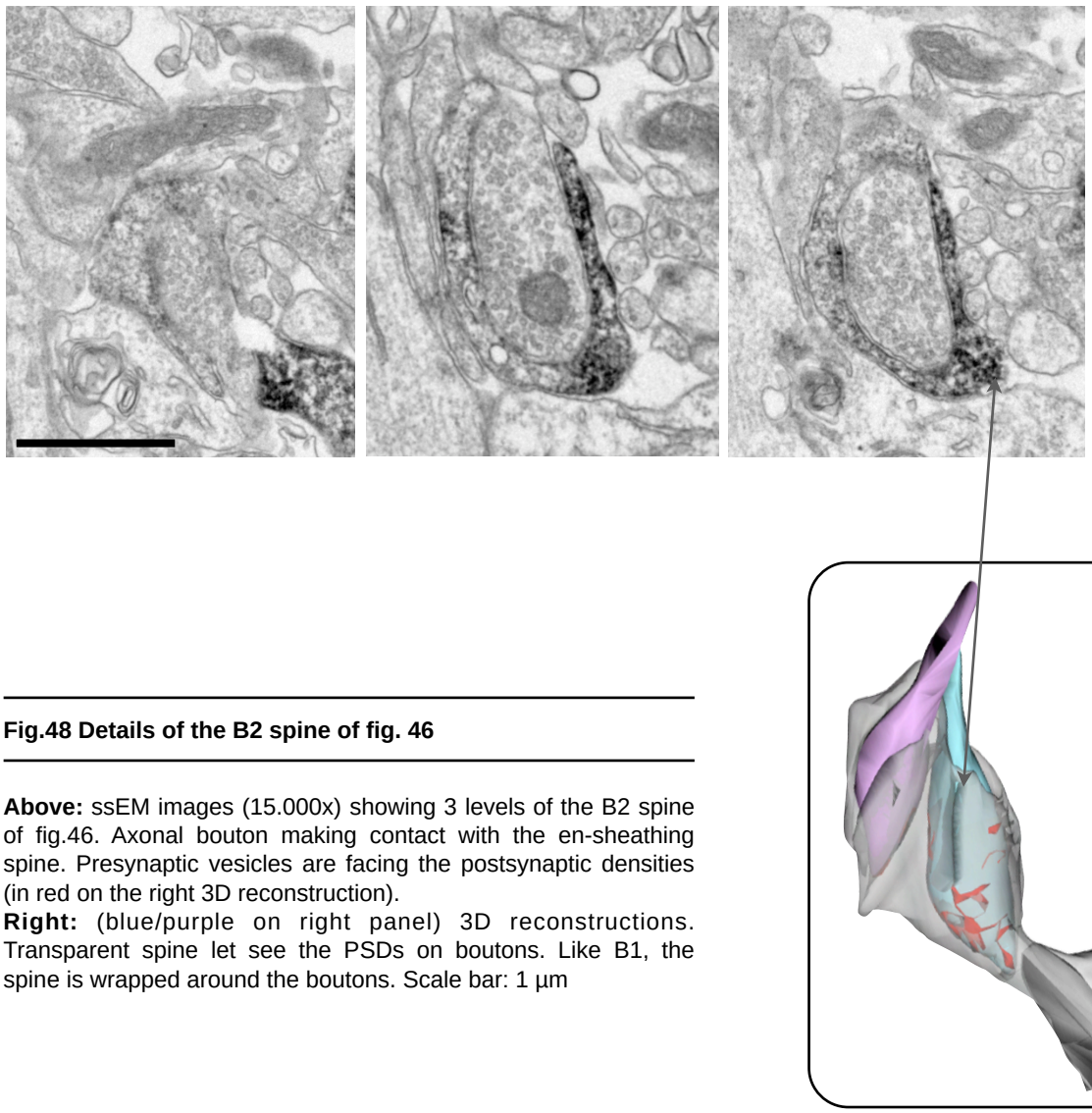


Fig.48 Details of the B2 spine of fig. 46

Above: ssEM images (15.000x) showing 3 levels of the B2 spine of fig.46. Axonal bouton making contact with the en-sheathing spine. Presynaptic vesicles are facing the postsynaptic densities (in red on the right 3D reconstruction).
Right: (blue/purple on right panel) 3D reconstructions. Transparent spine let see the PSDs on boutons. Like B1, the spine is wrapped around the boutons. Scale bar: 1 µm

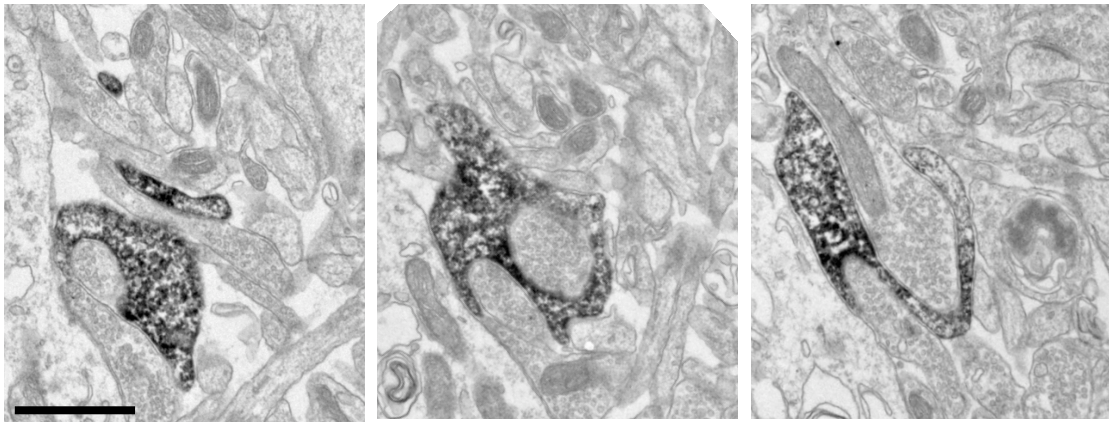


Fig.49 An other observation from ssEM images of a multiinnervated spine

Below: ssEM images (15.000x) showing 3 levels of a SAP97 transfected neuron spine. Axonal boutons are making contacts with the en-sheathing spine.
Scale bar: 1 µm

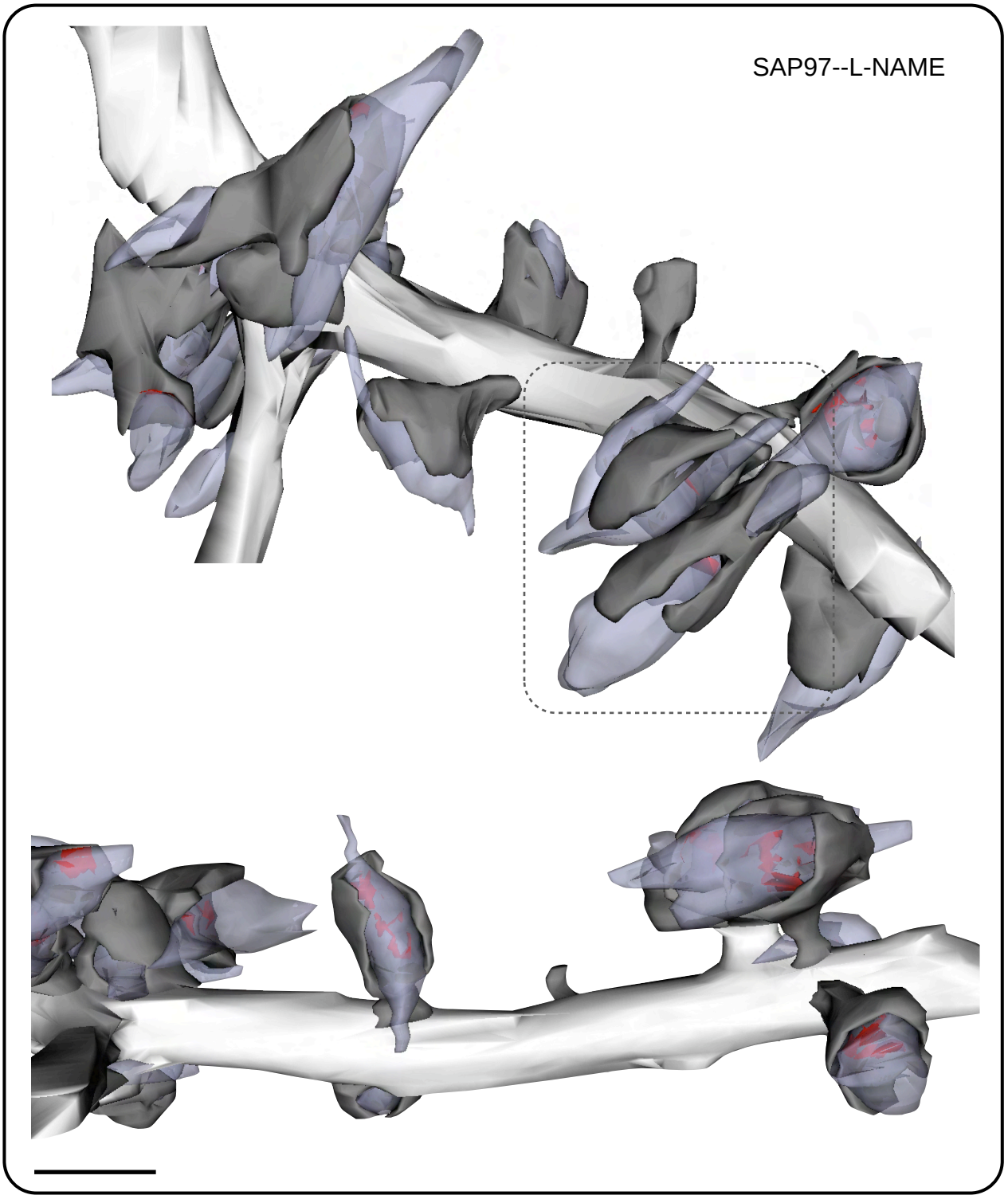
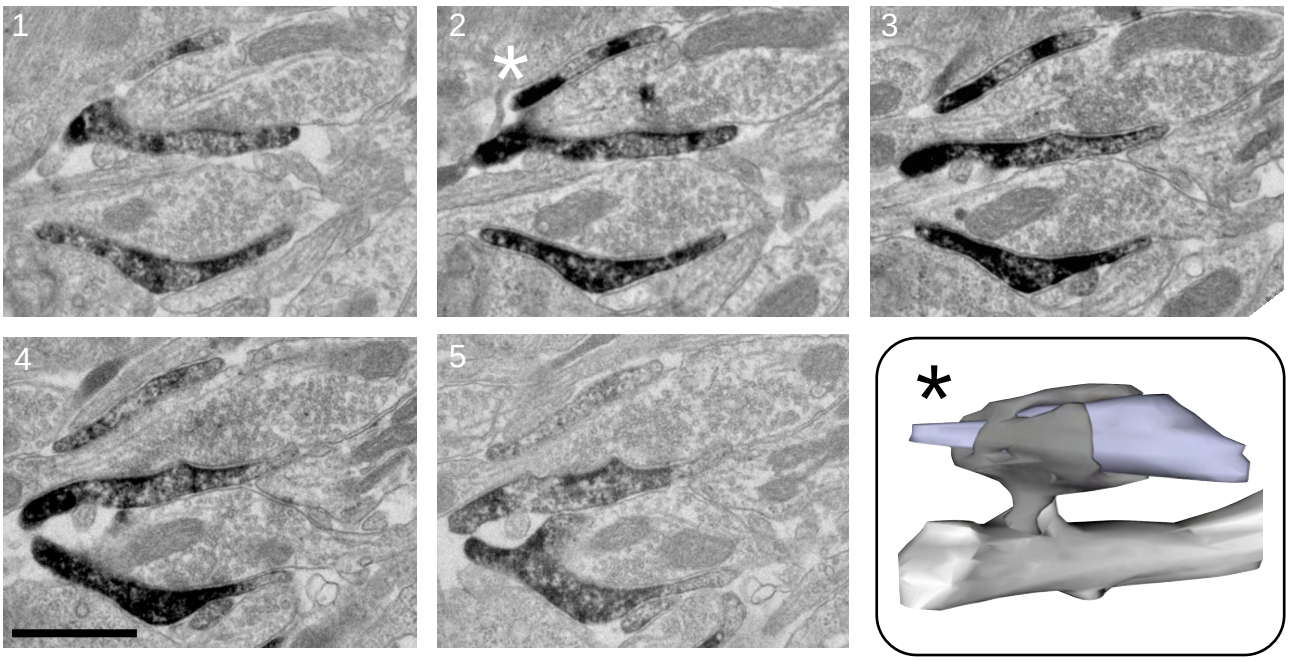


Fig.50 3D reconstruction from SAP97-eGFP transfected neurons treated with L-NAME

Dendritic segment reconstructed from SAP97-eGFP transfected neurons that were bathed for 2 days after transfection with L-NAME (NO synthase blocker).
These two views of the same dendritic segment, but from different angles, make visible the axonal boutons making contact with spines (PSDs in red). Dendrites, unlike for SAP97-eGFP transfected neurons, present only few multiinnervated spines, however boutons undergo partial or almost total en-sheeting by dendritic spines.
Scal bar: 1 µm



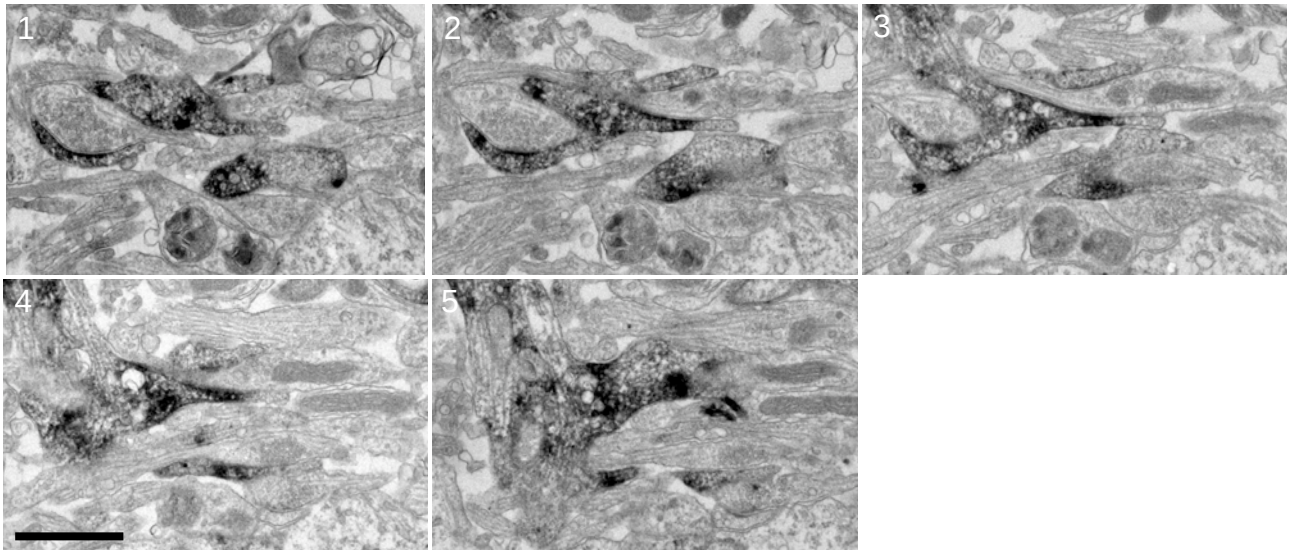


Fig.51 ssEM images of SAP97-eGFP transfected neurons treated with L-NAME

Above: ssEM images (15.000x) of the two spines framed by dashed square of the previous dendrite reconstructed in 3D. Spines run along axonal boutons making complex PSDs, and in some cases multiinnervated spines, but in a much smaller range than non-treated neurons.
Right: 3D reconstruction of the latest spine from ssEM images (W/B stars), spine (dark gray), bouton (purple).
Below: Bifurcation of a dendrite. Spines emerge from the dendrite as lamellar arms enwrapping adjacent axonal boutons. Several examples show spines with almost any spine neck.
Scale bar: 1 µm

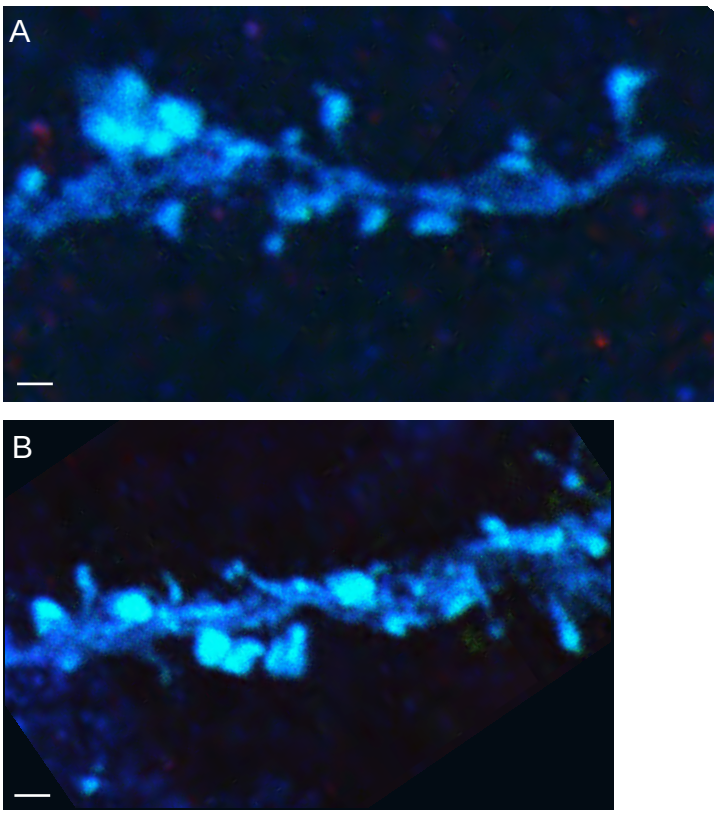


Fig.52 Immuno staining of PSD-95 in SAP97 transfected neurons

A & B: Two PSD-95 immuno stained dendritic segments showed that SAP97-eGFP overexpression (green) induced PSD-95 expression at spines (n=2). NB the PSD-95 weaker blue spots of the surrounding neurons (control) suggest that SAP97 increases PSD-95 recruitment at the SAP97 transfected spines. Thus PSD-95 may be the link between SAP97 overexpression and spine multiinnervation NO synthase dependent.
Scale bar: 1 µm

Fig.53a Spine volumes and PSDs surfaces comparison between eGFP and SAP97 from EM observations

A: Spine volumes of SAP97 (n=4, 45 spines analyzed 0.281 ± 0.043 µm^3) transfected neurons were bigger than eGFP control neurons (n=4 cells; 98 spines analyzed, 0.053 ± 0.0045 µm^3).
B: PSDs surfaces were also strongly increased in SAP97 transfected neurons (n=4, 41 spines, 0.333 ± 0.052 µm^2) similarly to PSD-95 transfected neurons (0.189 µm^2). (One-way analysis of variance).***, P< 0.0001. Data are mean ± SEM (error bars).

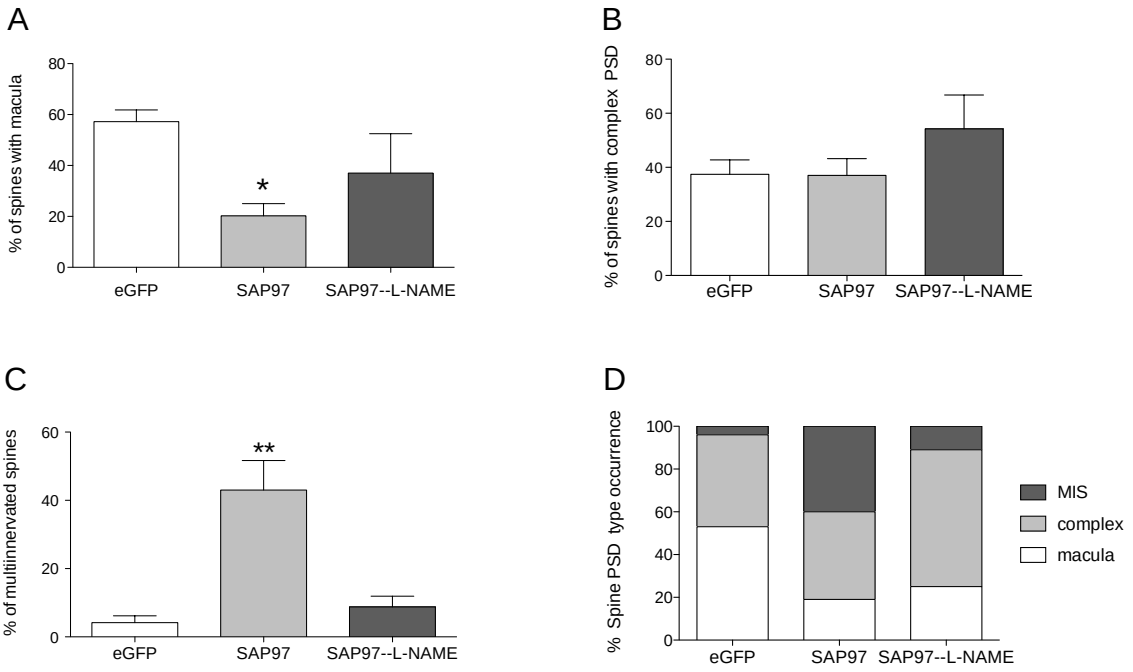


Fig.53b Spine PSD type occurrence

A: Percentage of spines with macular PSDs. Dendritic segments of SAP97-eGFP transfected neurons have less spines bearing macular PSDs than control eGFP expressing or SAP97--L-NAME treated neurons.
B: Percentage of spines with complex PSDs. Any noticeable differences in percentage are observed.
C: Percentage of multiinnervated spines (MIS). SAP97-eGFP transfected neurons show a dramatic increase in the number of multiinnervated spines. L-NAME prevents most of the apparition of MISs in SAP97-eGFP transfected neurons.
D: Summarized data for the proportion of spine PSD types (macular PSDs, white),(complex PSDs, light grey)(MISs. dark grey).
Observations of ssEM images, analyzed after 3D reconstructions: 11DIV cultures; eGFP (n=4; 98 spines analyzed), SAP97 (n=4; 42 spines analyzed), SAP97--L-NAME (n=4; 53 spines analyzed). (One-way analysis of variance).*, $P< 0.05$; **, $P< 0.001$. Data are mean ± SEM (error bars).

4. N-Cadherin

N-Cadherin is expressed at excitatory synapses and is localized on both sides of the synapse, possibly even forming a regular meshwork within the synaptic cleft and thus providing strong adhesion across the synapse. Interference with N-Cadherin signaling, through α or β-Catenins, the major intracellular partners of N-Cadherin, results in defects in spine morphology, characterized by an increased number of thin, elongated spines (Togashi et al., 2002), but also changes in spine motility or stability in hippocampal culture models (Togashi et al., 2002). N-Cadherin also affects spine numbers: interactions between N-Cadherin and the extracellular domain of AMPA receptors directly affects spine density (Togashi et al., 2002). Finally, N-Cadherin is directly implicated in synaptic plasticity. Inhibition of N-Cadherin by antibodies interferes with the late phase of LTP (Bozdagi et al., 2000). β -Catenin regulates synaptic strength and surface expression of N-Cadherin is modulated by NMDA receptors (Tai et al., 2007). Altogether these data clearly point to a possible important function of N-Cadherin in spine dynamics and a possible role in synapse stability.

In our experiments CA1 pyramidal neurons were transfected with a vector (pcDNA3.1) expressing EGFP together with either wild type N-Cadherin (WT-NCad) or a mutant N-Cadherin (Δ390-NCad) characterized by a deletion of the extracellular domain. This deletion renders the protein nonfunctional for intercellular adhesion and interaction. It also competes with endogenous N-Cadherin for intracellular signaling, thus exerting a dominant negative effect (Takeichi and Abe, 2005.

Neurons were transfected at day 11 in vitro and, after 2-3 days, imaged with a confocal microscope to check their morphology, state of preservation and level of fluorescence and then fixed and embedded for serial section electron microscopy.

Analysis of transfected neurons with confocal microscopy showed that spines on neurons transfected with the mutant Δ390-NCad exhibited an altered morphology and, in particular, features of immaturity (fig. 55): spines were characterized by smaller heads than in control or wild type N-Cadherin expressing neurons confirming previous observations (Togashi et al., 2002). In addition, the proportion of filopodia-like structures (protrusions without enlargement at the tip) were more frequent in mutant Δ390-NCad transfected cells than in control or wild type NCad transfected neurons (Figure 56, left; Ctrl, 5.3 ± 0.8%; WT-NCad 5.4 ± 1.7%; Δ390-NCad 14.2 ± 2.3% (mean±sem)). Figure 56 (right) shows the size distribution of spine head measurements under the three conditions revealing a significant shift to the left of spine heads values in Δ390-NCad mutant cells (mean head width: 0.60 ± 0.01 μ m, 0.64 ± 0.02 μ m and 0.52 ± 0.01μm (mean ±sem) in eGFP (control), WT-NCad and mutant D390-NCad transfected cells, respectively, $p<0.0001$). The average length of protrusions was however not significantly different between groups (1.09 ± 0.04 μm; 1.12 ± 0.03 μm; 1.19 ± 0.03 μm (mean±sem); for EGFP, NCad and D390-NCad groups, respectively, $p>0.1$).

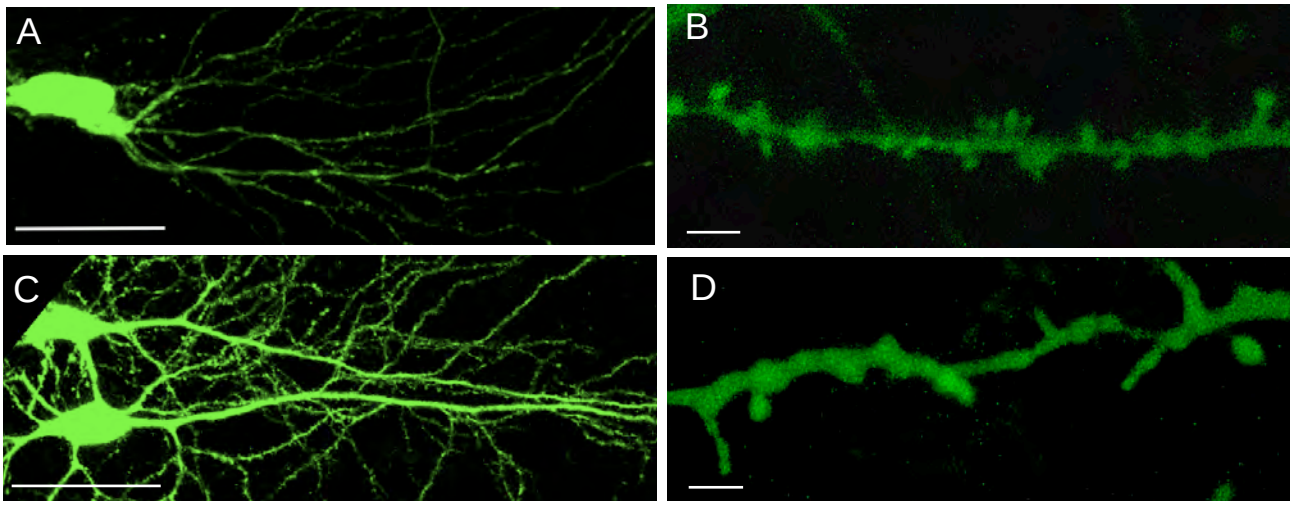


Fig.55 Δ390-NCad & WT NCad expressing neurons

A: WT NCad expressing neurons observed in confocal microscopy (40x).
B: WT NCad dendritic segments bearing spines with large heads, and stubby and relatively large necks. These spines look like mature spines (40x+10x digital zoom).
C: Δ390-NCad expressing neurons observed in confocal microscopy (40x).
D: Δ390-NCad dendritic segments bearing thin and elongated protrusions that look like immature spines (40x+10x digital zoom).
Scale bar (A, B: 50 µm; C, D: 2 µm)

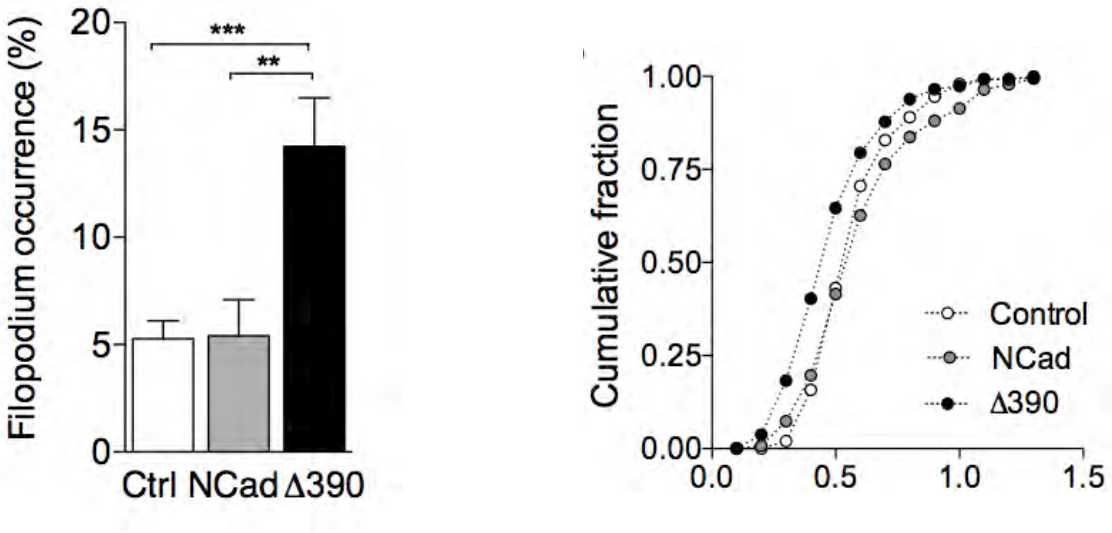


Fig.56 Filopodium occurrence and spine head size of WT-NCad and Δ390-NCad transfected cells

Left: Δ390-NCad transfected neurons show an increase in the percentage of filopodia (protrusions without enlargement at the tip), whereas NCad neurons have a similar percentage of filopodia than control neurons (Ctrl, 5.3 ± 0.8%; WT-NCad 5.4 ± 1.7%; Δ390-NCad 14.2 ± 2.3%).

Right: Size representation of spine head measurements revealing a significant shift for the Δ390-NCad neurons condition (mean head width: 0.60 ± 0.01 µm, 0.64 ± 0.02 µm and 0.52 ± 0.01µm in eGFP (control), WT-NCad and mutant D390-NCad transfected cells, respectively, $p<0.0001$).
Data obtained by Pablo Mendez from our lab.

Analysis of transfected neurons following fixation, embedding, serial sectioning and 3D reconstruction confirmed these observations but also revealed additional features. Cells transfected with the mutant Δ390-NCad were indeed characterized by many immature spines with elongated necks and small heads that give them an overall look of filopodia, whereas WT NCad transfected cells showed spines that could be considered as mature spines with a large spine head and often a stubby neck.

Spines on Δ390-NCad transfected cells had a significantly smaller volume than control neurons, while WT NCad transfected neurons had a significantly bigger volume than control neurons (mean±sem; n=4, 110 spines analyze, eGFP: 0.065 ± 0.006 μm^3; n=4, 58 spines analyzed, WT NCad: 0.1330 ± 0.021 μm^3; n=4, 127 spines analyzed, Δ390-NCad: 0.025 ± 0.002 μm^3) fig. 60, left, and Δ390-NCad transfected cells also exhibited smaller PDSs sizes while WT NCad had any significantly different PSDs sizes (n=4, 102 spines analyzed, eGFP: 0.045 ± 0.004 μm^2; n=3, 52 spines analyzed, WT NCad: 0.06369 ± 0.011 μm^2; n=4, 102 spines analyzed, Δ390-NCad: 0.02875 ± 0.002 μm^2), fig.60, right. Among all Δ390-NCad reconstructed protrusions, spines with PSDs represented 84%, while the proportion of the protrusions without PSD (mainly elongated protrusions) was 16%. These results thus indicate that interfering with N-Cadherin function results in spines that remain smaller and immature, and in a large proportion of protrusions without synaptic contacts.

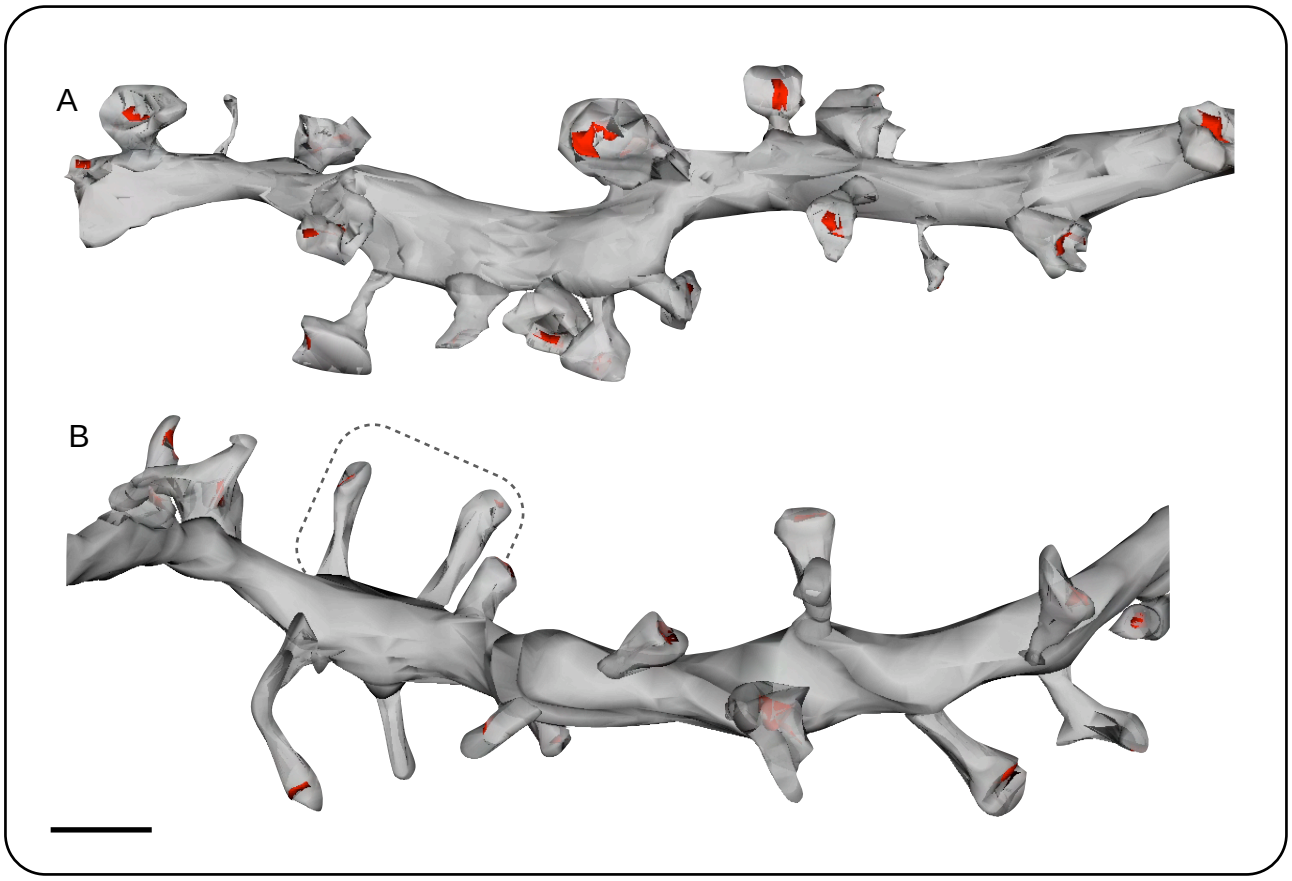


Fig.57 3D reconstruction of WT NCad and Δ390-NCad from ssEM images

A: 3D reconstruction from ssEM images of a typical WT NCad expressing neuron. Spines exhibit voluminous heads with proportional PSDs (red). Most of the spines express mature-like feature characterized by a large spine head. Spine necks are often short and stubby.
B: 3D reconstructed Δ390-NCad expressing neuron. Dendrites bear elongated protrusion that are filopodium-like shaped. Nevertheless, they mostly show a PSD (red) at their tips. The overall morphology is related with an immature feature characterized by thin and elongated protrusions with almost any salient spine head.
Scale bar: 1 µm

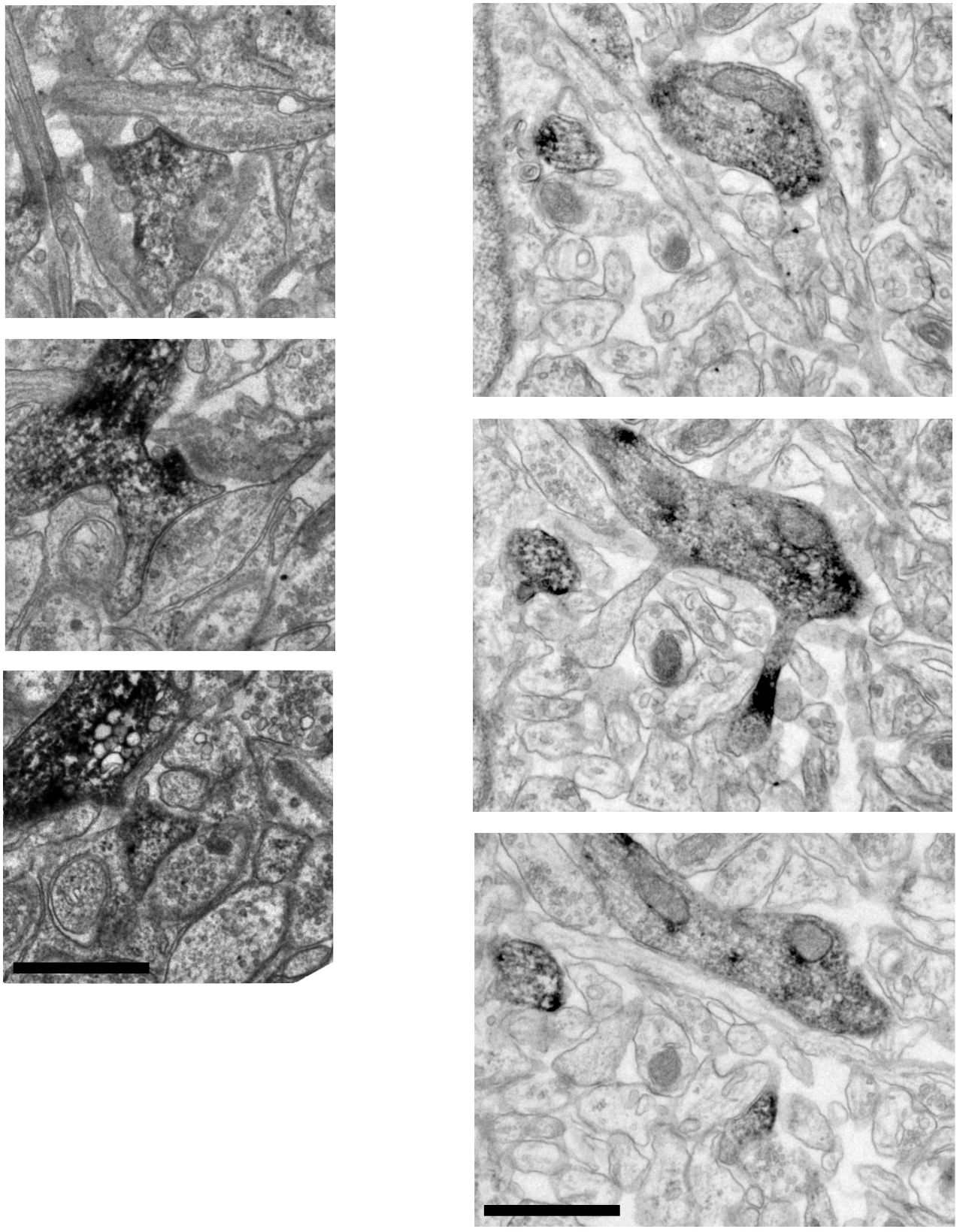


Fig.58 EM images of WT NCad and Δ390-NCad

Left: Spine of a WT NCad overexpressing neuron. Spine head is large with related PSDs. This spine show a mature feature that is underlined here by a double presynaptic innervation. The 3D reconstruction from the ssEM images.
Right: Spines of Δ390-NCad expressing neurons. Two protrusions making slight synapse with axon terminals. Same spines than those framed by the dashed line of the previous figure. Spines are elongated and have small head that look like immature spines or even like spine-like protrusions (see figure non synaptic protrusion).
Scale bar: 1 µm

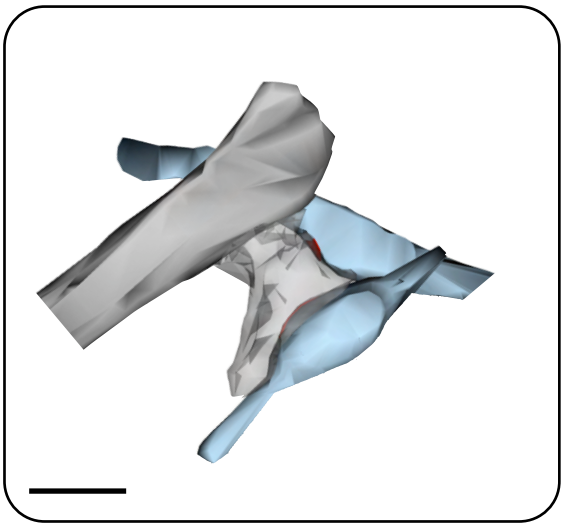


Fig.59 WT NCad 3D reconstruction

3D Reconstruction of the WT NCad spine showed in the left hand side of the previous figure. Two axonal boutons contact a spine by making synaptic contacts. However, MIS are barely observed in WT-NCad transfected neurons. Spine has a large and short neck.
Scale bar: 1 µm

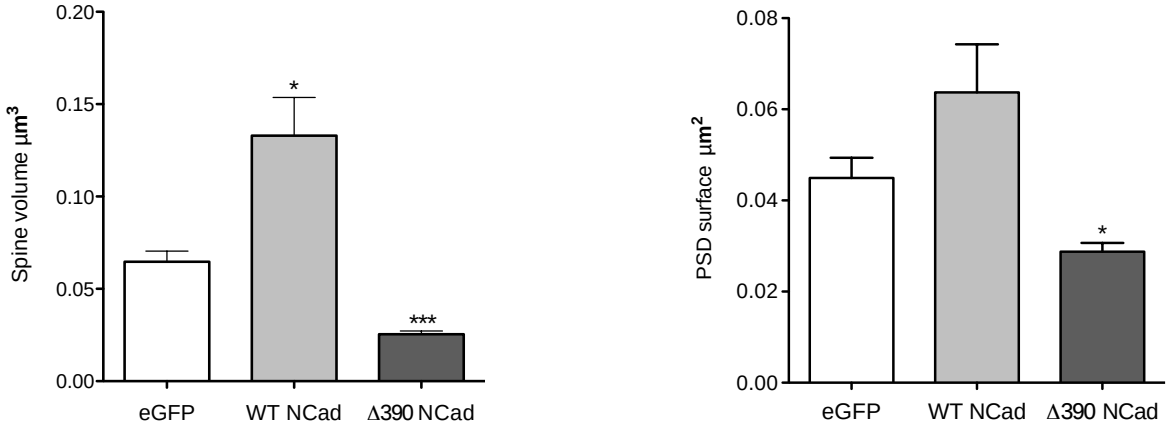


Fig.60 Spine volume and PSD Surface size of WT NCad and Δ390-NCad transfected neurons

Left: WT NCad overexpressing neurons present bigger spine volume than eGFP controls. Conversely, Δ390-NCad had a smaller spine volume.
Right: Δ 390-NCad expressing neurons present significant smaller PSDs surface size compared to eGFP or WT-NCad expressing neurons.

DISCUSSIONS

The morphology of spines and synapses vary as a function of development and activity (Buchs and Muller, 1996; Nikonenko et al., 2002; Matsuzaki et al., 2004) and these changes are believed to have functional implications and reflect differences in either strength and/or stability. As the molecular mechanisms regulating spine morphology are still essentially unknown, I tried in this study to focus on two molecules belonging to different families, SAP97 of the MAGUK family of scaffold proteins, and N-Cadherin, one of the main member of the adhesion molecule family, and examined through gain and loss of function experiments how these molecules affect spine and synapse morphology. The results reveal major differences in the morphological alterations produced by these two molecules, providing new insight in their function at the synapse.

1. Confocal - EM correlations of spine types and morphologies

As a first step in this analysis, I started by quantifying the characteristics of spines and synapses in hippocampal slice cultures. These experiments showed that under control conditions, most spines (94%) exhibited a PSD and were thus involved in the formation of a synaptic contact. A small percentage of protrusions were however devoid of PSD (6%), some with an elongated shape and no spine head, thus corresponding to the usual definition of filopodia, but others looking like spines, with a spine head and no PSD. In some cases these protrusions devoid of PSD did not even face a potential presynaptic partner. This result is therefore consistent with studies by Knott et al. and by Arellano et al. (Knott et al., 2006; Arellano et al., 2007) also showing the existence of spines devoid of PSD and strongly suggests that synapse formation may start with the growth of a protrusion that subsequently will acquire a PSD and a presynaptic partner. Consistent with this hypothesis, analysis of tagged PSD-95 expression in newly formed spines revealed the presence of clearly identifiable puncta only hours after appearance of the spine (De Roo et al, 2008). A recent study however indicates that new spines formed as a result of LTP induction are already functional within minutes after appearance, a result that is somewhat at odd with the previous studies and in particular with evidence obtained from in vivo experiments (Knott et al.2006). A difficulty in these studies however is to precisely define whether a PSD is present or not. Even if no specialized structure corresponding to the classical image of a PSD can be seen on EM pictures, it remains difficult to exclude that some components such as receptors are already expressed. The same argument can also be made with expression of tagged molecules. How many tagged molecules need to be expressed to become detectable as puncta is unclear and possibly expression of receptors and other PSD constituents can occur quite rapidly and increase progressively over the first hours after formation. These experiments at least reveal the complexity of analyzing the initial steps leading to the formation of a new spine synapse.

2. SAP97

These experiments showed that SAP97, when overexpressed at excitatory synapses, modifies the morphology of spines in a way that is very similar to what has been reported for PSD-95 (Nikonenko et al., 2008). Namely, SAP97 promotes the formation of multiinnervated spines (fig.53a). PSD-95 overexpression effects were mediated through activations of nitric oxide synthase (NOS), to which PSD-95 is directly linked, and release of NO. Multiinnervation spines were prevented when the SAP97 transfected neurons were treated by L-NAME (a NOS blocker) indicating that SAP97 overexpression induced MISs were also relying on the NOS. Interestingly, as SAP97 does not directly bind to NOS, the implication of NOS activity for the formation of multiinnervated spines suggests that this effect is very likely to be mediated through PSD-95 expression, as indicated by immunostaining experiments showing significantly increased PSD-95 puncta in SAP97 over-expressing neurons (fig.52). Overexpression of SAP97 also induced the apparition of large spines, with large and more complex PSDs in a more dramatic manner than for PSD-95 overexpression (fig 52/54). However, other EM observations suggest that SAP97 expression may produce specific effects that differ from those observed with PSD-95 expression. The morphological appearance of spine heads and their tendency to engulf and enwrap terminals is a feature that is not seen in a similar manner in PSD-95 expressing cells (fig 48-51). Thus MAGUK proteins, while they probably tend to recruit each other when overexpressed, may also exert specific effects through their different interactions with other PSD proteins. These experiments thus reveal the complexity of the regulation of spine morphology through the different components of the PSD.

Confocal observations of SAP97 transfected neurons showed spines with very peculiar shapes often difficult to interpret due to poorly defined contours. Ultrastructural observations and 3D reconstructions confirmed these impressions by demonstrating the existence of spines looking like leafs with extensions surrounding and ensheathing axonal boutons. In addition, spine volumes were much bigger than in control eGFP neurons (5 times) or even N-Cadherin overexpressing neurons. PSD's surfaces were also strongly increased in SAP97 overexpressing neurons by 8 times than eGFP control PSD's surfaces. Finally, unlike control eGFP and N-Cadherin overexpressing neurons, SAP97 overexpressing neurons were characterized by the presence of numerous multiinnervated spines representing a few less than half of the spine population. My results are in line with and provide an explanation for the results of Regalado et al., who found an increase in the intensity and number of synaptophysin, synapsin and Bassoon puncta, (Regalado et al., 2006), which can now be explained by the presence of multiinnervated spines. This result is also consistent with the recent description by I. Nikonenko that PSD-95 expression also leads to the formation of multiinnervated spines. Thus both PSD-95 and SAP97 appear to promote in a similar manner formation of multiinnervated spines. In the experiments made by Irina Nikonenko (Nikonenko et al., 2008), overexpressing PSD-95 induced multiinnervated spines with up to 7 presynaptic partners. Moreover, this phenomenon was prevented by the deletion of the second PDZ domain of PSD-95 which interacts with nitric oxide (NO) synthase (NOS). The role of NO in these mechanisms was further demonstrated by the possibility to block formation of multiinnervated spines by using small interfering RNA to down-regulate NOS expression or by bathing cultures with L-NAME, an NOS inhibitor (Nikonenko et al., 2008). Conversely, a NO donor or cyclic guanosine monophosphate analogue could induce MISs when applied to the hippocampal slice cultures. In addition, NOS blockade reduced spine and synapse density in developing hippocampal

cultures (Nikonenko et al., 2008). All together, Nikonenko's results indicated that postsynaptic sites trigger synapse formation with nearby axons via an NOS--PSD-95 interaction and NO signaling (Nikonenko et al., 2008). In my studies, blocking NOS by L-NAME also prevented multiinnervated synapse, although SAP97 is not known to directly interact with NOS. The interaction might however be indirect: for instance, SAP97 indirectly interacts with NOS through the plasma membrane Ca^{++}-ATPases via PDZ domains (Schuh et al., 2003). As observed by Cai et al., eGFP-PSD-95 transfection increases the clustering of SAP97 to spines by a factor of 3-4 in comparison with untransfected controls (Cai et al., 2006), indicating that expression of PSD-95 also drives SAP97 to the synapse. Such interactions between PSD-95 and SAP97 through their SH3 and the N-terminal respectively are important for AMPARs trafficking into dendritic spines (Cai et al., 2006) and therefore important for spine maturation and activity-depend synapse plasticity. Immuno-tagged PSD-95 showed enhance fluorescence in SAP97 overexpressed neurons. This result let think that overexpression of SAP97 may promote PSD-95 expression and its associated NOS activation, which in turn by releasing NO may possibly lead to multiinnervated spine formation.

Another interesting observation was that SAP97 transfected cells showed bigger PSD surfaces than PSD-95 expressing neurons. Besides the variability and small numbers of observed spines, the explanation may be given by a stronger or different impact of SAP97, in comparison to PSD-95, on the organization of the postsynaptic scaffold and cytoskeleton (Regalado et al., 2006). Indeed, SAP97, which is well known to promote receptor and channel trafficking from the ER to the synapse in a CaMKII dependent manner (Mauceri et al., 2004; Gardoni et al., 2007; Mauceri et al., 2007), can also promote the growth of the synapse by stabilizing glutamate receptors at the postsynaptic site through scaffold proteins like Shank1a, SPAR (spine-associated RapGAP), and proSAP2 (Regalado et al., 2006). This is consistent with the ubiquitous role of SAP97 at cellular junction as a promotor of post- junction synchronizer observed indeed many cell types (Muller et al., 1995).

Interestingly, SAP97 overexpression promotes a stronger and more extensive ensheathing of axonal boutons than PSD-95, an effect which is not prevented by NOS pharmacological blockade by L-NAME. Mechanisms that lead to spine remodeling in synapse formation and axo-spinal juxtaposition are barely known. Nevertheless, the stable ratio between synapse size and spine size (Harris and Stevens, 1989; Knott et al., 2006) argues for a close correlation between synapse regulation and spine sizes and shapes. In my studies, SAP97 showed a dramatic increase of PSDs size associated with a specific enwrapping of presynaptic partners. The lack of effect of L-NAME on axon ensheathing suggests that this process does not pass through NOS signalling. Actin cytoarchitecture plays an essential role in spine formation and remodeling. Balance between actin polymerization and depolymerization, and between elongating and branching actin defines spine shape. SAP97 overexpressing spines spread thin and lamellar extensions enveloping axons requesting bundle of actin running parallel to the spine apposition on the bouton rather than a branched network distributed in 3 directions which would only increase spine volume. This suggests that actin cytoskeleton may be controlled by the postsynaptic PSD's proteins promoting synapse stabilization and wrapping around axonal boutons. Among them, the kinase-anchoring protein (AKAP) 79/150, a protein kinase A (PKA)- and calcineurin-protein phosphatase 2B (CaN)-anchoring protein that is linked to NMDARs and AMPARs through PSD-95 and SAP97 (Colledge et al., 2000), is localized to the PSD via an N-terminal targeting domain that binds PIP2, F-actin, and cadherin adhesion complexes (Gomez et al., 2002). Therefore

AKAP could be one of the links between the overexpression of SAP97 and the cytoskeletal modification leading to spine ensheathing. In addition AKAP is involved in the stabilization of AMPARs during LTP, and CaN induction of LTD leads to rapid CaN mediated dephosphorylation of PKA-phosphorylated GluR1 receptors (Dell'Acqua et al., 2006). Therefore, a modification of the glutamate receptors at the membrane, and a change in the Calcium concentration by an overexpression of MAGUK proteins may induce the switch between the PKA or the CaN pathways. The Rac1 guanine-nucleotide exchange factor kalirin-7 is also linked to SAP97 via PDZ domains. Kalirin-7 activates Rac1 which in turn promotes spine enlargement through p21-activated kinase (PAK) phosphorylation (Carlisle and Kennedy, 2005). Phosphorylation of cofilin by PAK is one of the pathways whereby Rho GTPases regulate actin filament assembly (Carlisle and Kennedy, 2005). In conclusion therefore, interactions between SAP97 and specific molecular partners expressed in PSDs could account for the different morphologies observed upon expression of SAP97 versus PSD-95. While these two molecules belong to the same family of scaffold proteins and are usually believe to have overlapping functions, these results indicate that they also have distinct properties and participate in different ways to the regulation of spine morphology.

3. N-Cadherin

My results suggest that either N-Cadherin is required for the expression of a PSD on a new protrusion, thereby accounting for the increased proportion of immature spines and spine-like protrusions devoid of PSDs, or N-Cadherin plays a role in spine stability, so that in its absence, protrusion dynamics is increased resulting in an increased proportion of newly formed immature spines and filopodia. The results obtained through analysis of spine dynamics with repetitive confocal imaging point rather to the second possibility.

Interestingly, analysis of pyramidal neurons transfected with wild type N-Cadherin also showed prominent modifications of spine morphology. As illustrated in figures 57a, 58 left and 59, spines are on average larger, their volume increases by 2 times. PSDs tend also to be larger but to a smaller extend, 1.5 times. Concerning spine types, the proportion of filopodia in Δ390-NCad transfected neurons was increased by almost 3 times comparing to eGFP control neurons. Together, these results thus indicate that expression of wild type N-Cadherin promotes spine enlargement, without affecting PSD size, and that disruption of N-Cadherin leads to an increase of immature protrusions. As spine size correlates in many studies with spine stability, these results are thus consistent with the interpretation that a central role of N-Cadherin could be to promote spine maturation and their long-term stability.

Recent works of Xie et al., showed that spine size and N-Cadherin content were tightly coordinated. Indeed, N-Cadherin plays crucial roles in nervous system development and physiology (Bamji, 2005). Previous works showed that altering N-Cadherin attenuated synapse maturation and interfered with synapse plasticity, for instance in LTP (Tang et al., 1998; Bozdagi et al., 2000). Some studies showed that disrupting N-Cadherin provoked the emergence of thin and elongated protrusions that look like immature spines with confocal microscopy observations (Togashi et al., 2002; Abe et al., 2004; Xie et al., 2008)(Xie et al., 2008). The N-Cadherin complexes involving α- and β-catenins is another kalirin-7 partner (Xie et al., 2008). LTP dependent spine remodeling involves N-Cadherin clustering at the synapse, and activation of the Kalirin-7/ F-actin polymerization spine enlargement (Xie et al., 2008). This pathways is activated through the scaffolding

protein AF-6/afadin (Xie et al., 2008). By overexpressing the Δ390 N-Cadherin mutant, I also observed immature spines that were thin and elongated with a small spine-head. However, after ultrastructural serial sectioning EM analyses, I have observed that these immature spines were bearing PSDs and that they were facing active zones. Following the rule of proportion between the spine head size and the PSD surface, these immature spines had smaller PSD surfaces than eGFP control spines. Therefore, N-Cadherin seems important for spine and synapse maturation, but not necessary for synaptic contact formation. We cannot totally exclude however that the endogenous N-Cadherin still produced in dominant negative, mutant N-Cadherin transfected cells was sufficient to ensure proper synapse formation.

With regard to the role of N-Cadherin in spine morphology, two interesting conclusions emerge. First overexpression of the adhesion molecule promoted the growth of the spine head and of the PSD, but in a very different manner than SAP97 and PSD-95. The effect is clearly more marked on the volume than the PSD and the shape of the spine head is relatively preserved comparing to morphologies observed under control conditions. In fact, N-Cadherin expressing spines look very much like control spines, except that they are larger. This would be consistent with other observations obtained in the lab indicating that one of the central effect of N-Cadherin is to stabilize synapses, particularly as a result of induction of plasticity. As stability is linked to spine dimensions, N-Cadherin could represent that mediates this correlation affecting both stability and size. How N-Cadherin affects stability remains unknown, but this could be linked to the adhesive properties of the molecule and the possibility that they form complexes across the synaptic cleft (Zuber et al. 2005). The effects on spine dimensions could on the other hand be linked to the signaling mediated through N-Cadherin. The recent study by Xie et al. (Xie et al., 2008) indeed suggests that N-Cadherin could signal to rho GTPases, PAKs and the cytoskeleton to regulate spine dimensions. Cadherin expression at the synapse could therefore fulfill an ideal function by controlling two major aspects of spine properties: the changes in size and the stability.

The second interesting observation concerning the morphology of spine upon expression of N-Cadherin is that unlike SAP97 or PSD-95, no abnormal alterations of either PSDs or number of contacts can be found. This indicates that N-Cadherin plays a very different role than scaffold proteins at the synapse. Scaffold proteins probably directly control the expression of PSD constituents, and overexpressing one probably results in an overexpression of many PSD proteins such as for example NOS and receptors. This probably also explains why the main morphological effect of SAP97 or PSD-95 expression is on the size of the PSD and why this leads to the formation of very peculiar shapes of spines with multiinnervations and lamellar aspects such as with SAP97. In contrast, the absence of such effects with N-Cadherin suggests that the molecule is not part of the same PSD complexes and rather regulate spine properties instead of participating to the PSD architecture. This would be consistent with evidence suggesting a localization of the N-Cadherin at the periphery of the PSD as well as the evidence obtained in the lab that not all synapses express N-Cadherin. One could even think of N-Cadherin as a marker of stable synapses that would be expressed only if the synapse has to be maintained through for example induction of plasticity. In any case these experiments clearly illustrate that expression of synaptic proteins may affect in very different ways the morphology of dendritic spines and thus provide interesting information on their function at the synapse.

4. Comparison of the different spine phenotypes

SAP97, WT-NCad and PSD-95 transfected neurons all show an increase in spine size. Interestingly, spine characteristics are different in each conditions. SAP97 transfected neurons showed a larger spine volume and PSDs surface than PSD-95 transfected neurons (fig.63). SAP97 had an increased ratio PSD surface/ spine volume (n=4 cells, 41 spines analyzed, ratio: 2.170 ± 0.12 (mean±sem)) that was increased when compared to eGFP control neurons (n=4 cells, 80 spines analyzed, ratio: 0.8278 ± 0.063) (fig.64). A qualitative analyze of the spine shape, as illustrated in figure 61, shows that SAP97 spines tend to engulf axonal boutons, whereas PSD-95 make multiple cup-shaped spines without encapsulating axonal boutons. This special feature of the postsynaptic element, that is able to enwrap the presynaptic bouton, has also been observed in SAP97 spines treated with L-NAME (fig.61). Thus the ensheathing characteristic of SAP97 does not appear to be linked to NO production, contrary to the formation of multiinnervated spines. The mechanism of this effect remains unclear, but could be linked to the marked increase in PSD size that promotes enwrapping of the terminal. This could also be related to other binding partners of SAP97 and for example an increased expression of adhesion molecules or molecules interacting with the cytomatrix and thus promoting membrane/membrane contacts with the terminals.

Data Summary	eGFP control	SAP97	SAP97--L-NAME	Δ390-NCad	WT-NCad	PSD-95 (I.Nikonenko)
n (# of cells)	5	4	4	4	4	7
# of spines	108	45	53	118	57	234
Spine volume (μm^3)(mean±sem)	0.053±0.0045	0.281±0.043	–	0.025±0.002	0.133±0.021	0.144
PSD surface (μm^2) (mean±sem)	0.042±0.0042	0.333±0.052	–	0.0288±0.002	0.0637±0.011	0.189
PSD surface/spine volume (mean±sem)	0.828±0.0626	2.7±0.118	–	–	0.602±0.0558	–
multiinnervated percentage (mean±sem)	4.2±1.9	43±8.7	8.8±3.2	–	13.6±0.74	29.1±2.9

Summary of the values for all the studies

Interestingly, N-Cadherin transfected neurons showed spines with volumes than for control eGFP neurons but not as big as on SAP97 transfected neurons. Their typical spine shape presented a strong and relatively short spine neck and spine heads that looked like average eGFP control spines but with increased volumes (fig.63). In addition, PSDs surfaces were also much smaller than in the MAGUK proteins transfected neurons. PSD surface/ spine volume ratio (n=4 cells, 52 spines analyzed, ratio: 0.6016 ± 0.056 (mean ±sem)) was even smaller than for eGFP control neurons, fig.64.

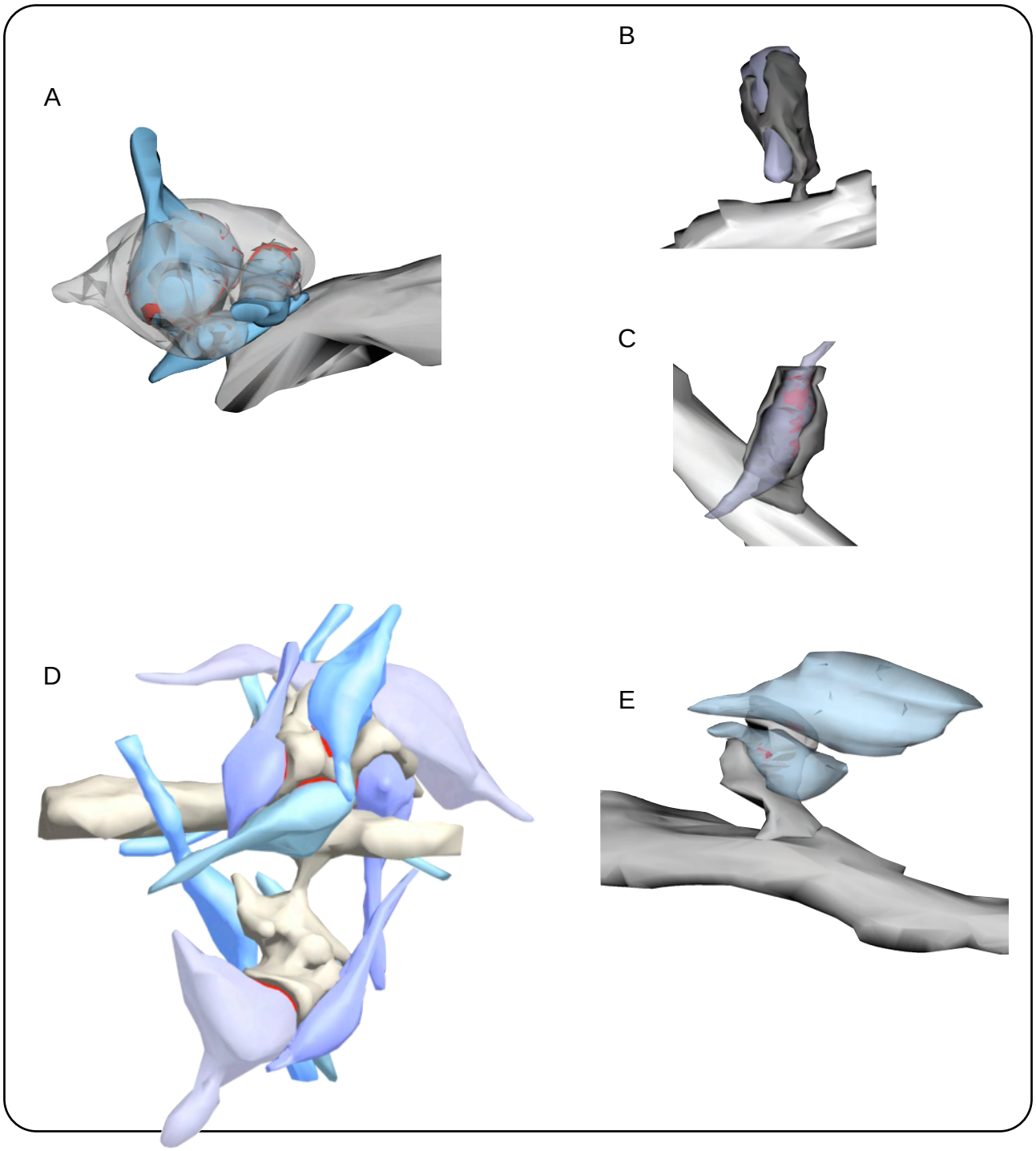


Fig.61 Comparison of large spines from SAP97, SAP97--L-NAME, PSD-95 and WT-NCad transfected neurons

A: SAP97 transfected neurons with 3 axonal boutons making synapses, PSDs in red. Boutons are ensheathed by the spine.
B, C: SAP97--L-NAME neurons ensheathing axonal boutons in a similar way than SAP97 not treated neurons.
D: PSD-95 transfected neurons with 2 multiinnervated spines. Spines make cup-shaped receptacle around boutons but without ensheathing.
E: WT-NCad transfected neuron with a large mutliinnervated spine (rarely observed), but any enwrapping and small PSDs (red). Spine neck is large. (courtesy from Irina Nikonenko, (Nikonenko et al., 2008))
Spines are fairly at the same magnification.

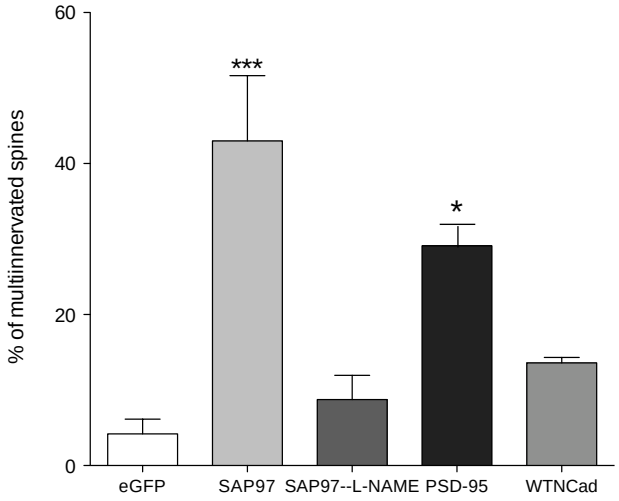


Fig.62 Multiinnervated spines in eGFP, SAP97, SAP97--L-NAME, PSD-95 and WT-NCad transfected neurons

SAP97 transfected neurons showed a significant increase (43 ± 8.7%) of multiinnervated spines versus eGFP controls (4.2 ± 1.9%), SAP97 effect was prevented by L-NAME treatment (8.8 ± 3.2%). PSD-95 (29.1 ± 2.9%,(Nikonenko et al., 2008, J Cell Biol, 183, 1115-27)) overexpresion experiments made by Irina Nikonenko also showed a significant increase in multiinervated spines. WT-NCad (13.60 ± 0.74%) showed a small increase that was not significant in comparison with eGFP control transfected neurons. ***, P< 0.0001.(One-way analysis of variance).*, P< 0.01 Data are mean ± SEM (error bars)

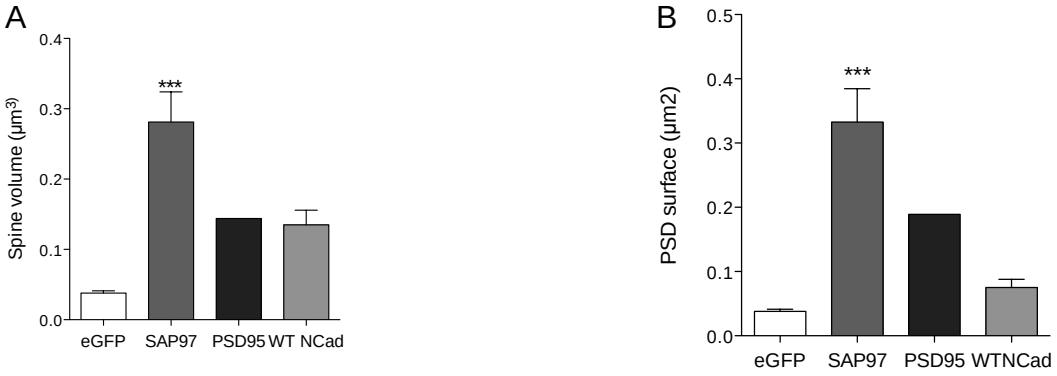


Fig.63 Spine volumes and PSDs surfaces comparison between SAP97, PSD-95 and WT-NCad

A: Spine volumes of SAP97 (n=4, 45 spines analyzed 0.281 ± 0.043 µm^3) transfected neurons were bigger than PSD-95 (PSD-95: 0.144 µm^3) and WT-NCad (n=4, 57 spines analyzed, 0.133 ± 0.021 µm^3) transfected neurons which were in turn bigger than eGFP control neurons.
B: PSDs surfaces were also strongly increased in SAP97 transfected neurons (n=4, 41 spines, 0.333 ± 0.052 µm^2) similarly to PSD-95 transfected neurons (0.189 µm^2), whereas WT-NCad (0.0637 ± 0.011 µm^2) showed a smaller increase. (One-way analysis of variance).***, P< 0.0001. Data are mean ± SEM (error bars).

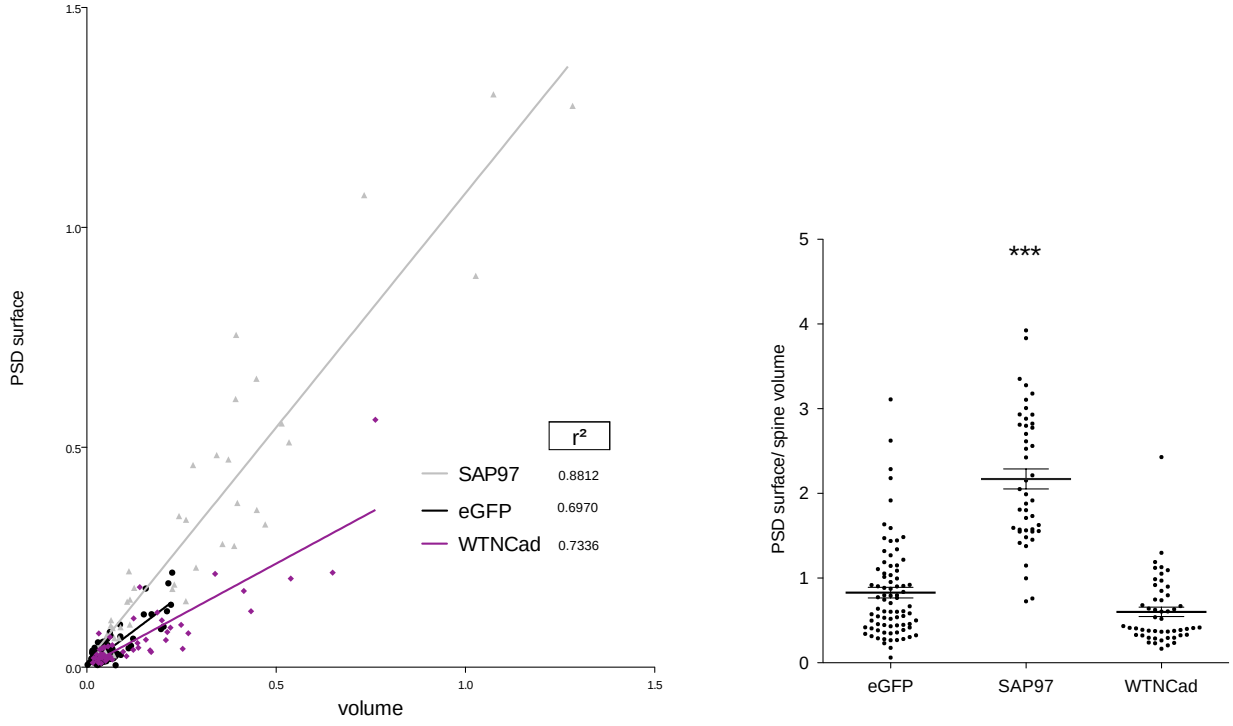


Fig.64 PSD surface/ volume ratio for eGFP, SAP97 and WT-NCad transfected neurons

Left: Linear regression between PSD surfaces and spine volumes showing that SAP97 transfected neurons made a shift of ratio towards PSD surface while WT-NCad made a shift towards spine volume in comparison with eGFP control neurons.
Right: A different representation of the PSD surface/ spine volume ratio that indicates the distribution for the three conditions. WT-NCad (n=4, 51 spines analyzed, 0.602 ± 0.0558) had a rather compact distribution versus the more spread distributions of SAP97 (n=4, 41 spines analyzed, 2.7 ± .118), and eGFP control transfected neurons (n=4; 81 spines analyzed, 0.828 ± 0.0626). (Kruskal-Wallis test) ***, P< 0.0001. Data are mean ± SEM (error bars).

Comparison of the morphologies of spines upon overexpression of different synaptic proteins leads to specific modifications probably reflecting the function and localization of these proteins at synapses. We found that overexpression of multi-modal scaffold proteins like SAP97 and PSD-95 induces an enlargement of spines and an enlargement of PSDs that is followed by peculiar morphologies specific to each molecule characterized by cup-shaped distortions of the spines at the point of contact with presynaptic boutons for PSD-95, and en-sheathing of spines around presynaptic boutons for SAP97. SAP97, as PSD95, promoted an increased PSD surface/spine volume ratio that may be explained by the important role of SAP97 and PSD95 in the architecture of the PSD. One might propose therefore that the primary effect of overexpressing these scaffold proteins was to enlarge the PSDs and that this was then accompanied by an enlargement of the spine volume. Enlargement of the PSD probably includes expression of a number of additional PSD proteins and not only SAP97 or PSD-95. This would account for the observation that in both cases, NOS expression is probably increased, leading to the formation of multiinnervated spines, a common feature of SAP97 and PSD-95 transfected cells. Interestingly however the shape of spines was not fully similar in cells transfected with SAP97 and PSD-95. Specifically, spines in SAP97 transfected neurons showed a characteristic ensheathing property that was not readily observed in PSD-95 transfected cells. This might eventually reflect the different binding partners of the two scaffold proteins and the possibility that SAP97 overexpression is associated with increased expression of other PSD components than with PSD-95. If this

is the case, then our data suggest that this effect does not involve NOS since spines ensheathed terminals were also observed following treatment with L-NAME, and not with PSD-95. Additional studies would however be required to identify the mechanism of this effect. Alternatively, these differences might also reflect the localization within the PDS of the two proteins. Localizations and binding partners of SAP97 and PSD-95 are similar but not completely the same in neurons and more specifically in dendritic spines. Therefore, SAP97 and PSD-95 act like homologue and complementary proteins at the level of spine and synapses, but with different specificities.

The results obtained with N-Cadherin expression also differ from those obtained with scaffold proteins PSD95 and SAP97. Spines were also enlarged, but the ratio of PSD surface/ spine volume was smaller for WT-NCad than for SAP97 or PSD-95 transfected cells and even than control neurons, this difference being especially noticeable for the big spines. Thus N-Cadherin affected more the volume than the PSD of spines. This suggests that N-Cadherin is more likely involved in signaling mechanisms regulating for example the spine cytoskeleton than the architecture of the PSD. This is consistent with recent evidence proposing that N-Cadherin is linked to the actin cytoskeleton regulatory pathway by signaling through RhoGTPases and PAKs and thus controlling spine size. Another interesting observation with N-Cadherin expression is that this did not result in multiinnervated spine formation, unlike expression of SAP97 and PSD-95. Again this result suggests that N-Cadherin expression does not result in increased expression of other PSD constituents. This could be accounted for by the fact that N-cadherin is not a primary constituent of the PSD and could actually be located at the periphery of the synapse. Through its transsynaptic signaling, N-Cadherin could rather have a regulatory function such as controlling size or stability of the synapse, but not represent a building block of the PSD.

The comparison between these large spines induced by different types of synaptic molecules provides evidence for the distinct functions of these proteins at the synapse and raise all sorts of new interesting questions. In particular they show the importance of ultrastructural analyses for better understanding how synaptic proteins contribute to shape synapse morphology and regulate its function.

The major information emerging from these studies is the importance to analyze the fine details of how expression of synaptic proteins affect spine morphology. The differences detected here using serial sectioning and 3D EM reconstruction would never have been and have not been observed by confocal microscopy despite the numerous studies that have been made. They reveal important aspects of the role of synaptic and PSD proteins in the regulation of synapse formation and persistence and open many new questions regarding the role and mechanisms through which these synaptic proteins might affect synaptic function and morphology. Considering that changes in spine morphology represent one of the hallmark of the defects reported in several developmental neuropsychiatric diseases and in particular in mental retardation, these studies point to the necessity to further and better characterized how synaptic proteins affects synaptic structures.

CONCLUSIONS

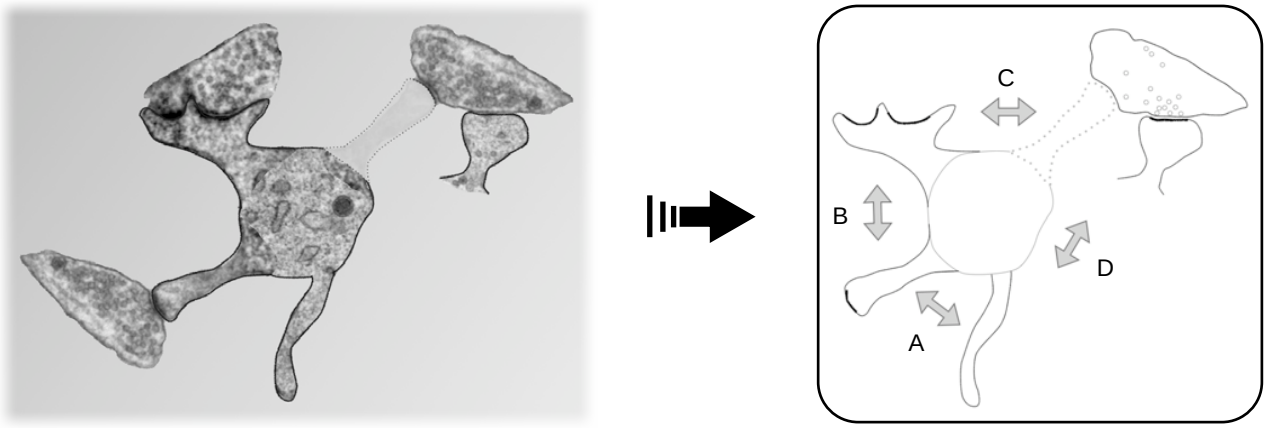


Fig. 65 Synopsis

Left: Photomontage of different protrusions stages observed in serial section Electron Microscopy **(**ssEM). Starting from the left clockwise: A small spine with almost no spine head but making a synapse with a presynaptic axon terminal (immature-like). Then a large and mature spine with perforated PSD making synapse with a unique presynaptic partner. Right, a sketch showing a disappearing spine which is, in some cases, in selective competition with an appearing spine. Below, a filopodia characterized by no PSD and no spine head and without active presynaptic partner.
Right: Schematic diagram resuming the macro changes occurring to dendritic protrusions.

A: Filopodial protrusions may switch to nascent spines after acquiring a PSD and making a synapse with a facing presynaptic partner.
-**The synapse formation requests scaffold proteins and GluRs recruited at the growing synapse as well as spine remodeling. My experiments suggest that SAP97 could be involved in such process, by promoting the formation of synaptic contact through the formation and enlargement of the PSD.**

B: Many works showed that spines adapt their size as a function of the activity they experience. Small spines are often enlarging after specific stimulation like LTP induction. Contrarily, large spines may also become smaller under certain conditions like LTD induction for instance, thus diminishing their volume.
-**SAP97 seems to be a central component of this process of activity-dependent spine maturation. My observations showed that SAP97 induces dramatic changes in spine morphology resulting in spines with complex PSDs that en-sheath the presynaptic partner, thus leading to the augmentation of the synapse surfaces.**
-Spine maturation also requests N-Cadherin. Upon expressing a non-functional, mutant N-Cadherin, spines remained immature and elongated, and thus could no longer become mature, while, contrarily, overexpression of N-Cadherin led to spine with large heads and stubby necks.

C: Neuronal remodeling is a dynamic process that includes formation and disappearance of spines or protrusions. Homeostatic and/or hebbian mechanisms modify this dynamic, thus remodeling the neuronal network in such order to fit to the new environmental stimuli.
- Our experiments show that SAP97 may be implicated in these processes through its indirect action on contact formation and the recruitment of scaffold proteins to the nascent and enlarging synapses. N-Cadherin is also critically implicated in spine dynamics, since the defects in spine maturation that we showed in mutant N-cadherin transfected cells leads to a compensatory mechanism that increases spine turnover via molecules linked to the actin-cytoskeleton regulating pathways.

D: Filopodia are versatile protrusions that appear quickly, but only a small percentage of new filopodia turn into stable protrusions (De Roo et al., 2008).
-Our experiments suggest that N-Cadherin is involved in the switch between a versatile protrusion and a stabilizing new spine.

PERSPECTIVES

Most current approaches use confocal imaging of spines in transfected neurons to examine whether and how interference with specific synaptic proteins affects global parameters such as spine number, length or spine head size. These parameters reveal however difficult to really appreciate at the confocal level and this probably explains why most of these studies failed to detect the important changes in spine morphology that we could identify through serial sectioning and 3D EM reconstruction.

An important message of this study is that a more careful analysis of the changes triggered by expression or mutation of synaptic proteins is highly valuable to better understand their synaptic role and function. It would be very interesting for example to extend this kind of analysis to a number of other constituents of the PSD and namely other scaffold proteins such as for example SHANK or HOMER, or other adhesion molecules such as integrins or NCAM. Another interesting open question could concern the signaling mechanisms implicated in some of these changes. For example one could wonder wether interfering with Rho GTPase signaling would affect the spine size changes observed upon overexpression of these PSD proteins. One could also use rescue approaches by expressing mutated variants of PSD proteins to test whether the structural changes reported here still occur. Knock down of the different binding domains of SAP97 would give informations on the type of binding necessary to induce a specific morphology, and on its associated molecule in particular mechanism. The ubiquitous binding sites borne by MAGUKs like the PDZ domains (Kurakin et al., 2007) would argue for a tagging or a knock down of their associated molecule for a fine understanding of their relationship.

A central question remains open concerning the orchestration of the molecular chain reactions triggered for instance by LTP-associated stimulation. Such event induces shift of type of molecules, like the replacement of GluR subunits and GluRs associated proteins (stargazin, GRIP1, AKAP), recruitment of scaffold proteins (PSD-95, SHANK), activation of specific kinases like c-AMP-PKA at the synapse. NMDARs may play a role of sensitive switch allowing the entry of calcium which would activate calcium related molecule like calmoduline/CaMKII and MyoVb (associated with recycling endosomes (REs)) which triggers rapid spine recruitment of endosomes and local exocytosis of AMPARs in spines (Wang et al., 2008). In the spine or at the synapse, what would be the concentration point of transition for a molecule to impose its associated function? Are the molecules interacting in a chaotic environment where the only important parameter is the concentration, or is there some very specific cascade of molecules that are recruited or activated in an Othello game mode as suggested by parallel kinases pathways observed in LTP studies (Wikstrom et al., 2003)?

Although several studies showed that synaptic molecules are important for spine and synapse formation and activity dependent spine remodeling, the exact function of each synaptic molecules and the hierarchy of their real importance is only starting to take place. Throughout species and cells, protein systems and solutions are either shared or recycled. Indeed, a protein can play a similar but slightly different role in some cases. Several explanations can be proposed for that, the most common one is the modification of the protein by a wobble of one or many amino-acids. However, the environment also plays a crucial role, a protein may express one of its functions, and not another, depending on the proteins or systems it will interact with. The shift of function is often observed with hormones, where depending on the level of

development, the protein will have a complete different function. An equivalent shift during development is also observed in neurons. GABA plays an excitatory role during a specific phase of neuron maturation, then it shifts into an inhibitory neurotransmitter. LTP induction is also subject to developmental switch (Yasuda et al., 2003).

A new era has come, where the questions concerning molecules will not only stay focused on a very punctual role of a molecule in a very simplified system, but will take account of the environment of the molecule, its number, the possible interactions with its potential partners. Thus, future technical means will allow to monitor more precisely molecules and system, for instance in their spatio-temporal evolution. It will then make an important steps toward the understanding of the major conditions that allow a molecule to play a specific role at a specific moment. Because of spatio-temporal resolution, we can bet that several studies (mainly unpublished for evident reasons) came to results impossible to interpret because of contradictory molecule behavior. This new dimension in molecule studies would help therapies that are focused on one or two molecules. Therapy are often counteracted by homeostatic or compensatory phenomenon that reduce the effect of a drug. The study of molecule mechanism could also take the advantage of the biodiversity. For instance the Drosophila discs-large (dlg) gene are members of the MAGUK family of proteins, as previously shown, a group of proteins involved in the localization, the transport and the recycling of receptors and channels in cell junctions, including the synapse. DLGA is similar to the vertebrate neuronal protein PSD-95, and DLGS97 is similar to the vertebrate neuronal and epithelial protein SAP97. DLGA is expressed in epithelia, neural tissue and muscle, whereas DLGS97 is expressed in neural tissue and muscle but not in epithelia. The distinctive difference between them is the presence in DLGS97 of an L27 domain (Lockwood et al., 2008; Mendoza-Topaz et al., 2008). The difference of cell type expression and presence of L27 domain between these variants is an example where the study of drosophila DLGS97 in comparison with vertebrates, or more specifically mammals, is of key relevance to collect precious informations on the role of synaptic MAGUKs in neurons.

My PhD work indicated that ultrastructural analyses may be of great help to sort the specific function of the numerous proteins that form the PSD and to understand how they affect the various functional characteristics of spines. Nevertheless, horizontal studies between species and different cell types may help to understand what are the gain or loss of function that occurred during the evolution in complex and very specific cells like neurons. By extrapolation, it would help to understand the molecular interactions in vertebrate neurons.

PERSONAL COMMENTS

This long lasting PhD gave me the opportunity to approach questions in neuroscience with interesting techniques like confocal microscopy, immuno-staining and even informatics. Nevertheless, the core of this PhD has been devoted to serial section electron microscopy. Electron microscopy brought substantial answers since the mid-fifties, in the 80's with molecular biology or confocal microscopy, EM turned to be put aside. Interestingly, the advances made in the field of confocal microscopy brought new questions and challenges for electron microscopists. Since a few years, automatization and informatics tools allow electron microscopists to produce large 3D reconstructions of neuronal tissue, meanwhile offering a precious view on the ultrastructure of a large number of neurons or synapses at one time. Clearly, 3D reconstruction will be a new gold standard in fundamental neuroscience, but also in the field of histopathology.

Nevertheless, the transition between the 2D electron microscopy to an automatized one is a big challenge. Indeed, 3D electron microscopy is a harsh work that need much patience, perseverance and chance sometime. When such observation technique is linked to tissue that has first to undergo experimental conditions, and when the object is not an entire tissue, but a specific cell, the game took proportion that seemed to be almost impossible to reach at the beginning of my thesis. Therefore, I am proud of two things: the abilities that I have acquired after years of training, and the fact that I have not abandoned my work despite the months and year of bad weather.

Last but not least, this bad weather led me to diversify my extra-lab activities to supply a positive and valorizing enthusiasm to my mood. Among them, my function as officer on call in case of radionuclear, biological or chemical events for the *Département du territoire de Genève* opened my mind on the risk of human technics on its close and global environment. In addition, my involvement in the organization of the european communication campaign for the Lemanic Neuroscience (FENS 08) in an institutional and inadequate context brought me a valuable experience for future jobs.

CURRICULUM VITAE

Lorenzo Pietro Poglia

PhD Candidate, Master's in Biology, Licentiate in Biology.

Date and Place of Birth: September 2nd, 1976, Neuchâtel, CH.

Educational Background

•**Ph.D.**
Department of Fundamental Neuroscience, Faculty of Medicine, University of Geneva. 2003-2009

•**Master's in Biology**
Department of Fundamental Neuroscience, Faculty of Medicine, University of Geneva. 2001-2003
Major in Neurosciences 5.5/6.

•**Licentiate in Biology**
Faculty of Sciences, University of Geneva. 1997-2001
Major in Molecular Biology 4.8/6.

Languages

•**French:** Mother Tongue.
•**Italian:** Mother Tongue (reading, speaking).
•**English:** Fluent - Cambridge Advanced Certificate.
•**German:** Intermediate.

Professional Experience

•**Research and Teaching Assistant**

Department of Fundamental Neuroscience, Faculty of Medicine, University of Geneva 2003-2009

Conducted research in neurosciences. Lectured in student seminars and lectured for children

Research topic: Brain remodeling at the level of neuronal network.

The aim of my studies is to understand what roles and functions may have few selected molecules and related pathways on dendritic spine synapse morphology. To address this question, genetic modifications are induced in neurons by biolistic transfection. Genes expression is observed by confocal microscopy. Finally, serial section electron microscopy is followed by 3D reconstruction and image analysis. The model used is the organotypic hippocampal slice cultures from rat brains.

Expertise

-Transfection: biolistic system, lentivirus.

-Immunostaining.

-Confocal microscopy on organotypic hippocampal slice culture. Previews and follow up

-Serial section electron microscopy for 3D neuron reconstruction (Mosaic Builder/Reconstruct)

-Serial section EM alignment using a new softwear (Mosaic Builder) made in collaboration with Spaltenstein Natural Image. Testing of alignment automatisation codes, under process.

•**Member of the NrBC unit Geneva Cantonal Nuclear/ Biological/ Chemical Unit (SIE) 2007-2009.**

-Officer on call in case of nuclear, biological or chemical events. Part of the cantonal inter-services security plan (OSIRIS); part time.

-Related to ABC lab specialist in the Swiss Army Spiez Laboratories. Conducted a project for a neurotoxic detection device on the basis of multielectrode chip.

•**Mosaic Builder. Electron Microscopy image analysis software by Spaltenstein Natural Image. 2007**

-Interface designing. As Electron Microscope Expert.

-Icon designing for the Mosaic Builder software.

•**European communication campaign for the Lemanic Neuroscience** Univesity of Geneva, University of Lausanne, Ecole Polytecnique Fédérale de Lausanne.2008

Actively involved in the conception and management of a new image.

-Logo and website design: worked with a graphic designer (Superposition design).

-Video clip: writing of the scenario, acting. Filmed with a redactor of the german television RTL2 (Daniel Boehm).

•**FENS Forum Geneva 2008.** European Forum of the Federation of the European Neuroscience Societies.
Organization (Comity) of satellite communication events for young scientists.2008

-Creator of the JUMP-THE-FENS! 08 concept.
-Maker of the JUMP-THE-FENS! 08 website.
-3 nights at the United Nation Beach.

Extra-Curricular Activities

•**Astronaut recruitment selection for ESA** (European Space Agency) 2008
Invited by ESA in Hamburg for second round tests after first round selection. Among the 750/8500.

•**Ski teacher Sports University of Geneva**. Winter 2007-2008
Taught ski to students of the University of Geneva

•**High-school Biology teacher Ecole de Culture Générale Jean-Piage**t. 2001-2002
Taught biology to four classes; part time 40%.

•**Wattman Geneva Mass Transport Company** (Transports Publics Genevois -TPG). 1999-2001
Worked as tram driver; part time 20%.

•**Boat pilot Geneva water ski club** (Le Reposoir). summer 1998
Worked as boat pilot for water skiers and wake boarders.

•**University of Geneva**

-President of the Biology Student's Association 1998-2000
-Member of the Biology Department Council 1999-2001
-Member of the University Tuition Fees Management Committee 1999-2001
-Fuchs Major and President of the Student Society Stella Genevensis. 1999-2002

Interests & Hobbies

Nanbudo, Judo, skiing, snowboarding, wake boarding, kite surfing, diving, football.
History and Geopolitic. Theatre - main roles in plays of the University of Geneva Theatre Troupe.

Personal bibliography

Role of SAP97 in multi-innervated spines formation.
Poglia L, Nikonenko N, Muller D
Paper in preparation.

N-cadherin participates in synapse stabilisation.
PM.Garcia, Poglia L, De Roo M, Klauser P, Muller D
Paper in preparation.

Spine dynamics and synapse remodeling during LTP and memory processes.
De Roo M, Klauser P, Garcia PM, Poglia L, Muller D.
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APPENDIX

Niels Stensen (Discours sur l'anatomie du cerveau)

Warning: As the text of Stensen is in french I will write the next section in french.

Niels Stensen

Fig.65
Niels Stensen (1638-1686)

Niels Stensen (1638-1686; Nicolas Sténon en français, Niccolò Stenone en italien) grandit dans un cercle aisé. Il est le fils de Sten Pedersen un orfèvre et joaillier de la court qui est lui-même fils d'un prêcheur de Skåne. Sa nature fragile lui empêche dès son plus jeune âge de vaquer aux loisirs quotidiens des enfants de son âge. Ceci le pousse à l'écoute des adultes, en particulier lorsqu'ils parlent de religion. L'atelier de son père devient son premier laboratoire où jusqu'à son départ à l'âge de 21 ans, il explore une multitude de thèmes. Au delà des mesures de l'or qu'il effectue, il décrit les machines hydrauliques. Sur la même voie de la curiosité qui le pousse à tous les mystères de la nature, il construit un microscope avec deux lentilles, il étudie le salpêtre et le sulfure et produit un colorant rouge au vitriol. Stensen commence ses études de médecine à l'université de Copenhague en 1656. Il part pour Rostock en Allemagne, puis à Amsterdam où il décrit le conduit salivaire (*Ductus stenonianua*). Cette découverte lui vaut un conflit avec son ancien professeur, le professeur Blassius. Passant rapidement sur les époques de sa vie, Stensen arrive en France dans les cercles éclairés. C'est à Paris, en 1665, dans une réunion du cercle de Thévenot (le chambellan et libraire de Louis XIV) future Académie royale des Sciences que Stensen partage son élégante et légendaire présentation sur l'anatomie du cerveau: ¨Discours sur l'anatomie du cerveau¨. Cette présentation en français ne lui pose pas de problème lui qui parle danois, allemand, dutch, français, italien, latin, grec, arabe et hébreu.

Bien qu'ici ne sera évoqué que son penchant pour l'anatomie, il ne faut pas oublier que Stensen s'est intéressé de près à la géologie et à bien d'autre sujets. En 1669, après de perspicaces observations lors de ses nombreux voyages en Italie, Stensen publie un ouvrage fondamental en géologie et thèmes liés : "*De solido intra solidum naturaliter contento dissertationis prodromus*". De ce recueil de recherche, on peut tirer quatre avancées majeures dans autant de voies de recherche (néologismes pour l'époque).

1. En cristallographie : Il démontre que les cristaux restent constant en proportion, ce qui se traduit par l'invariabilité des angles des faces de cristaux de quartz en rapport à leur taille et leur aspect.
2. En paléontologie : Il suggère que les fossiles représentent les restes des organismes ayants disparus.

3. En géologie : Il apporte des arguments importants concernant la formation des montagnes. Pour lui, ce sont les mouvements de la croûte terrestre ainsi que l'érosion qui façonne le paysage.
4. En géologie toujours : Il avance la notion de strates découlant du phénomène de sédimentation.

Par l'articulation des différentes voies de recherche et grâce aux études approfondies sur les couches sédimentaires et les fossiles, Stensen prouve qu'il est possible de reconstituer l'histoire géologique d'une région. Ce travail sera effectué en Toscane. Cette capacité à remettre en doute les acquis et de prendre de nouveaux angles d'attaque sur les sujets qu'il étudie lui sera très utile pour transformer l'étude de l'anatomie.

Une nouvelle approche méthodologique pour l'anatomie

Il ne sera traité ici que de l'anatomie du cerveau bien que Stensen s'intéressa à l'anatomie en général.

A son époque, comme il souligne dans son discours, il est d'usage de faire des leçons d'anatomie qui s'inspirent fortement par leurs méthodes et leurs connaissances des textes d'Aristote et de Galien. Sa curiosité, sa liberté intellectuelle, et son habilité dans la pratique expérimentale le mène à modifier clairement les us-et-coutumes du monde de l'anatomie. Les morceaux choisis du texte découlant du ¨Discours sur l'anatomie¨ sont ventilés au fil du texte.

Commençons par une brève radiographie du milieu dans lequel sa pensée évolue

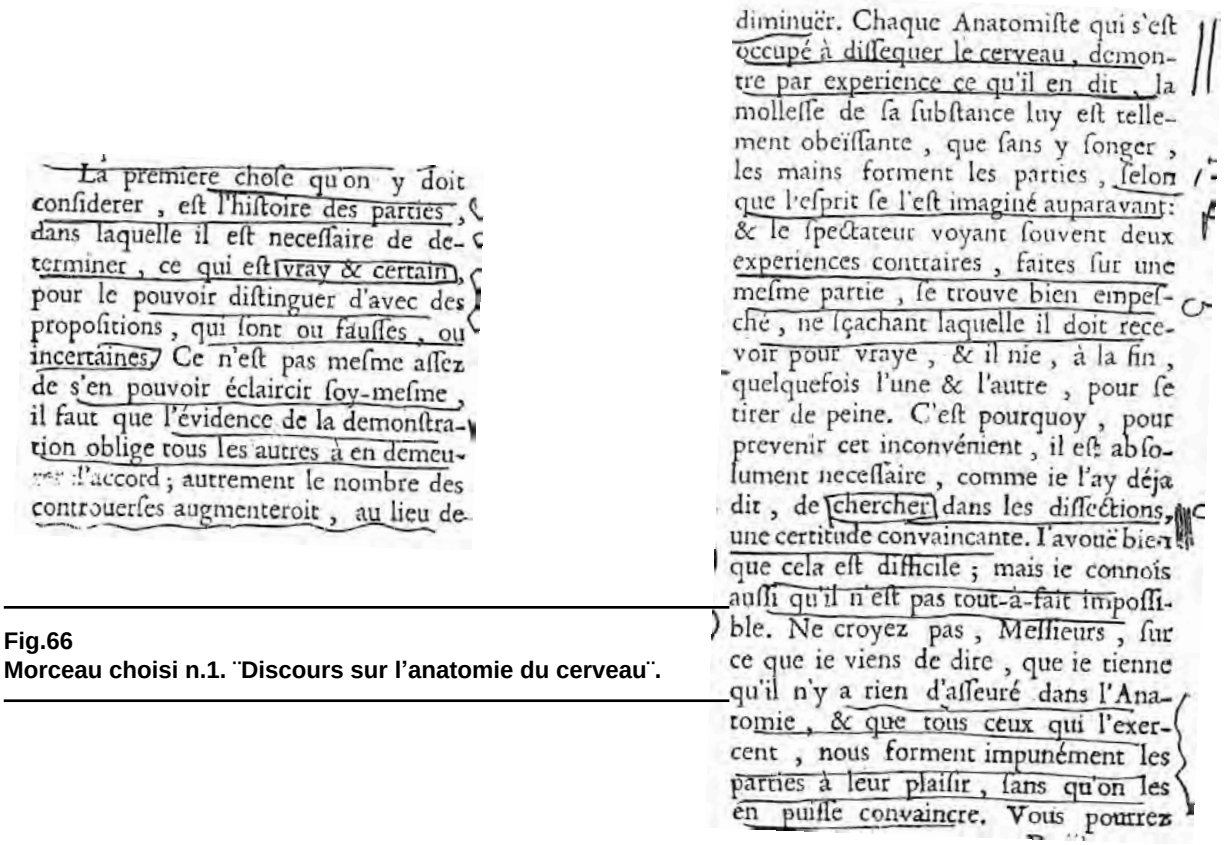
Comme mentionné plus haut, il est présent dans une multitude de discipline. Son nom court dans les réseaux de pointe de l'époque, il participe entre autre à :

- Le cercle de Thévenot qui plus tard deviendra l'Académie royale des Sciences sous l'impulsion de Louis XIV qui dans cette époque de naissance d'une nouvelle méthodologie générale en science est convaincu de devoir se trouver au centre du progrès.

-*L'Accademia del Cimento (Accademia dell'esperimento)*, est la première société savante scientifique à utiliser la méthode expérimentale galiléenne en Europe (1657). Elle est fondée à Florence en 1657 par Léopold de Médicis et le Grand-duc de Toscane Ferdinand II de Médicis et compte parmi ses membres Galileo Galilei, Evangelista Torricelli, Vincenzo Viviani et Giovanni Borelli.

Très croyant et nommé prêtre en 1675, chose à laquelle il consacrera le restant de ses jours. Il essaie même de convaincre Spinoza (1632-1677) de se rallier à se idées religieuses. Il fréquente donc les sphères les plus éclairées de l'époque. Stensen représente l'homme savant et très cultivé de son époque préfigurant les encyclopédistes. Il fait de brillantes études d'anatomie et de sciences naturelles sous la conduite de Thomas Bartholin (1616-1680) et chez l'anatomiste et botaniste Simon Pauli (1603-1680). Un livret de Stensen retrouvé en 1949 dont le titre est ¨Chaos¨ (peut-être en regard à la mixité de son contenu) révèle qu'il a lu des centaines d'ouvrages scientifiques écrit par au moins huit auteurs. Stensen est fortement influencé par cette époque qui voit l'émergence des sciences d'observation et de l'expérimentation. Pour ne

se limiter qu'à la méthode, Francis Bacon et Robert Boyle sont considérés comme les fondateurs de la méthode expérimentale. On notera ici l'importance du texte philosophique de Bacon de 1620, *Novum Organum*. Ce titre qui se traduit par ¨nouvel instrument¨ fait écho à l'oeuvre d'Aristote , ¨Organon¨ qui traite de logique et de syllogisme. Dans *Novum Oganum,* Bacon détaille un nouveau système de logique qu'il croit être supérieur à l'ancienne voie du syllogisme. Pour Bacon, trouver l'essence d'une chose est un simple processus de réduction et d'utilisation d'un raisonnement d'induction. Il est capital de tenir compte de ces influences pour comprendre le processus psychologique qui permet à Stensen de participer à une nouvelle méthodologie en anatomie.

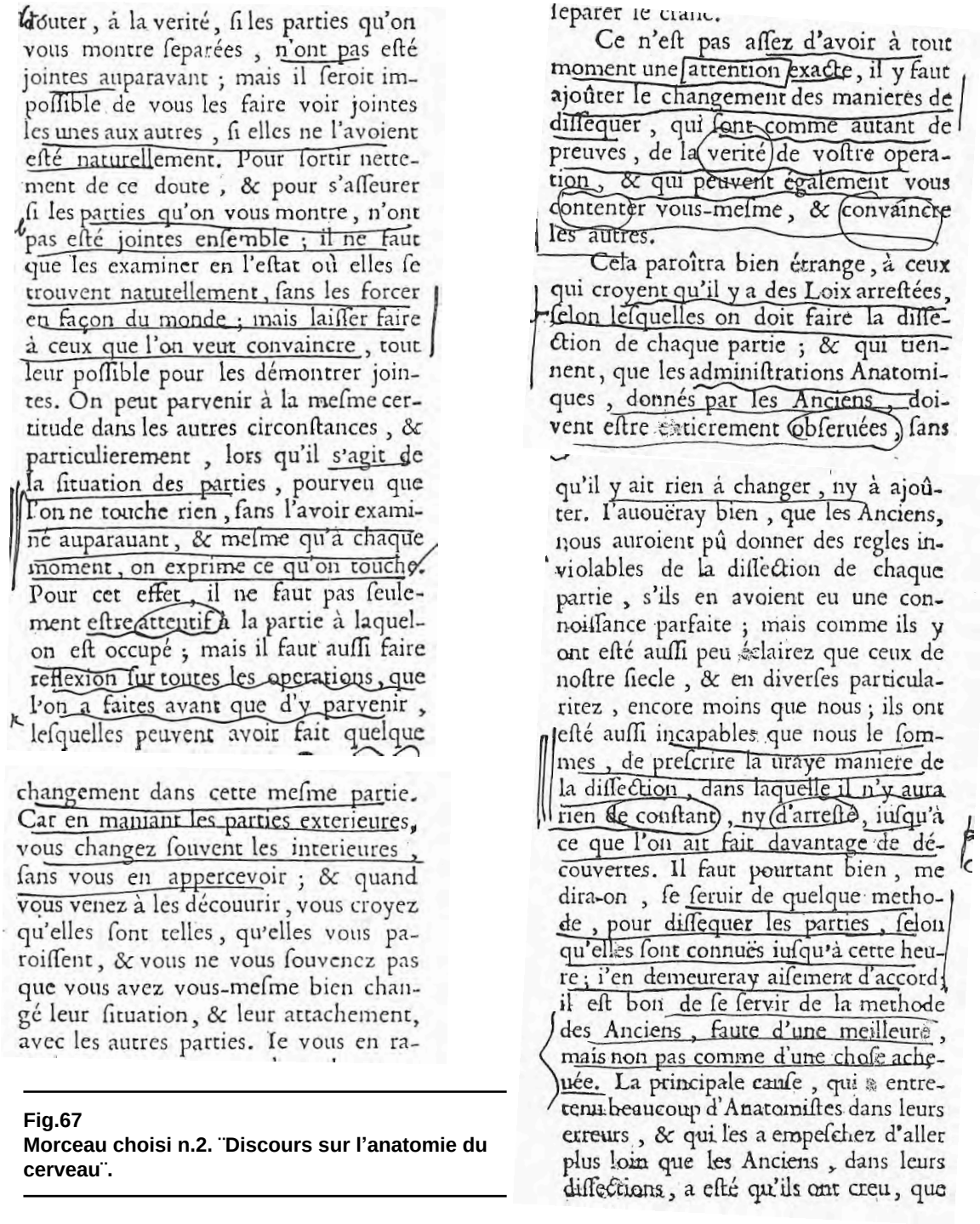


La premiere choſe qu'on y doit conſiderer , eſt l'hiſtoire des parties, dans laquelle il eſt neceſſaire de determiner , ce qui eſt vray & certain, pour le pouvoir diſtinguer d'avec des propoſitions , qui ſont ou fauſſes , ou incertaines. Ce n'eſt pas meſme aſſez de s'en pouvoir éclaircir ſoy-meſme , il faut que l'évidence de la demonſtration oblige tous les autres à en demeurer d'accord ; autrement le nombre des controuerſes augmenteroit , au lieu de diminuër. Chaque Anatomiſte qui s'eſt occupé à diſſequer le cerveau , demontre par experience ce qu'il en dit , la molleſſe de ſa ſubſtance luy eſt tellement obeïſſante , que ſans y ſonger , les mains forment les parties , ſelon que l'eſprit ſe l'eſt imaginé auparavant: & le ſpectateur voyant ſouvent deux experiences contraires , faites ſur une meſme partie , ſe trouve bien empeſché , ne ſçachant laquelle il doit recevoir pour vraye , & il nie , à la fin , quelquefois l'une & l'autre , pour ſe tirer de peine. C'eſt pourquoy , pour prevenir cet inconvénient , il eſt abſolument neceſſaire , comme ie l'ay déja dit , de chercher dans les diſſections, une certitude convaincante. I'avouë bien que cela eſt difficile ; mais ie connois auſſi qu'il n'eſt pas tout-à-fait impoſſible. Ne croyez pas , Meſſieurs , ſur ce que ie viens de dire , que ie tienne qu'il n'y a rien d'aſſeuré dans l'Anatomie , & que tous ceux qui l'exercent , nous forment impunément les parties à leur plaiſir , ſans qu'on les en puiſſe convaincre. Vous pourrez

Fig.66
Morceau choisi n.1. ¨Discours sur l'anatomie du cerveau¨.

Critères du retour à l'objet naturel (critique des artefacts)

L' anatomie se veut être une science descriptive qui se base sur l'observation des corps. Cette observation des parties du corps requiert que l'on altère l'intégrité du corps par les outils de dissection. Ce qui demande donc une méthode dont les résultats de la dissection en découle. C'est pourquoi Stensen met en garde contre le fait de croire trop vite ce que l'on vous présente. Lui dit de lui même qu'il ne ¨sait rien¨. Ce qui le place dans une approche ouverte sur le sujet plutôt qu'enfermé dans une prison de préjugés. De plus, il ne faut pas uniquement se convaincre soi-même, mais également les autres. Il souligne également l'importance de décrire chaque pas qui est fait pendant la dissection. En effet, la méthode de dissection influence directement les parties à décrire. C'est pourquoi, il propose de se libérer des méthodes des anciens, non pas en le rejetant simplement, mais en s'y inspirant et en les dépassant sans taboo.



ſouter, à la verité, ſi les parties qu'on vous montre ſeparées, n'ont pas eſté jointes auparavant ; mais il ſeroit impoſſible de vous les faire voir jointes les unes aux autres, ſi elles ne l'avoient eſté naturellement. Pour ſortir nettement de ce doute, & pour s'aſſeurer ſi les parties qu'on vous montre, n'ont pas eſté jointes enſemble ; il ne faut que les examiner en l'eſtat où elles ſe trouvent naturellement, ſans les forcer en façon du monde ; mais laiſſer faire à ceux que l'on veut convaincre, tout leur poſſible pour les démontrer jointes. On peut parvenir à la meſme certitude dans les autres circonſtances, & particulierement, lors qu'il s'agit de la ſituation des parties, pourveu que l'on ne touche rien, ſans l'avoir examiné auparauant, & meſme qu'à chaque moment, on exprime ce qu'on touche. Pour cet effet, il ne faut pas ſeulement eſtre attentif à la partie à laquelle on eſt occupé ; mais il faut auſſi faire reflexion ſur toutes les operations, que l'on a faites avant que d'y parvenir, leſquelles peuvent avoir fait quelque

changement dans cette meſme partie. Car en maniant les parties exterieures, vous changez ſouvent les interieures, ſans vous en appercevoir ; & quand vous venez à les découurir, vous croyez qu'elles ſont telles, qu'elles vous paroiſſent, & vous ne vous ſouvenez pas que vous avez vous-meſme bien changé leur ſituation, & leur attachement, avec les autres parties. Ie vous en ra-

ſeparer le crane.

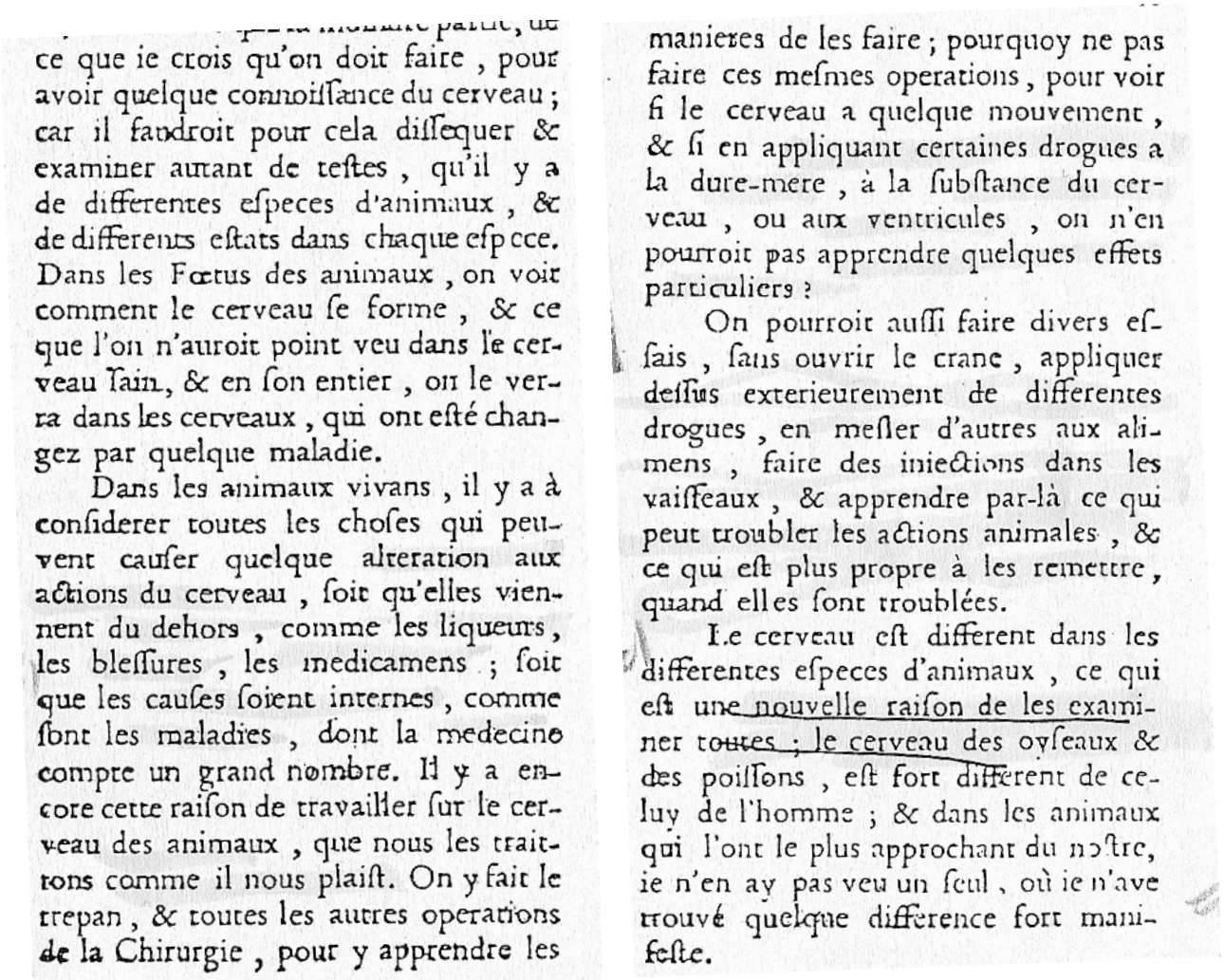
Ce n'eſt pas aſſez d'avoir à tout moment une attention exacte, il y faut ajoûter le changement des manieres de diſſequer, qui ſont comme autant de preuves, de la verité de voſtre operation, & qui peuvent également vous contenter vous-meſme, & convaincre les autres.

Cela paroîtra bien étrange, à ceux qui croyent qu'il y a des Loix arreſtées, ſelon leſquelles on doit faire la diſſection de chaque partie ; & qui tiennent, que les adminiſtrations Anatomiques, donnés par les Anciens, doivent eſtre entierement obſeruées, ſans

qu'il y ait rien á changer, ny à ajoûter. I'auouëray bien, que les Anciens, nous auroient pû donner des regles inviolables de la diſſection de chaque partie, s'ils en avoient eu une connoiſſance parfaite ; mais comme ils y ont eſté auſſi peu éclairez que ceux de noſtre ſiecle, & en diverſes particularitez, encore moins que nous ; ils ont eſté auſſi incapables que nous le ſommes, de preſcrire la uraye maniere de la diſſection, dans laquelle il n'y aura rien de conſtant, ny d'arreſté, iuſqu'à ce que l'on ait fait davantage de découvertes. Il faut pourtant bien, me dira-on, ſe ſeruir de quelque methode, pour diſſequer les parties, ſelon qu'elles ſont connuës iuſqu'à cette heure ; i'en demeureray aiſement d'accord ; il eſt bon de ſe ſervir de la methode des Anciens, faute d'une meilleure, mais non pas comme d'une choſe achevée. La principale cauſe, qui a entretenu beaucoup d'Anatomiſtes dans leurs erreurs, & qui les a empeſchez d'aller plus loin que les Anciens, dans leurs diſſections, a eſté qu'ils ont creu, que

Fig.67
Morceau choisi n.2. ¨Discours sur l'anatomie du cerveau¨.

En plus de promouvoir un nouvel état d'esprit qui mène à remettre en cause les méthodes, et d'adapter celles-ci pour qu'elles puissent faire transparaître les parties du cerveau en les altérant le moins possible; il propose d'ouvrir le champ de l'anatomie aux autres espèces animales. Ceci facilite l'accès aux cerveaux (sous-entendu autres que des cerveaux d'humains qui sont par éthique à l'état de mort) et à l'expérimentation animale, à savoir qu'il est possible de tester par des lésions ou d'autres moyens les fonctions liées à l'anatomie, ce que nous appelons aujourd'hui l'anatomie fonctionnelle. Mais tout comme pour la géologie, il est important de considérer différents paysages pour se faire une idée des mécanismes qui régissent leur formation.



ce que ie crois qu'on doit faire, pour avoir quelque connoiſſance du cerveau; car il faudroit pour cela diſſequer & examiner autant de teſtes, qu'il y a de differentes eſpeces d'animaux, & de differents eſtats dans chaque eſpece. Dans les Fœtus des animaux, on voit comment le cerveau ſe forme, & ce que l'on n'auroit point veu dans le cerveau ſain, & en ſon entier, on le verra dans les cerveaux, qui ont eſté changez par quelque maladie.

Dans les animaux vivans, il y a à conſiderer toutes les choſes qui peuvent cauſer quelque alteration aux actions du cerveau, ſoit qu'elles viennent du dehors, comme les liqueurs, les bleſſures, les medicamens; ſoit que les cauſes ſoient internes, comme ſont les maladies, dont la medecine compte un grand nombre. Il y a encore cette raiſon de travailler ſur le cerveau des animaux, que nous les traittons comme il nous plaiſt. On y fait le trepan, & toutes les autres operations de la Chirurgie, pour y apprendre les manieres de les faire; pourquoy ne pas faire ces meſmes operations, pour voir ſi le cerveau a quelque mouvement, & ſi en appliquant certaines drogues a la dure-mere, à la ſubſtance du cerveau, ou aux ventricules, on n'en pourroit pas apprendre quelques effets particuliers?

On pourroit auſſi faire divers eſſais, ſans ouvrir le crane, appliquer deſſus exterieurement de differentes drogues, en meſler d'autres aux alimens, faire des iniections dans les vaiſſeaux, & apprendre par-là ce qui peut troubler les actions animales, & ce qui eſt plus propre à les remettre, quand elles ſont troublées.

Le cerveau eſt different dans les differentes eſpeces d'animaux, ce qui eſt une nouvelle raiſon de les examiner toutes; le cerveau des oyſeaux & des poiſſons, eſt fort different de celuy de l'homme; & dans les animaux qui l'ont le plus approchant du noſtre, ie n'en ay pas veu un ſeul, où ie n'ave trouvé quelque difference fort manifeſte.

Fig.68 Morceau choisi n.3. ¨Discours sur l'anatomie du cerveau¨.

De la même manière, la comparaison des structures du cerveau de différents animaux peut expliquer leurs différences et leurs similitudes. L'étude des foetus revêt aussi pour Stensen une approche nécessaire, tout comme l'étude des maladies qui permettent de faire un lien entre le symptôme et les parties du cerveau touchées. Encore une fois, il promeut l'observation parallèle qui permet de ne pas limiter la réflexion à une seule voie. Il propose également d'utiliser des drogues qui soient administrées à la dure-mere, à la substance du cerveau, aux ventricules et d'en observer les effets. Sur la même lancée, il suggère d'introduire par les aliments ou par les veines différentes drogues aux animaux. Ceci évite de blesser le cerveau, et cela permet de constater les troubles induits pour mieux comprendre les effets positifs ou négatifs des drogues sur le cerveau.

Stensen décrit la matière blanche, la matière grise et les fibres qu'il voit se ramifier entre les parties du cerveau. Il s'interroge sur la continuation de ces fibres. Il introduit donc la notion de fonctionnalité. La vie est donc toujours forme, structure et fonction. Intéressé pendant son jeune âge par la description des machines hydrauliques, il voit comme certains de ses contemporains éclairés, le cerveau comme une machine dont on doit comprendre les parties pour en comprendre le mécanisme général. Stensen suggère donc de consacrer beaucoup temps à la recherche, ce que les médecins et les chirurgiens ne font pas selon

lui. Car une démonstration prend qu'une demi-heure ou plus, alors que les éléments nécessaires à la démonstration demandent parfois des années entières à être découverts.

Conclusion

Niels Stensen est un précurseur dans toutes les disciplines dans lesquelles il s'essaie. Sa force réside dans son immense culture, ainsi que dans la méthodologie scientifique nouvelle qu'il applique à tous les domaines de recherches. Au delà des avances qu'il a amenées en géologie, Stensen relit l'anatomie de son temps : le coeur prend sa fonction et son statut de muscle pompe, le cerveau prend sa véritable importance, il est le l'hôte de l'âme, et de ce fait mérite d'être étudié en détail. Cependant, il considère le cerveau comme une machine, donc il le démystifie, et permet son étude dans sa fonctionnalité. En avançant l'idée que des drogues, des lésions puissent altérer les fonctions du cerveau, il introduit la notion de compartiments fonctionnels au sein du cerveau. Stensen fait montre d'une grande force de proposition.

Les critiques

Il critique durement l'état d'esprit des anatomistes de l'époque. ¨Je ne sais rien¨ dit-il, ¨Mais ils n'en savent pas plus¨ ajoute-t-il en références aux anciens. Les connaissances anatomiques ne sont pas solides pour Stensen, car trop de précipitation et d'imagination influence les anatomistes. Il plaide pour moins d'autosuffisance et une recherche sérieuse qui prenne son temps et s'assume, et n'ait pas peur de ce qu'elle trouve. En résumé, il milite pour une rénovation de l'anatomie du cerveau.

Propositions

- ¨Il faut étudier la forme, les relations entre les parties, et les insertions et connections des fibres.¨
- ¨Les écoles de dissection sont utiles, cependant il faut innover et s'en libérer.¨
- ¨Les opérations de l'anatomie du cerveau sont créatrices d'artéfacts (obstacle de l'objet, du vocabulaire, des instruments et des opérations elles-mêmes). ¨
- ¨L'imagination est un guide trompeur (critiques des anatomistes théoriciens et des cartésiens- la glande pinéale de Descartes)

Et plus fondamentalement encore :

- ¨Les observations et démonstrations doivent être claires et évidentes et reposer sur des opérations critiques. ¨
- ¨Il faut appliquer l'analyse cartésienne sans les préjugés de Descartes.¨

Finalement, c'est un véritable programme de recherche qui est proposé.

Commentaire personnel

Stensen est d'une certaine façon le ¨père¨ de l'anatomie fonctionnelle. Actuellement, l'anatomie fonctionnelle fait des pas de géant grâce aux techniques non invasives. De même, à l'autre extrémité, la morphologie des acteurs de la plasticité synaptique est aussi à l'ordre du jour. Rappelons-nous qu'il a fallut un centenaire depuis Cajal et ses compères pour qu'enfin l'on dépasse la simple description des éléments et que l'on puisse donner des fonctions aux différentes morphologies que l'on trouve à ce niveau là d'anatomie.