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TSPAN5 is a key player in dendritic spines formation and AMPA receptors recycling

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Abstract

Abstract

TSPAN5 is a brain enriched protein member of the tetraspanin superfamily, a group of transmembrane proteins some of which have been shown to fundamentally regulate the development of mammalian nervous system. This class of proteins presents the peculiar ability to clusterize forming specialized membrane region called Tetraspanin Enriched Microdomains (TEMs) where they can accumulate other proteins.

We found that in developing neurons TSPAN5 was mainly present at the surface membrane while it was concentrated in an intracellular compartment in the postsynapse of mature neurons. We hypothesized that these different localisations could be due to different functions. To deepen the first function of the protein, we knocked down the expression of the protein and found that this led to a dramatic reduction in the number of dendritic spines. We, thus, hypothesized that TSPAN5, through the formation of TEMs, could be responsible of dendritic spines formation. We observed in differential lysis of developing rat hippocampal neurons that two proteins, fundamental for dendritic spines formation, Neuroligin-1 and GluA2 AMPA receptor subunit, were associated with TSPAN5 TEMs. We found that the knockdown of TSPAN5 led to increased mobility of Neuroligin-1 and GluA2 AMPA receptors suggesting the loss of clusterization typical of the first moments of spines formation.

To understand the second function of TSPAN5 we identified AP-4 complex as an interactor of the C-terminal intracellular tail of TSPAN5. This complex is known to act on AMPARs trafficking through direct binding of Stargazin, an AMPARs auxiliary subunit.

We observed that the knockdown of TSPAN5, carried out after the majority of the synaptogenesis was occurred, caused a strong decrease in surface and total level of GluA2. Different evidences suggested an involvement of TSPAN5 in vesicular transport of GluA2 and we demonstrated that TSPAN5 was necessary for the correct recycling of this receptor.

These results highlight multiple roles of TSPAN5 in the regulation of both synapse formation and synaptic functioning in mammalian brain through two distinct mechanisms of action.

1. Aim of the work

TSPAN5 is a protein conserved in mammals belonging to the tetraspanin superfamily and presents the highest level of expression in brain in rodents.

TSPAN5 transcript has a peculiar expression pattern (Garcia-Frigola et al., 2000; 2001) suggestive of roles in central nervous system development more precisely for hippocampus and neocortex and thus it was of interest to study this protein function.

Moreover, as different tetraspanins have been implicated in various functions of neuronal development or in direct regulation of neurotransmitter receptors, we were interested in understanding whether TSPAN5 could have similar roles.

In the first part of the project we studied the expression and subcellular localisation of TSPAN5 protein identifying a very interesting pattern that highly differs between immature and mature neurons. This was suggestive of two possibly distinct functions of TSPAN5 at different developmental stages.

Thus, in the second part of the project, we decided to investigate the role of TSPAN5 and, in particular, of the Tetraspanin Enriched Microdomains formed by this protein on plasma membrane in immature neurons. We used small hairpin RNA (shRNA) to knockdown the protein expression in a model of rat primary hippocampal cultures and identified a profound defect in dendritic spines number. We thus subsequently characterized TSPAN5 function in this compartment by means of biochemical and super-resolution live imaging technique. Our results suggest that the protein could directly act in dendritic spines formation, a process that is far from being fully understood, despite the great amount of work done during past years to elucidate its various steps.

The third part of the project was aimed to unravel the function of TSPAN5 in mature synapses where the protein seemed redistributed to intracellular compartments. We used a Yeast Two-Hybrid Screening to identify intracellular partners of the protein and isolated AP-4, an adaptor complex involved in AMPA receptors trafficking. We thus investigated this process with various biochemical and imaging techniques and demonstrated a direct role of TSPAN5 in regulating GluA2 AMPA-Rs subunit recycling. This complex series of events regulated by a plethora of proteins is necessary to maintain the correct balance of receptor on neuronal plasma membrane and as a result the proper functioning of brain circuits.

Our results identify TSPAN5 as a regulator of both synapses formation and function with two distinct activities that are separated by their developmental stage of action. This makes TSPAN5 a new fundamental player in excitatory synapses and increases our level of knowledge in brain functioning.

2. Introduction

2.1 Tetraspanins

2.1.1 Tetraspanin superfamily

The tetraspanins are a superfamily of evolutionarily conserved transmembrane proteins with a molecular weight varying from 25 to 50 KDa. They are abundantly expressed almost in every cell type and tissue. The whole superfamily shares the basic structure that is composed of four transmembrane domains, two extracellular loops (EC1 or Small Extracellular Loop (SEL) and EC2 or Large Extracellular Loop (LEL)) and short intracellular N-terminal and C-terminal tails (Figure 1) (Wright & Tomlinson, 1994) (Maecker *et al.*, 1997) (Kovalenko *et al.*, 2005).

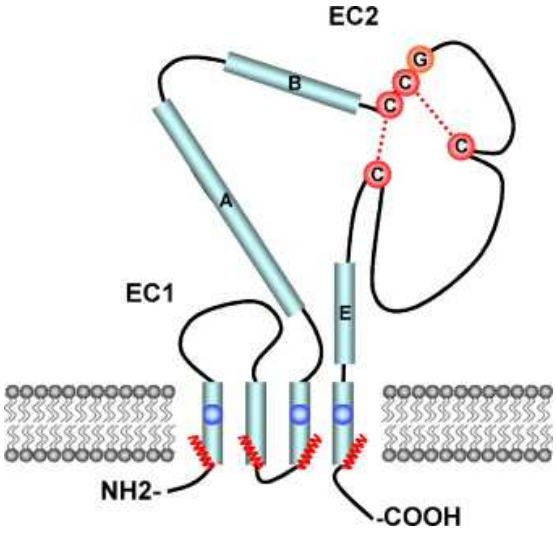


Figure 1. Tetraspanin structure. Tetraspanins are characterized by four transmembrane domains (with conserved charged residues; blue circles), a small (EC1 or SEL) and a large extracellular domain (EC2 or LEL), a very small intracellular loop, and short cytoplasmic N- and C-terminal tails. Sites of conserved palmitoylation at juxtamembrane residues are depicted in red. One CCG motif and 2–8 additional cysteine residues are found in the LEL domain, forming intramolecular disulfide bridges (dotted lines). (Bassani & Cingolani, 2012)

The extracellular domain LEL is generally divided in two regions: one is conserved and contains α-helices A, B and E and is likely involved in dimerization and/or binding to other tetraspanins; the second is comprised between helices B and E and differs among different tetraspanins conferring interaction specificity (Berditchevski *et al.*, 2001) (Stipp *et al.*, 2003).
Other conserved regions found in tetraspanins are a CCG motif right after the B helix, a PXXC motif and a cysteine proximal to the fourth transmembrane domain. These cysteines form disulphide bridges that allow the correct folding of the LEL. However, some tetraspanins possess additional cysteines in the LEL with a maximum number of eight.
SEL domain function is unknown but it is thought to mainly promote and stabilize LEL folding.

The transmembrane domains are the most conserved among the superfamily and contain polar residues that could form hydrogen bonds thus stabilizing the structure of the whole protein (Bienstock & Barrett, 2001). Residues on the intracellular side but positioned closed to the membrane could undergo palmitoylation modification as shown in Fig 1.

The short intracellular C-terminal tail presents the highest degree of divergence between family members. These tails are known to participate in some signalling cascades modulating many biological processes such as proliferation, migration, differentiation and cell-cell adhesion through interaction with other proteins. In particular, a strong association has been found between the tetraspanins CD63-CD81 and the phosphatidylinositol-4 kinase likely acting on cell motility (Yauch *et al.*, 1998) (Berditchevski *et al.*, 1997) (Zhang *et al.*, 2001) (Stipp *et al.*, 2003).

Thirty-three different tetraspanins are known in *Homo sapiens*; they do not have receptor function even if they are exploited by some microbes to enter the cells: hepatitis C virus protein E2 binds CD81 (Pileri *et al.*, 1998) and FimH of uropathogenic bacteria binds the tetraspanin Uroplakin 1A (Wu *et al.*, 1996). This family is involved in the pathogenesis of many diseases. Mutations, usually occurring on the EC2 domain, in peripherin/RDS lead to several retinal disorders probably disrupting photoreceptor morphogenesis (Kohl *et al.*, 1998) (Figure 2).

Table 1 | **Genetic analysis of tetraspanin functions**

Species	Tetraspanin	Associated phenotype*
Human	TM4SF2	X-linked mental retardation
	CD151	Skin, kidney, platelet malfunctions; deafness, β-thalassemia
	Peripherin/RDS	Retinal degeneration
	ROM	Digenic retinal degeneration
Mouse	CD9	Sperm–egg fusion, monocyte fusion, brain and peripheral nerve defects
	CD81	Immune regulation, monocyte fusion, brain enlargement, malaria infection
	CD37	Mild lymphocyte defects
	Tssc6	Mild lymphocyte defects
	CD151	Lymphocyte proliferation, platelet clotting, keratinocyte migration
	Peripherin/RDS	Retinal degeneration
	ROM	Mild retinal degeneration
Fly	late bloomer	Delayed synapses in embryos
	Sun (CG12143)	Light-induced retinal degeneration
	Tsp68C*	Suppresses abnormal haemocyte proliferation
Worm	TSP-15	Disrupted epidermal integrity
Fungi	MgPLS1	Host leaf penetration
	BcPLS1	Host leaf penetration

Figure 2. Genetic analysis of tetraspanins function up to date (Hemler, 2005).

Many members of this family have important functions in the immune system: the CD81-CD19 complex is fundamental for B and T cells (Maecker & Levy, 1997) (Miyazaki *et al.*, 1997) (Deng *et al.*, 2000). However, mutations directly related to pathologies are generally rare in genes of the tetraspanin superfamily likely due to possible functional redundancy.
Recently tetraspanins have also been suggested as promising drug targets in numerous illnesses considering that they could be easily reached on their extracellular domains possibly interfering and modulating the activity of their intracellular partners (Hemler, 2008).

2.1.2 Tetraspanin Enriched Microdomains (TEMs)

All tetraspanins are able to form specialized membrane domains named Tetraspanin Enriched Microdomains (TEM) where they interact each other and with other proteins (Figure 3).

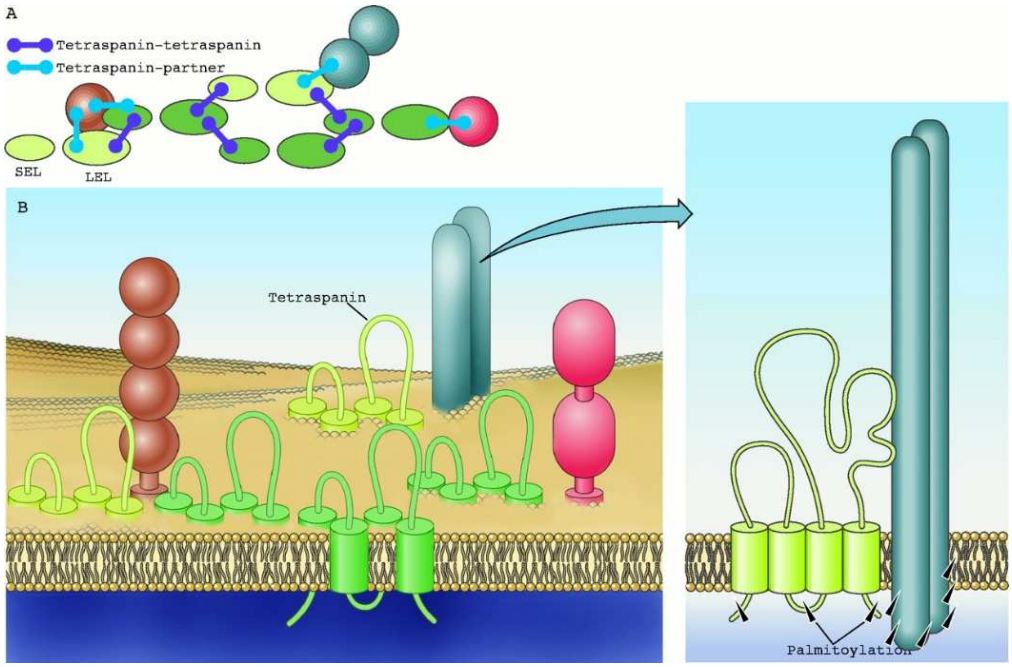


Figure 3. Representation of the Tetraspanin Enriched Microdomain (TEM), Tetraspanins are shown in shades of green; partners of the immunoglobulin superfamily are shown in magenta and brown, and those of the integrin are in blue. (Levy & Shoham, 2005).

In particular TEMs have been found to accommodate many different proteins such as Integrins, members of the Ig family (e.g. MHC I and II), proteoglycans, growth factor receptors etc. (Boucheix & Rubinstein, 2001).
Accordingly, the main hypothesis for tetraspanins function is that they could act as molecular facilitator connecting the different actors of many processes in the TEMs; these interactions are unlikely to happen randomly and the ability to concentrate proteins in small membrane domains would result in increased likelihood (Maecker *et al.*, 1997). The domain responsible of these interactions is the EC2 loop (Boucheix & Rubinstein, 2001).

These domains are highly enriched in cholesterol and the palmitoylations that occur as post-translational modifications are involved in the direct binding of tetraspanins to this lipid (Charrin *et al.*, 2003) (Silvie *et al.*, 2006). This interaction contributes to the physical separation of TEMs from the rest of the membrane due to different lipidic composition. This composition also causes the TEMs to be very insoluble in numerous lysis buffer leading to a variety of problems in studying this family of proteins and in particular in the definition of specific interactors.

Indeed, the lysis in mild detergent buffer (containing CHAPS or Brij as detergents) is able to solubilize only partially the domains and co-immunoprecipitation experiments performed in this condition would reveal not only specific interactors but also proteins that are present in the TEMs due to interaction with other tetraspanins. The high cholesterol concentration, however, gives us also a tool to circumvent this problem.

The lysis in buffer containing digitonin, a detergent that exerts its action by binding to cholesterol, and the subsequent centrifugation of the samples gives the possibility to completely remove TEMs from the preparation allowing to detect specific interactors of the selected tetraspanin that would have been extracted as monomer from the TEMs.

Tetraspanin interactions are generally divided in three levels:

1) When the interaction is resistant to strong detergent (e.g. Triton X-100) suggesting direct binding that occurs at high stoichiometry. These interactions are the only ones believed to occur directly;

2) When the interaction is less robust and not resistant to very strong detergent but occurring in hydrophobic reagents (such as Brij 96). These interactions usually involve other tetraspanins;

3) When the interaction is detectable only in very mild detergent (e.g. CHAPS) that would completely fail in solubilizing TEMs.

Level 2 and 3 interactions are thought to occur mainly through TEMs.

A very recent study based on super-resolution imaging techniques studied the organization of TEMs formed by tetraspanins (Zuidscherwoude *et al.*, 2015).

This study proposed that different tetraspanins organize separate TEMs with distinct mobility characteristics and localisations instead of having domains where many different tetraspanin members can cohabitate. These domains appear to have a medium size of 120 nm containing less than 10 molecules per domain. This result is comparable with previous ultrastructural observation of TEMs that have suggested an area of 0,2 μm^2 (Nydegger *et al.*, 2006). The authors also proposed that these domains on the surface membrane form a dynamic network that is regulated by the weak heterotypic interaction between the different tetraspanins of different TEMs or with other proteins.

Different studies suggest that it is possible to act on TEMs formation, either inducing or preventing, by applying monoclonal antibodies directed against extracellular epitopes with consequent downstream effects (Hemler, 2008).

2.1.3 Tetraspanins in the brain

Several tetraspanins have been found in the nervous system, including CD9, CD81, TM4SF2/A15, TSPAN2, TSPAN3/OAP-1, TSPAN5 and neurospanin, where some of them act on neurite extension (Schmidt *et al.*, 1996) (Banerjee *et al.*, 1997) and synapse maturation (Kopczynski *et al.*, 1996) and two members have been involved in forms of mental retardation: TSPAN6 (Vincent *et al.*, 2012) and TSPAN7 (Zemni *et al*., 2000).

CD81 has been involved in astrocyte and microglia functioning as knockout mice for this gene showed enlarged brain with increased number of astrocytes and microglia (Geisert *et al.*, 2002).

Tetraspanin-7 (TSPAN7) has been found mutated in forms of X-linked Intellectual disabilities (XLID) (Zemni *et al.*, 2000) (Bassani *et al.*, 2012). The protein, almost ubiquitously expressed but most strongly in brain (Hosokawa *et al.*, 1999), was found mutated in different ways (a X;2 balanced translocation, a premature stop codon and a missense substitution) in patients affected by intellectual disability (Zemni *et al*., 2000). Bassani and colleagues has characterized TSPAN7 as active in dendritic spine stabilization and filopodia formation during rat hippocampal neurons development leading to a functional maturation of the synapses (Bassani *et al*., 2012). Silencing TSPAN7 leads to a phenotype characterized by normal spine density but with a reduced spine width and reduced expression of synaptic markers. It also causes a faster spine turnover dynamics. TSPAN7 appears to interact, through its C-terminal domain, with Protein Interacting with C-Kinase 1 (PICK1), integrin and GluA2/3, a subunit of AMPA receptor (α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid, AMPA-Rs). This complex seems to be important to stabilize AMPA receptors on dendritic spines considering that the silencing of TSPAN7 leads to faster internalization of the receptor while the silencing of PICK1 decreased it. Both TSPAN7 and GluA2/3 bind PICK1 at its PDZ (Postsynaptic density protein (PSD-95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (zo-1)) domain likely competing for this site; this could explain the opposite effects of TSPAN7 and PICK1 on GluA2/3 internalization.

2.1.4 Tetraspanin 5 (TSPAN5)

Human TSPAN5 gene (alternative name: NET-4; tetraspan 5; TM4SF9; transmembrane 4 superfamily member 9; transmembrane 4 superfamily, member 8) is located on chromosome 4q23 and has a 3365 bp long sequence divided in 8 exons; it encodes for a 268 amminoacids long protein (Entrez Gene).

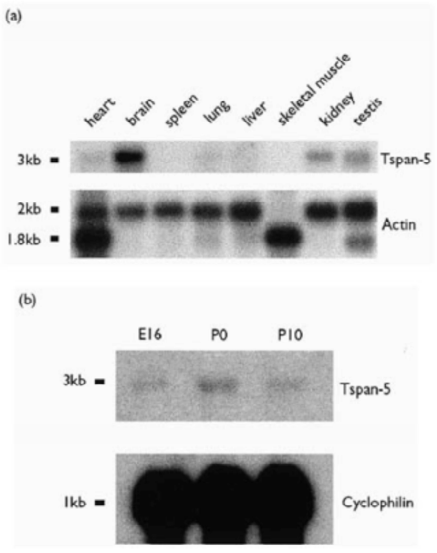
It was first identified by Todd and colleagues in 1998 (Todd *et al.*, 1998) where they showed a prevalent presence of the mRNA transcript in human brain.

It is conserved in chimpanzee, rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, and *C.elegans* (tsp-12) and it is a typical member of the tetraspanin superfamily with which it shares the structural characteristics as it is inferred by sequence homology. It is part of the C8 subgroup of tetraspanins, clustered by the presence of eight cysteine residues in the LEL.

The mouse protein sequence contains four N-glycosilation and six myristoylation sites and a putative Protein Kinase C (PKC) phosphorylation site at the intracellular N terminal tail (Garcia-Frigola *et al.*, 2000).

Very little is known about this protein. It has been found involved, together with another tetraspanin, NET6, in osteoclastogenesis where it seems to promote the cell fusion that is necessary to produce osteoclasts. In fact the TSPAN5 mRNA appears to be up-regulated upon activation of RANKL (Receptor activator of nuclear factor kappa-B ligand), the system that specifically induces the osteoclastogenesis in mononuclear macrophage/monocyte lineage precursor (Iwai *et al.*, 2007).

TSPAN5 mRNA presence in mice has been extensively studied and it is detected at high level in the brain, at lower level in heart, kidney, testis and weakly in lung and liver. In mouse brain the transcript is first detected at embryonic day 13-14 and it reach his peak at P0 strongly decreasing at P10 (Figure 4).



	E14	E16	P0	P5	P10	P15	P21	Adult
Telencephalon								
Olfactory bulb								
Glomerular cell layer			–	+	+	+	+	+
Mitral cell layer	++	++	+++	+++	+++	+++	+++	+++
Granular cell layer		+	+	+	+	+	+	+
Neocortex	++++	+++++	+++++	++++	++++	++++	++++	++++
Piriform cortex	+	++	++++	++++	++++	++++	++++	++++
Entorhinal cortex		–	++	+++	++++	++++	++++	++++
SVZ/RMS	+	+	++	++	++	++	++	++
Hippocampus								
CA1, CA2, CA3 region	++++	++++	++++	++++	++++	++++	++++	++++
Dentate gyrus	++++	++++	++++	++++	++++	++++	++++	++++
Basal forebrain								
Ganglionar eminence	++	+						
Caudate putamen			+	+	+	+	+	+
Globus pallidus			++	++	++	++	++	++
Medial septum/diagonal band		+++	++++	++++	++++	++++	++++	++++
Dorso-lateral septum		+	+	+	+	+	+	+
Lateral nucleus olfactory tract			++	++	++	++	++	++
Amygdaloid complex		++	+++	+++	+++	+++	+++	+++
Hypothalamus								
Estria terminalis nuclei	+++	+++	++	+	+	+	+	+
Ventral nuclei	+	++	++	++	+	+	+	+

Figure 4. A) Northern blot analysis of mouse TSPAN5 mRNA within different mouse tissues. B) Whole brain northern blot at different developmental stages. Table) Level of expression of mouse TSPAN5 mRNA in different brain areas and at different developmental stages. (+/−) very low, (+) low, (++) moderate, (+++) high, (++++) very high (Garcia-Frigola *et al.*, 2000) (Garcia-Frigola *et al.*, 2001).

At prenatal and postnatal stages, the transcript is highly regulated with its expression pattern strongly varying in time and in different brain areas. It remains expressed from E14 to adulthood at very high level in the whole hippocampus and in the neocortex. In the adulthood, high signal is present also in the cortical structures such as the layer II-III and IV-V of the cortex, the piriform cortex and the entorrhinal cortex interneurons, and in the amygdala, in the medial sectum and in the olfactory bulb. In the cerebellum the transcript is preferentially expressed in the Purkinje cells. (Garcia-Frigola *et al.*, 2000, Garcia-Frigola *et al.*, 2001). The expression pattern strongly suggests that mouse TSPAN5 could have a relevant function in the Central Nervous System and specifically in the development and functional maturation of hippocampus and neocortex.

A recent paper has shown that Tsp-12, the TSPAN5 ortholog in *C. elegans*, is a key regulator of Notch activity likely promoting its cleavage by γ-secretase (Dunn et al, 2010). Similar results have been published later confirming these findings also in mammals and pointing out TSPAN5-dependent activation of ADAM10 (A Disintegrin And Metalloprotease domain 10), another metalloprotease involved in Notch activation (Haining *et al.*, 2012) (Dornier *et al.*, 2012).

Notch is an extensively studied protein that mediates cell-cell interactions involved in the specification of the cell fate in animal development. It is fundamental in the regulation of migration, morphology, synaptic plasticity and survival of immature and mature neurons as well as in the development of the brain. Notch signalling is involved in Allagile, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), Hajdu–Cheney syndromes, Down's syndrome and Alzheimer's disease (Ables *et al.*, 2011). Notch is present at the cell membrane as heterodimer between its amino and carboxy terminal. The binding of its ligand, Delta/Serrate/LAG-2 family, causes the cleavage at the juxtamembrane portion by ADAM disintegrin and metalloprotease. This event creates the substrate for γ-secretase that cutting the protein in its transmembrane domain releases the intracellular active domain. The domain reaches the nucleus where it interacts with CBF1/Suppressor of Hairless/ LAG-1, Mastermind/SEL-8 and other DNA binding proteins to activate transcription of specific genes.

In a silencing screening of 21 tetraspanins in a *C. elegans* germ line with Notch constitutive active, tsp-12 came out as the sole able to suppress the sterility of the line induced by Notch overactivity. A null allele of tsp-12 synergized in causing embryonic lethality with glp-1 (one of the Notch ortholog protein in *C. elegans*) and in enhancing the phenotype caused by the

loss of function of ADAM10 and ADAM17/TACE, both involved in Notch processing. Tsp-12 has many human orthologs: TSPAN17, TSPAN14, TSPAN15, TSPAN10, TSPAN33 and TSPAN5. Human Notch1 is constitutively active in forms of T cell acute lymphoblastic leukemia (T-ALL). Using HeLa cells carrying the mutations responsible for this constitutive activity and silencing the different Tsp-12 human orthologs the authors identified TSPAN33 and TSPAN5 ability to reduce Notch1 transcript abundance. The use of truncated forms of Notch resembling the cleaved protein by ADAM or by γ- secretase showed that the silencing of TSPAN33 reduced the active form of Notch only in the ADAM cleaved form demonstrating that this tetraspanin is involved in γ-secretase cleavage. The silencing of TSPAN5 synergized with the silencing of TSPAN33 in reducing the membrane bound, constitutively active form of Notch. The authors thus proposed that the modulation of TSPAN5 was able to modify Notch cleavage and then its activity. This role of TSPAN5, however, seems not to be highly specific as other members of the tetraspanin family can exert the same or at least very similar function on metalloproteases; for instance all the C8 subgroup, (Haining *et al.*, 2012) (Dornier *et al.*, 2012), Tetraspanin 12 (Xu *et al.*, 2009) and Tetraspanin 15 (Prox *et al.*, 2012) have been shown to have such activity.

2.2 Dendritic spines formation

This paragraph will shortly describe the sequential phenomenons required for the correct formation of dendritic spines, the postsynaptic compartment of excitatory synapses highly present in hippocampus, as we identified TSPAN5 as a key player in this cascade of events.

Synapse formation is a very complex process that requires many different steps to occur in a precise spatial and temporal scale. Of course, the correct chain of all these events is crucial for establishing connections between neurons and thus for the formation of brain circuits and for its proper functioning. Glutamatergic synapses are the most abundant excitatory synapses existing in the brain; they have the peculiarity of being built, on the postsynaptic side, on specialized membranous protrusions called dendritic spines (Nimchinsky *et al.*, 2002, Sheng & Kim, 2002). The importance of number and shape of dendritic spines is underlined by the fact that alterations in these parameters have been found in many neurological and psychiatric diseases and in particular in many forms of intellectual disability and autism spectrum disorders (Figure 5) (Fiala *et al.*, 2002).

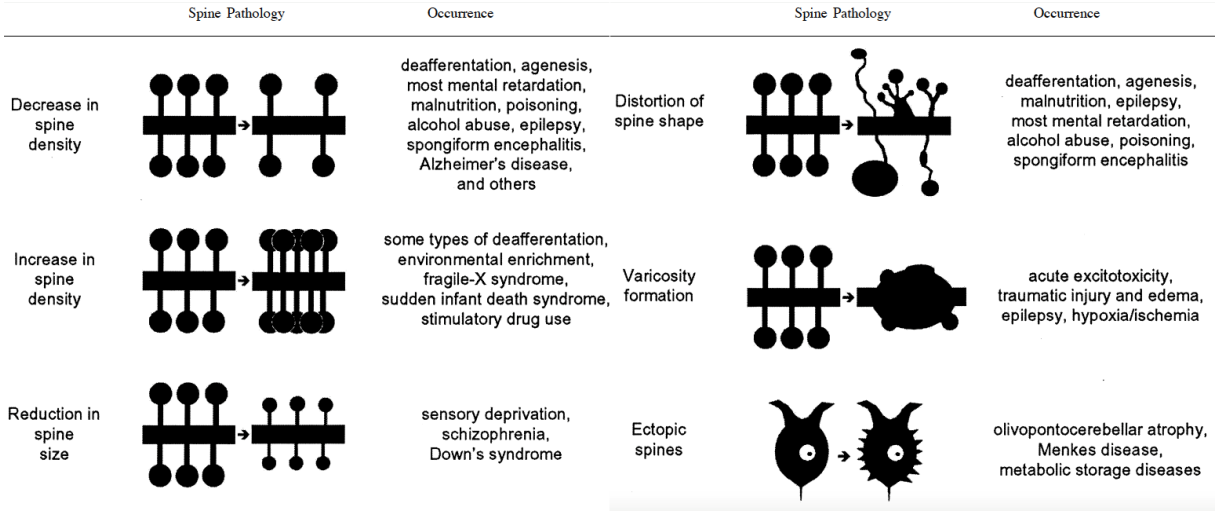


Figure 5. Dendritic spines number (both reduction and increase), shape (both in length and width) and positions defects are associated with many different forms of intellectual disability, epilepsy and other neurological conditions (Fiala *et al.*, 2002).

These organelles are extremely dynamic, as they can be formed very rapidly and very rapidly removed (in the order of minutes) (Bonhoeffer & Yuste, 2002) but they can also remain stable for days or months. They are thus thought to be the major repositories of long-term memory in the brain and are in fact found at very high density in hippocampal pyramidal neurons (Segal, 2005). Despite the extensive studies that have addressed the process of spines formation in the past years, the picture is far from being complete.

Different hypotheses exist for the very first steps of spines formation and in particular on the definition of the place where a spine is going to be formed (for a review see (Yuste & Bonhoeffer, 2004). The hypotheses differ mainly on the relevance of axonal contact for the formation of the spines and on the importance of filopodia in this process. However, we are not going deeper in this topic as it is out of the specific importance of our work.

Nevertheless, it is necessary that an intracellular cascade takes place to have the formation of the complex postsynaptic network of proteins composed of receptors, adhesion molecules and scaffold proteins. Adhesion molecules are thought to be crucial in the initial signalling as they can bind physically the dendritic spine to the forming presynaptic bouton. Neuroligins are for sure among major players in this process. This family of type 1 transmembrane proteins, that in humans counts five members, Nlg-1, Nlg-2, Nlg-3, Nlg-4 and Nlg-4Y (Jamain *et al.*, 2003), has been associated with postsynaptic formation in numerous studies. This role is related to their ability to bind presynaptic Neurexins (Sudhof, 2008) thus forming a stable bridge across the synaptic cleft and to the interaction with intracellular scaffolding proteins (Figure 6).

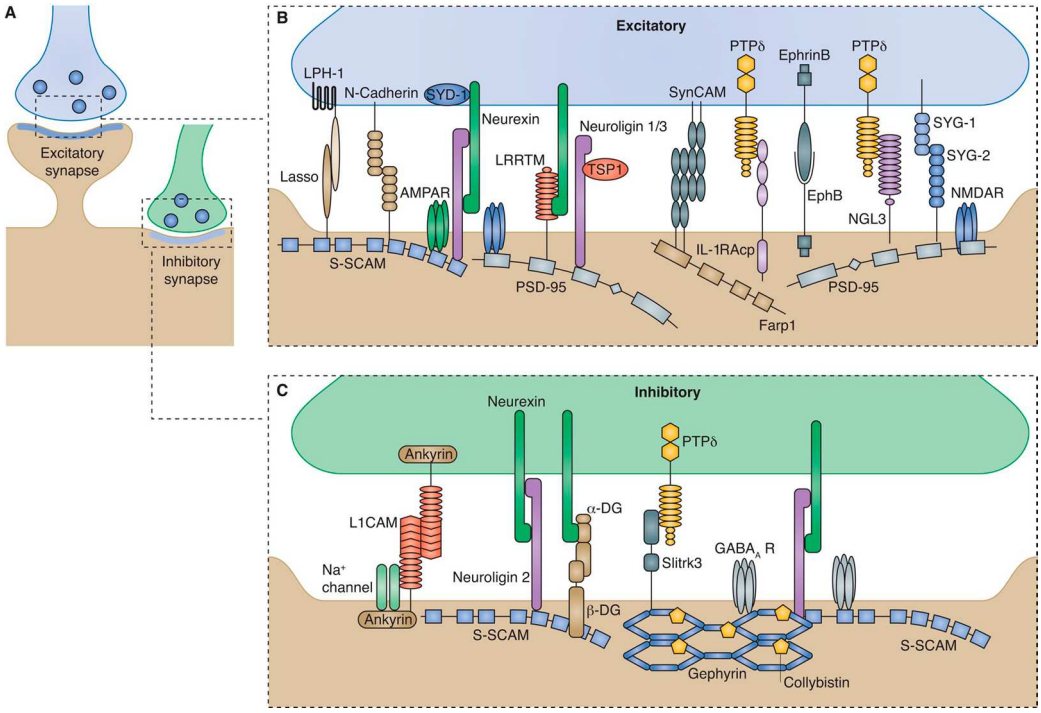


Figure 6. Neuroligins extracellular and intracellular interactors in excitatory and inhibitory synapses (A). Neuroligin 1 is the major trans-synaptic adhesion molecule that organizes scaffold proteins (PSD-95, S-SCAM etc.) and thus all receptors in excitatory synapses. Neuroligin 3 and other adhesion molecules (SynCam, EphrinB, etc…) participate in the formation of this complex (B). Neuroligin 2 is instead the major organizer of inhibitory postsynapse. (Chia *et al.*, 2013).

Neuroligins present a specificity for excitatory or inhibitory synapses with Neuroligin 1 being the main trans-synaptic adhesion molecule for excitatory (Song *et al.*, 1999) and Neuroligin 2 for inhibitory (Graf *et al.*, 2004) (Varoqueaux *et al.*, 2004) but they can exchange position to some extent.

The importance of Neuroligins for brain functioning is pointed out by the finding that mutations in Neuroligin 3 and 4 genes are associated with autism spectrum disorders and intellectual disability (Jamain *et al.*, 2003) (Chih *et al.*, 2004) (Comoletti *et al.*, 2004) (Laumonnier *et al.*, 2004) (Talebizadeh *et al.*, 2006).

On the intracellular side Neuroligin 1 is able to bind PSD-95 (Postsynaptic Density-95), the main excitatory scaffold protein, on one of its PDZ domains but also many other scaffolding such as other MAGUKs (Membrane-Associated GUanylate Kinases), S-SCAM (Synaptic SCaffolding Molecule), SHANK (SH3 And multiple ANKyrin repeat domains protein) and PICK1 (Irie *et al.*, 1997) (Iida *et al.*, 2004) (Meyer *et al.*, 2004). This ability to recruit scaffolding essential for postsynapse assembly suggests that Neuroligin 1 can be the first protein that localises in the forming dendritic spines. It creates homo-multimers through its catalytically inactive acetylcholinesterase (AChE)-homologous domain (Comoletti *et al.*,

2003) (Dean *et al.*, 2003) thus increasing the strength and the stability of the synapse via binding to neurexins.

The relevance of Neuroligins in spines formation is demonstrated by the fact that the overexpression of Neuroligin 1 leads to increase number of dendritic spines in cultured neurons (Levinson *et al.*, 2005) and conversely its knockdown decreases spines density (Chih *et al.*, 2005). Moreover, the ectopic expression of Neuroligins in HEK (Human Embrionic Kidney) cells is sufficient to induce the formation of mature presynaptic terminals in contacting neuronal axons (Scheiffele *et al.*, 2000) (Fu *et al.*, 2003) (Sara *et al.*, 2005) and on the other side the expression of neurexins in HEK cells gives rise to postsynapses in associated dendrites (Graf *et al.*, 2004). These effects are so strong that they can be mimicked using microspheres coated with Neuroligins or Neurexins respectively (Dean *et al.*, 2003) (Graf *et al.*, 2004).

Neuroligin 1 is present on the surface of neurons and it is organized in clusters even before the contact event between dendrite and axons. However, the recruitment of Neuroligin 1 to sites of contact occurs very rapidly, in the order of 1-3 minutes, mainly from the freely diffusing pool (Barrow *et al.*, 2009). An extensive study of the dynamics of the events occurring after axon-dendrite contact made by Barrow and colleagues (Barrow *et al.*, 2009) observed that the clusterization of Neuroligin 1 at this site is sufficient to induce the recruitment of PSD-95 in 30-60 minutes in a palmitoylation dependent manner. PSD-95 appears to be mobilized from existing synapses to be redirected to the newly formed ones (Mondin *et al.*, 2011). The arrival of NMDA receptors (N-methyl-D-Aspartate receptor) appears to be almost concomitant to the clusterization of Neuroligin 1 but the transport seems to be happening in intracellular vesicles somehow linked to surface Neuroligin 1. AMPA receptors are instead recruited with slower dynamics showing a peak accumulation after 1-2 hours from the axon-dendrite contact (Friedman *et al.*, 2000). Moreover, different works have analysed at super-resolution level the diffusion of AMPA-Rs in physiological condition and upon different stimulation (Heine *et al.*, 2008) (Mondin *et al.*, 2011) (Nair *et al.*, 2013) (Constals *et al.*, 2015).

It seems that AMPA-Rs are recruited to Neurexin-Neuroligin contact sites thanks to PSD-95, which can bind Stargazin, the main TARP (Transmembrane AMPA receptor Regulatory Protein) at its PDZ binding site.

Consistently with these observations, the knockdown of either Neuroligin 1 or PSD-95 decreases AMPA-Rs clusters and increases its diffusion; reverse effects are observed upon overexpression of the two proteins suggesting that Neuroligin 1, through PSD-95, act as a trap to immobilize freely diffusing AMPA-Rs (Mondin *et al.*, 2011) (Figure 7).

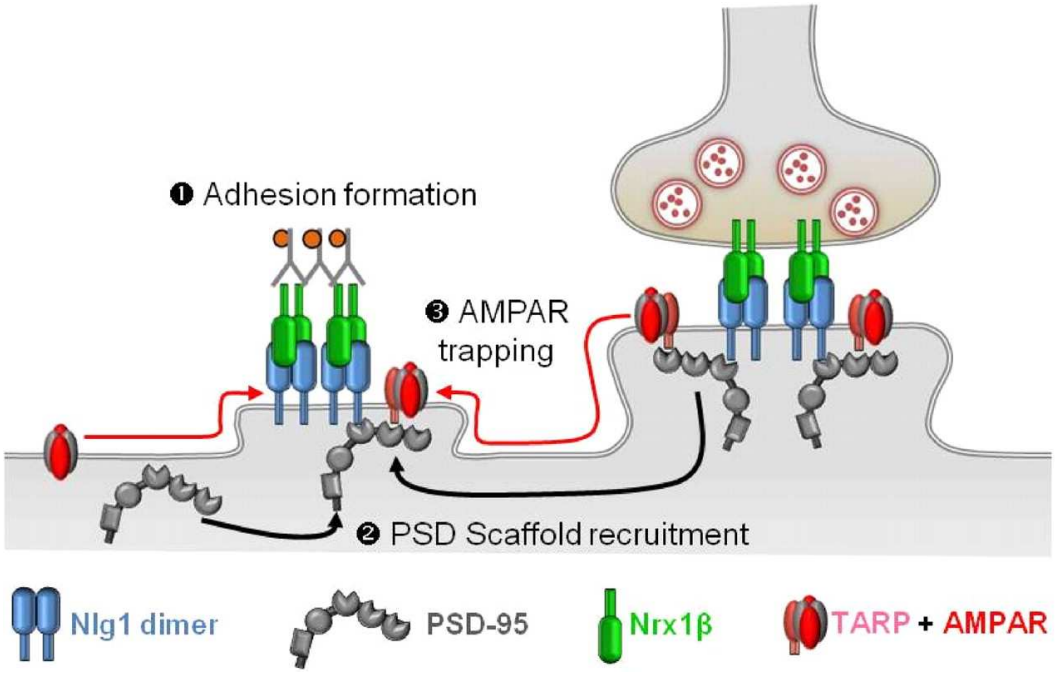


Figure 7. Possible model of formation of the postsynaptic proteins complex with the depiction of Neuroligin-neurexin contacts (1.Adhesion formation) and the sequential recruitment of PSD-95 (2. PSD scaffold recruitment) and AMPA-Rs through Stargazin binding and trapping (3. AMPAR trapping) (Mondin *et al.*, 2011).

Moreover, it appears that AMPA-Rs dimers are differentially regulated at Neurexin-Neuroligin contact sites. In particular, GluA2 is accumulated at these locations whereas GluA1 is not. This discrepancy is likely due to differential requirement of neurotransmitter release from the presynaptic side. In fact, GluA2 recruitment is independent from NMDA activation, as it occurs also in presence of Tetradotoxin (TTX) and APV (DL-2-Amino-5-phosphonoPentanoic acid), very well-known blockers of action potentials and NMDA receptors respectively (Heine *et al.*, 2008). In contrast, GluA1 accumulation appears to require glutamate release, as it does not occur in conditions where the presynaptic side is not directly present but only mimicked by using latex beads coated with Neurexins. In addition, when a real axon-dendrite contact site is examined the recruitment of GluA1 is blocked by application of TTX/APV.

2.3 Adaptor Protein Complex 4 (AP-4)

This section will provide a brief explanation of the structure and function of AP-4 as, with our study, we identified this protein as a new TSPAN5 direct interactor.

AP-4 is a heterotetrameric complex conserved in mammals, chicken, *Dycostelium discoideum* and *Arabidopsis taliana* (Boehm & Bonifacino, 2001) that was discovered 15 years ago (Dell'Angelica *et al.*, 1999) (Hirst *et al.*, 1999). It belongs to the family of Adaptor Proteins (APs). This family of protein complexes counts five members (AP-1-5) all composed of four subunits: two large subunit (β1-5 and γ, α, δ, ε and ζ), one medium (μ1-5) and one

small subunit (σ1-5) (Figure 8) (Hirst *et al.*, 2013). These subunits are strictly associated and they cannot be observed in monomeric form probably due to high rate of degradation.

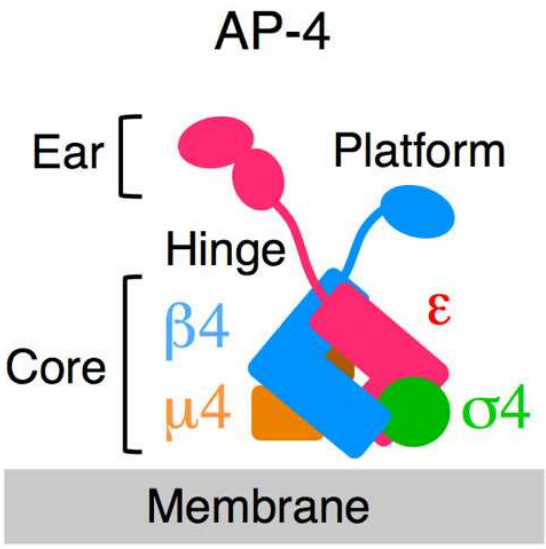


Figure 8. AP-4 complex structure. The four subunits are represented with different colours: ε in magenta with the hinge linker and the ear (or appendage) domain, β4 in blue with the hinge and the ear domain, μ4 in orange and σ4 in green. As depicted, μ4 and σ4 together with the main part of ε and β4 represent the core of the complex that is in close proximity with membranes. (modified from (Mattera *et al.*, 2015).

AP-1, AP-2 and AP-3 have been extensively studied in past years and different roles have been established. On the opposite, AP-4 and AP5 have been more recently described and little is known about their function.

In general AP complexes are able to form protein coat (mainly clathrin-coat) on different membranous organelles and, through the recognition of signal motifs on cargoes protein, to associate them to vesicles that are then transported to specific compartments, mainly through motor proteins (Owen *et al.*, 2004).

For example, AP-1 is involved in vesicular transport between Trans Golgi Network (TGN) and endosomes and in sorting membrane proteins to the basolateral compartment, AP-2 mediates internalization from the plasma membrane of many different proteins and AP-3 can direct proteins from endosomes to lysosome-related organelles such as melanosomes (Bonifacino & Traub, 2003) (Dell'Angelica, 2009).

At least AP-1 and AP-4 has been involved in sorting proteins in polarized cells, such as epithelial cells and neurons, to the basolateral compartment (named somato-dendritic in neurons) (Farias *et al.*, 2012) (Guo *et al.*, 2013) (Matsuda *et al.*, 2008).

The signal motifs recognized by AP complexes are quite various with the two main being dileucine based ([DE] XXXL [LIM]) and tyrosine based (YXXØ with Ø being a bulky hydrophobic amminoacid). The first is generally bound by γ and σ1 for AP-1, α and σ2 for AP-2 and δ and σ3 for AP-3 (Janvier *et al.*, 2003) (Chaudhuri *et al.*, 2007) (Doray *et al.*,

2007); the second instead is recognized by C-terminal domain of μ (1-3) (Ohno *et al.*, 1995) (Ohno *et al.*, 1996).

α of the AP-2 complex interacts with many regulators of coat machinery or vesicles assembly (Owen *et al.*, 2000) and β 1, 2 and 3 interact directly with clathrin (Gallusser & Kirchhausen, 1993) (Shih *et al.*, 1995) (Dell'Angelica *et al.*, 1998). σ1 and 3 are required for functional integrity of the complex (Shim *et al.*, 2000) (Mullins *et al.*, 2000).

Compared to the other AP complexes AP-4 presents some peculiarities: first of all no association with clathrin has been observed and signal motif differs. Indeed, it is capable of binding only YXXØ motif for example of CD63, LAMP-1, LAMP-2 (Lysosomes-Associated Membrane Proteins 1 and 2) and TGN38 (Trans Golgi Network integral protein 38) (Hirst *et al.*, 1999) (Stephens & Banting, 1998) (Aguilar *et al.*, 2001). However, these interactions are weak and do not seem to be fundamental for proteins localisation, as the removal of AP-4 does not alter it (Simmen *et al.*, 2002) (Janvier & Bonifacino, 2005). μ4, instead, preferentially binds signals motif such as F YD[PR]F (Aguilar *et al.*, 2001) or YX [FYL] [FL] E (Burgos *et al.*, 2010).

AP-4 can interact with ARF1 (ADP rybosilation factor), a protein that cycles between GTP (Guanosine-3-Phosphate) and GDP (Guanosine-2-Phosphate) bound state through the action of GAPs (GTPase Activating Protein) and GEFs (Guanine nucleotide Exchange Factors) (Goldberg, 1998) (Roth *et al.*, 1999) (Menetrey *et al.*, 2000) (Pasqualato *et al.*, 2001). ARFs is fundamental in regulating membrane recruitment of AP-4, a mechanism shared with AP-1 and AP-3 (Liang & Kornfeld, 1997) (Ooi *et al.*, 1998) (Zhu *et al.*, 1998) (Drake *et al.*, 2000) but not with AP-2 that instead takes advantage of the binding with synaptotagmin for nucleation of endocytic clathrin-coated pits (Slepnev *et al.*, 2000). The interaction between AP-4 and ARF1, differently from AP-3 and AP-1, does not occur through the β subunit but it is based on μ (nucleotide independent) and ε (when ARF1 is in the GTP bound state) subunits (Boehm *et al.*, 2001).

Moreover, an ENTH (Epsin-N-Terminal Homology) domain-containing protein, tepsin, was found to interact with AP-4 in a mass spectrometry screening and represents the only cytosolic protein found to be associated with the complex (Borner *et al.*, 2012). This protein is similar to epsinR and CALM (Clathrin Assembly Limphoid Myeloid leukemia protein) that have been found associated with AP-1 and AP-2 complexes respectively. The interaction between AP-4 and tepsin, however, is very low at steady state, explaining the difficulty of observing it in immunoprecipitation experiment, but it becomes fundamental when vesicle formation is occurring. The interaction takes place on the appendage domain of ε and the C-terminus of β4. This binding was also suggested to induce the clusterization of multiple AP-4 complexes increasing the likelihood of AP-4 coat formation (Mattera *et al.*, 2015).

AP-4, at least in epithelial cells, has been suggested to act as a sorting complex from the TGN to the basolateral compartment similarly to AP-1. However, a synthetic sequence (DLYYDPM) that is selectively bound by μ4 and not by other AP complexes was found to localise in the TGN and to accumulate in lysosomes when lysosomal inhibitor leupeptin was applied. This suggested that AP-4 can also mediate sorting from TGN to late endosomes-lysosomes (Ruben *et al.*, 2001). Another very recent paper demonstrated the interaction between AP-4 μ and the protein NAGPA, an enzyme that synthetizes mannose-6-phosphate signals that sort acid hydrolases to lysosomes strengthening the hypothesis of a role of AP-4 in lysosomal degradation (Raza *et al.*, 2015).

Many different lines of evidence propose a role for AP-4 in neurons. AP-4 is expressed in neuronal cell lines (Dilaver *et al.*, 2003) and in many regions of the brain (Yap *et al.*, 2003).

Burgos and colleagues have demonstrated that the direct binding of μ4 to Amiloyd Precursor Protein (APP) is necessary for its biosynthetic transport from the TGN to endosomes observing that depletion of AP-4 increases its TGN retention due to increased transient residence. This action of AP-4 has been suggested as protective from amyloid plaques formation as it removes APP from the site where γ-secretases produces Aβ peptide promoting instead caspase-cleavage and production of C31 non-pathogenic peptides (Burgos *et al.*, 2010).

In addition, AP-4 directly interacts through μ subunit with δ2 glutamate receptor, a receptor exclusively expressed in Purkinje cells of the cerebellum (Yap *et al.*, 2003). δ2 glutamate receptor is fundamental for cerebellum functioning as knockout mice show motor learning impairment. In these cells, AP-4 is localised in soma and dendrites and in particular in Golgi region as shown by co-localisation with TGN38 and immunogold Electron Microscopy. The binding occurs through different motifs on the receptor: di-aromatic residues (FXF), phenylalanine-based motifs (FGSV) and FR motifs.

A knockout (KO) mouse model of AP-4 β has been generated (Matsuda *et al.*, 2008): these mice, despite showing a complete loss of the whole AP-4 complex, do not present any visible impairment, as they are viable, fertile, with normal gait and no anatomical defects. However, in the cerebellum of these animals AMPA receptors were observed in calbindin positive structures (axon marker) in the DCN (deep cerebellar nuclei) that receive projections from Purkinje cells suggesting an incorrect transport of these receptors. Electron microscopy analysis showed aberrant structures in the axons resembling autophagosomes, suggestion confirmed by the finding of increased LC3-II (microtubule-associated protein 1A/1B-Light Chain 3, phosphatidylethanolamine conjugated, an autophagosomes marker). These structures appear to contain AMPA receptors as demonstrated by co-localisation with LC3-II. Surprisingly no differences were found in somato-dendritic GluA1 and GluA2 levels and surface localisation. Overexpressed GluA1 was also mislocalised in hippocampal neurons

cultured from KOs. Similar mis-sorting effects were observed for other cargos of AP-4 such as δ2 glutamate receptor and LDLR (Low Density Lipoprotein Receptor) but not for mGluR1, Transferrin Receptor (TfR), NR1 (NMDA Receptor 1) that do not count on AP-4 for correct sorting. GluA1 and GluA2 are unable to bind directly μ4 but they are bound through TARPs (γ2 or stargazin, γ3, γ4 but not γ5) and at least γ3 was found mis-sorted in KO animals. This binding occurs at the C-terminal tail of TARPs at YRYRF motif. The authors thus hypothesized that, after AMPA-Rs are bound by TARPs at the Endoplasmic Reticulum, AP-4 assembles at TGN or at some post Golgi compartment inducing correct somato-dendritic sorting of AMPA receptors. As some AMPA-Rs are present at presynapses they must escape AP-4 sorting possibly by phosphorylation of residues in the C terminal tail of TARPs (Matsuda *et al.*, 2013).

AP-4 importance in the brain is also suggested by the recent findings of AP-4 loss of function mutation in different patients affected by a complex set of symptoms, which has been named AP-4 deficiency syndrome (Verkerk *et al.*, 2009) (Abou Jamra *et al.*, 2011) (Moreno-De-Luca *et al.*, 2011) (Kong *et al.*, 2013) (Tuysuz *et al.*, 2014) (Hardies *et al.*, 2015) (Figure 9).

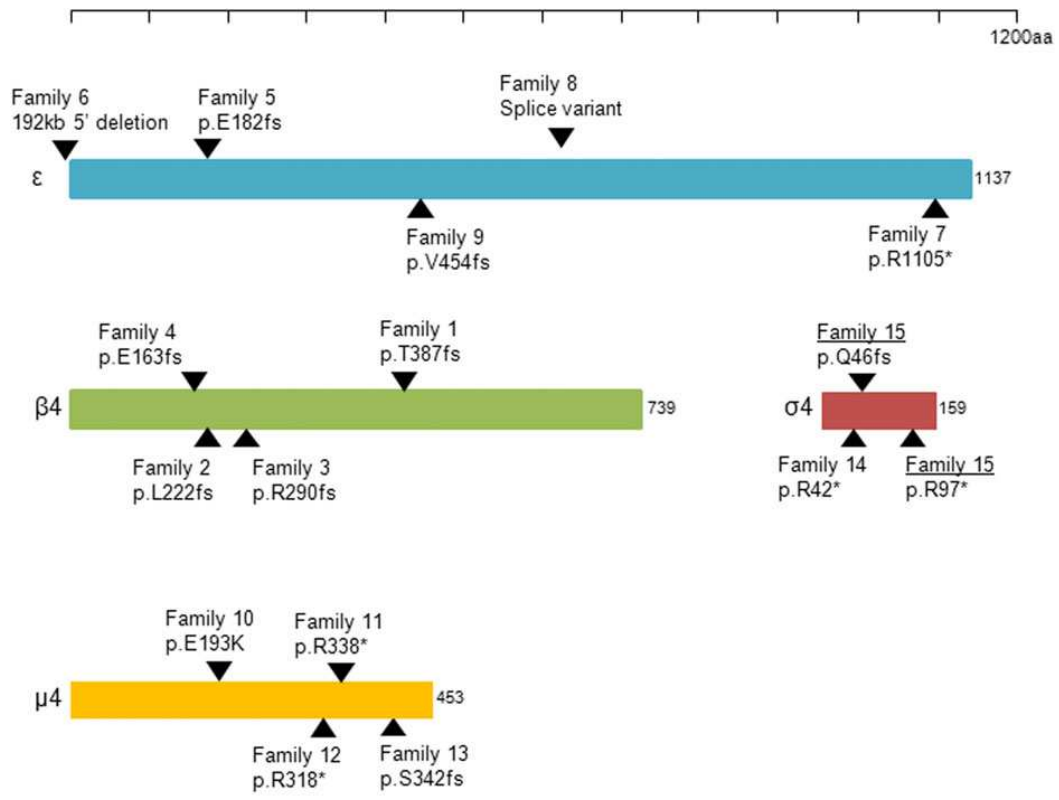


Figure 9. Scheme representing the mutations found in the four subunits of AP-4 complex causative of AP-4 deficiency syndrome (modified from (Hardies *et al.*, 2015).

This syndrome is characterized by microcephaly, muscular hypotonia that evolves to hypertonia, hyperreflexia, spastic paraplegia, inability to walk, severe cognitive deficit, marked speech delay, adaptive impairment, high palate, peculiar facial shape with wide

nasal bridge, short stature, hyperlaxity, genu recurvatum, pes planus or talipes equinovarus, waddling gait, stereotypic laughter, shy character. Epilepsy was observed in some cases. Neuroimaging techniques can show ventriculomegaly, hippocampal globoid formation, flat and thin hippocampus, thin splenium of corpus callosum and reduced white matter in temporal region.

Interestingly, mutations on all subunits of AP-4 complex have been found to cause very similar phenotype as the loss of any of them will lead to the disruption of the complex and to degradation of the remaining subunits. Different types of mutation have been found such as nonsense and frameshift mutations that usually result in premature truncation of the protein.

Very recently a variant of AP-4E1, encoding for the ε subunit, in heterozygosis, was found in individuals affected by stuttering (Raza *et al.*, 2015).

2.4 AMPA receptors

This paragraph will provide a brief description of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or AMPA receptors as they are fundamental proteins for synapse assembly and functioning which trafficking to the plasma membrane is regulated by AP-4 adaptor complex that we identified as TSPAN5 interactor.

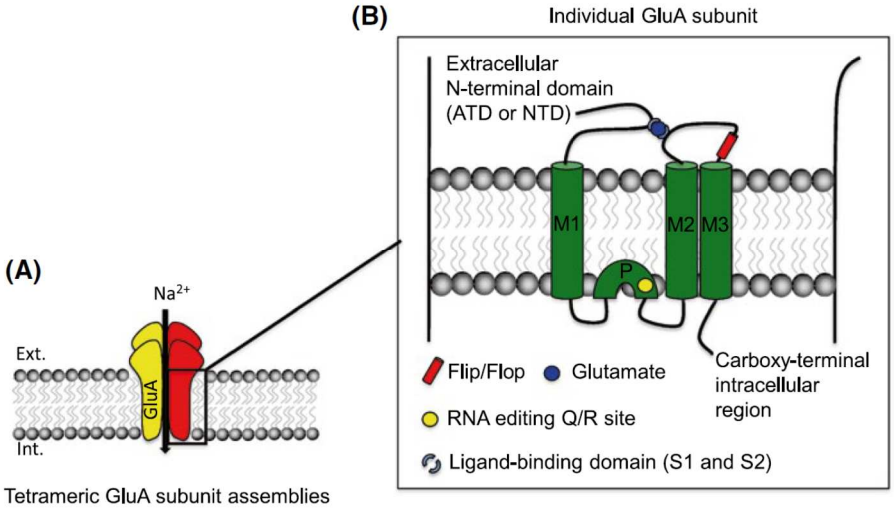


Figure 10. Illustration of A) AMPA-R heterotetramer in the membrane and of B) individual GluA subunit with the membrane domains in green, glutamate in its binding site in blue, Flip/Flop editing site in red and RNA editing Q/R site in yellow (Bassani *et al.*, 2013).

AMPA receptors are among the major glutamate receptors of the brain. They mediate most fast excitatory synaptic transmission thus being fundamental for correct brain functioning with specific roles in learning, memory and cognition. Since their discovery they have been extensively studied in many different models and their importance is now well established for what concerns basal transmission and for mechanisms of plasticity including Hebbian (both

Long Term Potentiation, LTP, and Long Term Depression, LTD) and homeostatic (referred as synaptic scaling).

These ionotropic receptors are tetrameric complexes assembled from four subunits (GluA1-4) (Traynelis *et al.*, 2010) with GluA1-GluA2 and GluA2-GluA3 being the most expressed receptor in adult hippocampus and cortex (Craig *et al.*, 1993).

From a structural point of view all the subunits are composed of an N-terminal extracellular domain (ATD or NTD) followed by a ligand binding domain (LBD), three transmembrane domains (M1, M2 and M3), one re-entrant loop on the cytosolic side and an intracellular C-terminal tail that presents the highest divergence between the four members (Figure 10) (Rosenmund *et al.*, 1998) (Hollmann & Heinemann, 1994) (Dingledine *et al.*, 1999).

Membrane trafficking constitutes one of the main mechanisms of regulation of these receptors with two principal processes involved: removal (or endocytosis) and recycling. These events are strictly interconnected and we will analyse them with major focus on the recycling traffic as it mainly concerns the purpose of this study (Figure 11).

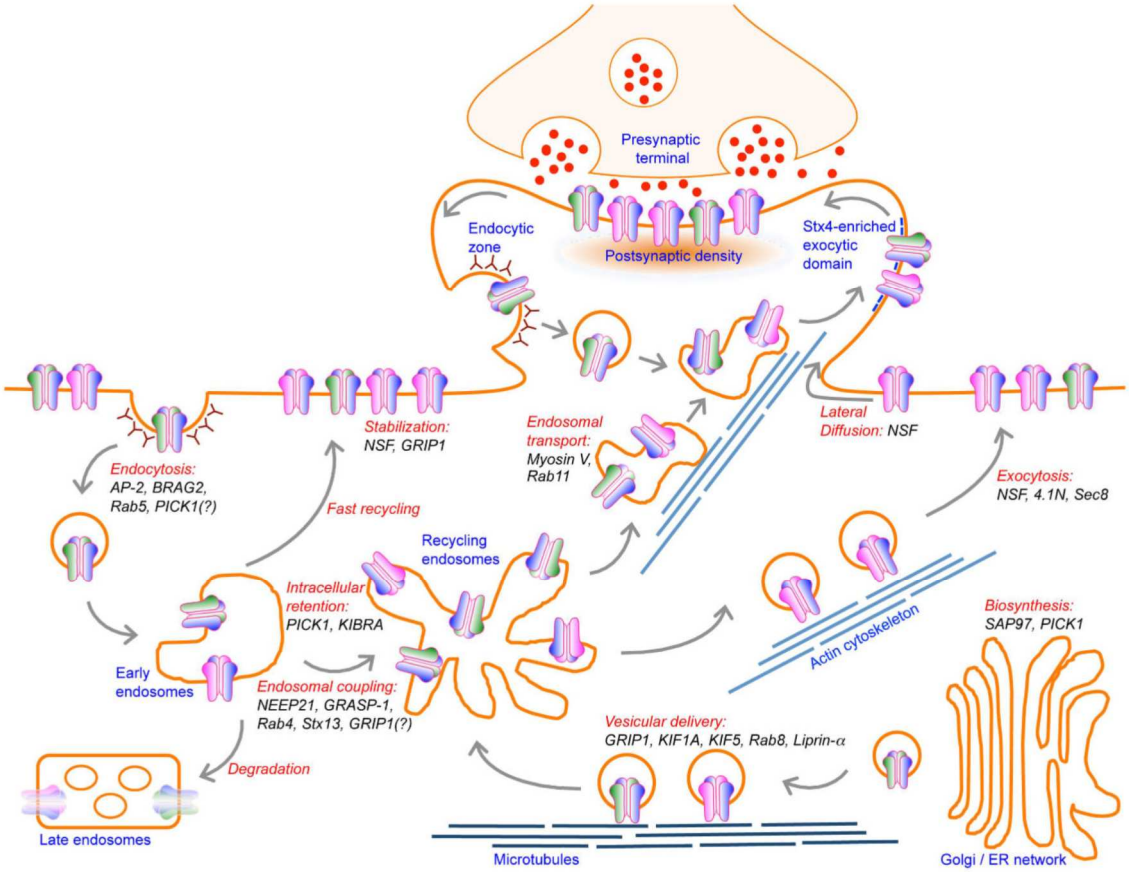


Figure 11. AMPA-Rs membrane trafficking. The receptors are present at the PSD but they can escape laterally diffusing to presynaptic sites where they are endocytosed. The vesicles than reach the endosomal system that will direct them to lysosome for degradation or to recycling endosomes from where they exocytose in perisynaptic sites and then travel back to the PSD via lateral diffusion. Newly synthesized receptors travel from the ER/Golgi network via microtubules and then enter in the endosomal circuit for delivery. Many different interactors involved in these processes are depicted (Anggono & Huganir, 2012).

Two main factors affect AMPA-Rs trafficking: interacting partners and phosphorylation or other modifications; we will cite many proteins that can bind or modify AMPA-Rs and that are involved in these circumstances.

2.4.1 AMPA-Rs endocytosis

AMPA receptors removal is functionally related to LTD and to homeostatic response to sustained activation. It occurs through endocytosis mainly mediated by clathrin-coated pit.

One of the first direct evidence that LTD plasticity induces AMPA-Rs endocytosis come from the observation of GluA2 tagged with pHluorin, a mutant GFP sensitive to pH that is fluorescent only in neutral pH while it is switched off in acidic pH that is found in all the intracellular vesicular compartments, endosomes included. In this condition, the stimulation of LTD through NMDA application induces a decrease in the signal arising from this construct suggesting a rapid endocytosis of GluA2 (Ashby *et al.*, 2004).

One of the major player that acts on AMPA-Rs endocytosis is AP-2, or adaptor protein complex 2, that we discussed earlier in this introduction. It is able to bind AMPA-Rs both directly and indirectly with stronger affinity through stargazin, one of the major TARPs, and to induce internalization of the receptors (Lee *et al.*, 2002) (Kastning *et al.*, 2007) (Matsuda *et al.*, 2013). This event seems of particular importance in NMDA-induced LTD where AMPA-Rs removal is one of the major system to reduce synaptic strength. The site of binding for AP-2 on AMPA-Rs was found to interact with another protein involved in receptors trafficking, NSF (N-ethylmaleimide Sensitive Fusion protein), an ATPase concurring to membrane fusion. The overlapping of the two binding sites produces the competition for AMPA-Rs binding between AP-2 and NSF with the latter being able to stabilize postsynaptic membrane preventing endocytosis (Beretta *et al.*, 2005) (Araki *et al.*, 2010).

AP-3A adaptor was also involved in LTD, in fact it was demonstrated that it is part of a ternary complex with stargazin and AP-2. The formation of this complex is promoted by calcineurin phosphatase activation that dephosphorylates the C-terminus of stargazin. After AP-2 mediated internalization, AP-3A participates in directing AMPA-Rs to lysosomes for degradation (Matsuda *et al.*, 2013).

Another important player in AMPA-Rs endocytosis is PICK1 (Protein Interacting with C Kinase 1). This protein interacts with the C-terminus of AMPA-Rs with greater affinity when calcium ions increase, such as during LTD, and promote endocytosis (Hanley & Henley, 2005). The phosphorylation of GluA2 on residue Serine 880 by PKC increases the binding of PICK1 that was also shown to stabilize AMPA-Rs in intracellular compartments after endocytosis (Lin & Huganir, 2007) (Hanley, 2008) (Citri *et al.*, 2010).

Phosphorylation and dephosphorylation of the C-terminal tail play a major role in receptor endocytosis with differences between the subunits: to simplify, it is predicted that GluA1

unphoshorylated state is more prone to endocytosis whereas GluA2 is preferentially removed from cell surface when it is phosphorylated.

PICK1 is also able to inhibit Arp2/3 (Actin Related Protein 2 and 3) thus blocking actin polymerization that results in spine shrinkage, another important feature of LTD (Rocca *et al.*, 2008) (Nakamura *et al.*, 2011). PICK1 is also regulated by a mechanism of competition, in fact, as described earlier in this introduction, it can be bound by TSPAN7 preventing the association with AMPA-Rs and thus blocking endocytosis (Bassani *et al.*, 2012).

Another player in GluA2 endocytosis is BRAG2 (Brefeldin-Resistant Arf-GEF 2), a guanine-nucleotide exchange factor of the Arf-GEF family. Its binding to AMPA-Rs is enhanced by phosphorylation of tyrosine 876 and it promotes the recruitment of Arf 6, a protein involved in coat recruitment (Scholz *et al.*, 2010).

2.4.2 AMPA-Rs recycling

Recycling is the process through which endocytosed receptors can be redirected to plasma membrane. This pathway is fundamental as it creates a pool of AMPA-Rs that are in close proximity with the postsynaptic membrane and thus ready to be exocytosed rapidly upon request. The events that require this pool of receptors are for example LTP and homeostatic up-scaling. From an experimental point of view it could be difficult to distinguish the recycling from the insertion of new receptors as they follow similar routes and are regulated by similar pathways; thus, we are going to deepen the recycling taking into account also mechanisms that have been proposed for newly synthesized receptors.

In general, it is thought that LTP stimulates the membrane insertion of GluA1-GluA2 receptors that would normally be excluded from synapses, whereas GluA2-GluA3 receptors traffic constitutively in and out the PSD.

Newly synthetized receptors are thought to travel through vesicular transport mediated by either kinesin or dynein (Setou *et al.*, 2002) (Perestenko & Henley, 2003) (Kapitein *et al.*, 2010).

AMPA-Rs recycling is mainly mediated by the adaptor RAB11 (RAs-related protein in Brain 11), which is indeed used as a marker for recycling endosomes, and requires MyosinVa or b motor that transports the vesicles to the plasma membrane on actin filaments routes (Correia *et al.*, 2008) (Wang *et al.*, 2008) (Stenmark, 2009).

The exact site of exocytosis is still under investigation but the most accepted model hypothesizes that it would be out of the PSD in the perisynapse. The receptors then diffuse laterally on the membrane to reach the correct site where they are trapped by scaffolding proteins (Borgdorff & Choquet, 2002) (Ehlers *et al.*, 2007) (Yudowski *et al.*, 2007) (Heine *et al.*, 2008) (Makino & Malinow, 2009).

However, the requirement of AMPA-Rs and the specific tetramers necessary for LTP are still matter of debate. Nevertheless, LTP generates an important entry of calcium that would activate a series of kinases such as CAMKII (Calcium/Calmodulin-dependent protein Kinase II), PKC and PKA (Protein Kinase A) that can phosphorylate GluA1 (Lee, 2006) (Lisman *et al.*, 2012). Many different residues of GluA1 can be phosphorylated: serines 816, 818, 831, 845, 880 and threonine 840 (Barria *et al.*, 1997) (Boehm *et al.*, 2006) (Lee, 2006). For example, the protein 4.1N (protein 4.1 Neuronal) interacts with GluA1 with greater affinity when PKC phosphorylates Ser816 and 818 leading to the insertion of the receptor in the membrane (Shen *et al.*, 2000) (Lin *et al.*, 2009). An atypical PKC isoform, the PKMζ (a truncated form of PKCζ), increases AMPA-Rs levels, GluA2 in particular, at the surface membrane through NSF and its inhibition blocks the maintenance phase of LTP without affecting the induction (Yao *et al.*, 2008). GRIP1/2 (Glutamate receptor interacting protein 1 and 2) association with GluA2 is also regulated by phosphorylation (Kulangara *et al.*, 2007); in fact, the phosphorylation of Ser880 decreases the affinity of this binding that is thought to be necessary for both delivery of newly synthesized receptors, through kinesin heavy chains binding (Wyszynski *et al.*, 2002) (Shin *et al.*, 2003) and recycling of internalized receptors through the interaction with NEEP21 (Steiner *et al.*, 2005). The knockdown of either GRIP1 or NEEP21 results in the accumulation of GLuA2 in early endosomes and lysosomes diminishing its surface expression and thus blocking LTP (Alberi *et al.*, 2005).

GRIP1 also interacts with GRASP-1 (GRIP-associated protein 1), an effector of RAB4 expressed specifically in neurons, which regulates its association with recycling endosomes through syntaxin 13. The removal of GRASP1 also inhibits the late phase of LTP by reducing AMPA-Rs recycling (Ye *et al.*, 2000) (Hoogenraad *et al.*, 2010).

Interestingly, in patients affected by autism, missense mutations of GRIP were found to cause faster recycling and increased surface expression of GluA2 (Mejias *et al.*, 2011).

NSF, which was already cited above in this introduction, is also a regulator of GluA2 insertion in surface membrane and it also act in its stabilization at synapses by competing with the binding of both AP-2 and PICK1.

Exocytosis is also triggered in homeostatic synaptic up-scaling, that is when prolonged activity deprivation, for example by twenty-four hours bath application of TTX, produces recruitment of receptors and increase in currents to counterbalance the chronic inhibition.

On one side, a major role has been attributed to calcium permeable AMPA-Rs, thus lacking GluA2 subunit, but the results are still under debate. It has been observed that TTX induces local synthesis of GluA1 from mRNA in dendrites by effect of retinoic acid signalling. The receptor then reaches the membrane in a pathway dependent on CAMKIIβ and phosphorylation at Ser845 of GluA1 itself (Goel *et al.*, 2011) (Groth *et al.*, 2011). On the other hand, many evidences support a role for GluA2 in synaptic scaling. For example,

knockdown of GluA2, or the overexpression of its C-terminal tail, was enough to block completely the scaling whereas knockdown or the C-terminus of GluA1 had no effect (Gainey *et al.*, 2009). Moreover, also PICK1 was found to be released from GluA2/3 binding upon activity deprivation to increase its delivery to synapses (Anggono *et al.*, 2011).

3. Materials and methods

3.1 cDNA constructs

The shRNA sequence for human TSPAN5 was obtained from (Dunn *et al.*, 2010) and modified to knockdown the rat TSPAN5 mRNA (CAGGACAATTTAACCATTGTG). The Scrambled (SCR) sequence was designed using InvivoGen software available online (www.invivogen.com/sirnawizard/scambled.php) (GCAAATTCGTGTCGTATAACA). Both sequences were designed to be inserted in a short hairpin RNA and cloned into pLVTHM vector. As the shRNA targets specifically the rat sequence all the rescue experiments were performed co-expressing the human cDNA of TSPAN5.

TSPAN5-GFP was a kind gift of Professor Eric Rubinstein (Dornier *et al.*, 2012). TSPAN5 sequence was sub-cloned into pMH4-SYN-tdimer2-RFP; gift of Dr R. Tsien.

RAB4-GFP, RAB7-GFP and RAB11-GFP were kind gift of Professor Giampietro Schiavo.

Neuroligin 1-AP and BirA-ER were kind gifts of Dr Olivier Thoumine.

3.2 Cell cultures, transfection and lentiviral infection

Human Embryonic Kidney 293FT cell line for generating lentivirus was grown in Dulbecco's modified Eagle medium plus 10% fetal bovine serum, 1% penicillin/Streptomycin and 1% G418, an aminoglycoside antibiotic. Lentiviruses were prepared as by Lois and colleagues (Lois *et al.*, 2002). Primary hippocampal neurons were prepared from embryonic rat (E18) brains (Brewer *et al.*, 1993) and plated on coverslips coated with poly-L-lysine (0,25mg/ml) at 75.000/well for immunochemistry and 300.000/well for biochemistry, co-immunoprecipitation and lentivirus infection. Cultured neurons were transfected using Lipofectamine 2000. Immature neurons were transfected or infected at DIV 5 and fixed or lysed at DIV 12 whereas mature neurons were transfected at DIV12 and analysed at DIV18-20.

3.3 Fractionation (PSD and Vesicles)

To prepare synaptic plasma membrane fractions we used the protocol described by Perez-Otano and colleagues (Perez-Otano & Ehlers, 2004).

Rat cortices and hippocampi were homogenized in a buffer composed of 0.32M sucrose and HEPES 4mM, pH 7.4 plus protease inhibitor cocktail with a glass-teflon homogenizer on ice. All the following steps were performed at 4℃. The homogenate was then centrifuged at 1000g for 10 minutes to isolate P1 fraction corresponding mainly to nuclei. The supernatant (S1) was further centrifuged at 10000g for 15 minutes. The resulting supernatant (S2) contains cytosol and light membranes. The pellet (P2), containing crude synaptosomes, was resuspended in the same HEPES/sucrose buffer and centrifuged again at 10000g for 15 minutes to wash out contaminants. P2 fraction was then lysed with hypo-osmotic shock

adding 9 volumes of ice-cold H20 plus protease inhibitors and with a further homogenization in teflon-glass homogenizer. HEPES 1M was added to restore the initial concentration. Complete lysis was obtained by constant mixing for 30 minutes.

The lysate was then centrifuged at 25000g for 20 minutes to remove the crude synaptic vesicles (S3). The pellet (P3) was resuspended in HEPES/sucrose buffer and loaded on a discontinue sucrose gradient made of three phases: 0.8, 1.0 and 1.2 M. The gradient was centrifuged at 150000g for 2 hours without break. The purified synaptic plasma membranes were collected from between 1.0 and 1.2 M layers. 2.5 volumes of 4mM HEPES were added to restore the initial sucrose concentration.

The fraction was further centrifuged at 150000g for 30 minutes (Syn). To further separate the membranes the Syn fraction was resuspended in 50mM HEPES, 2mM EDTA, pH 7.4 plus protease inhibitor cocktail. Triton X-100 was added to a final concentration of 0.5% and the preparation was rotated for 15 minutes.

A centrifugation at 32000g for 20 minutes yielded the PSD-1T fraction. This latter fraction was resuspended in 50mM HEPES, 2mM EDTA, pH 7.4. Triton X-100 was added at final concentration 0.5% to half of the preparation and it was rotated for 15 minutes and then centrifuged at 200000g for 20 minutes to collect the PSD-2T fraction.

PSD-1T+S was obtained incubating the other half of the PSD-1T fraction with 3% sarcosyl for 10 minutes and centrifuging at 200000g for 1 hour.

All the fractions were loaded on poly-acrylamide gels in equal volumes and underwent standard western blotting procedure.

The fractionation of vesicles followed the protocol from Rao and colleagues (Rao *et al.*, 2011) with minor modifications.

Briefly, crude synaptosomes were prepared as described above and lysed with hypo-osmotic shock with ice cold H20. The resulting vesicles were loaded on the top of a continuous gradient of sucrose (from 50mM to 1M) and centrifuged at 65000g for 3 hours at 4℃. Ten fractions of equal volume were collected from the top (Fraction 1) to the bottom (Fraction 10). Protein content was concentrated by precipitation with 6% trichloroacetic acid (TCA) and 0.02 % sodium deoxycholate.

All the fractions were loaded on poly-acrylamide gels in equal volumes and underwent standard western blotting procedure.

3.4 BS3 crosslinking

The experiments were carried out adapting the protocol from Boudreau and colleagues (Boudreau *et al.*, 2012). Cultured neurons at different DIV were first washed with PBS supplemented with 0.1 mM $CaCl_2$ and 1mM $MgCl_2$ (PBS c/m). Non-membrane permeable BS3 cross-linker was added at 1mg/ml concentration in PBS c/m and incubated for 10 min at

4℃. The crosslinking was then quenched washing the cells with 50mM glycine in TBS c/m. A final wash with TBS c/m removes the glycine. The cultures were then collected and lysed with mechanical homogenization in a buffer composed of 50mM Tris, 150mM NaCl, 1mM EDTA, 1% SDS, pH 7,4. The lysates were then loaded on acrylamide gel and underwent standard western blotting procedures to analyse GluA2/3, GluA1, TSPAN5, Stargazin, Tubulin and Transferrin receptor.

3.5 Immunoprecipitation

For immunoprecipitation experiments, cultured neurons and rat brains homogenates (homogenization buffer: 50mM TRIS-HCl, 200 mM NaCl, 1 mM EDTA or 0,1 mM $CaCl_2$ and 1mM $MgCl_2$ pH 7.4, protease inhibitor cocktail supplemented with either 1%NP40 and 1% Triton X-100 or 1% Digitonin) were centrifuged at 10000 g for 30 min at 4℃ and supernatants were incubated with appropriate antibodies at 4℃ overnight. Protein A-agarose beads (Santa Cruz Biotechnology, US) were then incubated with homogenates at 4℃ for 2 hours. The beads were washed three times with lysis buffer, resuspended in 3X sample buffer and bound proteins were separated by SDS-PAGE. The following antibodies were used for immunoprecipitation: rabbit anti: -TSPAN5 (rabbit, SIGMA Aldrich), -GluA2 surface epitope (mouse, Millipore), -GluA2/3 (rabbit, gift of Prof. Gotti), -Stargazin (mouse, Millipore), -AP-4S1 (rabbit, gift of Prof. Hirst), -AP-4E (rabbit, gift of Prof. Hirst), -MyosinVa (rabbit, Sigma Aldrich).

3.6 SDS-PAGE, western blot analysis

Proteins were separated through SDS-PAGE on poli-acrilamyde gels and electro-blotted onto nitrocellulose membranes (GE Healtcare) in buffer containing 0.025 M Tris-HCl, 0.192 M glycine, 20% methanol, SDS 0.05% at 400 mA for 120 min. Immunoblotting reactions were performed by incubating with the primary antibodies (RT, 2-3h in 5% milk TBS-Tween 0.1%) anti: -α tubulin (1:10000, mouse, SIGMA), -TSPAN5 (1:500, rabbit, SIGMA Aldrich), -GluA2 (1:1000, mouse, NeuroMab), -GluA2 surface epitope (1:1000, mouse, Millipore), -GluA2/3 (1:2000, rabbit, gift of Prof. Gotti), -GluA1 (1:1000, rabbit, Millipore), -AP-4S1 (1:250, rabbit, gift of Prof. Hirst), -AP-4E (1:500, rabbit, gift of Prof. Hirst), -Stargazin (1:500, mouse, Millipore), -Transferrin Receptor (1:1000, mouse, Invitrogen), -Neuroligin 1 (1:500, rabbit, Synaptic Systems), -EEA1 (1:500, mouse, Sigma Aldrich), -MyosinVa (1:500, rabbit, Sigma Aldrich), Rab11 (1:500, mouse, BD), -VGlut1 (1:500, rabbit, Synaptic Systems), -GFP (1:1000, rabbit, MBL).

Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:2000 GE Healtcare) were used as secondary antibodies (RT, 1h in 5% milk, TBS-Tween 0.1%).

Immunoreactive bands were visualized by enhanced chemiluminescence (ECL,

PerkinElmer). Band intensity was measured using ImageJ and statistical analysis performed with GraphPad Prism 6 software. Data are expressed as mean ± SEM.

3.7 Immunofluorescence, surface staining

Cultured Hippocampal neurons were fixed either in 4% paraformaldehyde- 4% sucrose for 10 minutes at 37℃ or with methanol 100% for 10 minutes at -20℃ and incubated with anti: -TSPAN5 (1:50, rabbit, SIGMA Aldrich), -PSD-95 (1:400, mouse, NeuroMab), -GluA2 (1:200, mouse, NeuroMab), -GluA2 surface epitope (1:100, mouse, Millipore), -GluA2/3 (1:200, rabbit, gift of Prof. Gotti), -GluA1 (1:200, rabbit, Millipore), -Bassoon (1:100, mouse, Neuromab),) -AP-4S1 (1:100, rabbit, gift of Prof. Hirst), -AP-4E (1:100, rabbit, gift of Prof. Hirst) in GDB1X solution (2X: gelatin 0.2%, Triton X100 0.6%, 30mM Na2HPO4 pH 7.4, 0.9M NaCl) for 2 hours at room temperature.

For surface staining, live DIV18-20 hippocampal neurons were incubated for 10 min at 37℃ with anti GluA2 surface epitope antibody in culturing medium. After washing (PBS supplement with 1mM MgCl2 and 0.1 mM CaCl2), neurons were fixed for 10 minutes at room temperature in 4% paraformaldehyde/ 4% sucrose.

Cells were then washed and incubated with Alexa 488 (1:400, Invitrogen), Alexa 555 (1:400, Invitrogen) or DyeLight-649 (1:200, Jackson Laboratories) coupled secondary antibodies anti-mouse diluted in GDB1X solution for 1 hour at room temperature.

3.8 Membrane trafficking imaging assays (internalization and recycling)

Internalization experiments were performed as by Bassani and colleagues (Bassani *et al.*, 2012).

Neurons were incubated with the anti-GluA2 surface epitope antibody at 10 µg/ml in culture medium for 10 minutes at room temperature. The excess antibody was then removed by washing with PBS c/m. The antibody-bound receptors were then allowed to undergo internalization for 0, 5 or 10 minutes at 37℃. After paraformaldehyde fixation, a secondary antibody labelled with AlexaFluor 555 was incubated in non-permeabilizing condition (PBS supplemented with 10% goat serum) for 1 hour at room temperature. After washing, the coverslips were incubated with a secondary antibody labelled with DyeLight-649 in permeabilizing condition (GDB1X) for 1 hour at room temperature.

Recycling assay was adapted from the one of Raynaud and colleagues (Raynaud *et al.*, 2013). The antibody anti-GluA2 surface epitope was incubated as above but at 37℃ to potentiate the internalization that was then allowed to occur for 30 minutes. To mask the antibody-bound receptor that was not internalized, a secondary antibody labelled with AlexaFluor 488 was incubated for 10 minutes at 37℃. The unlabelled, internalized, antibody-bound receptor was then allowed to be recycled back to the surface of neurons for 0 or 10

minutes. After paraformaldehyde fixation the coverslips were incubated with secondary antibodies as described above.

3.9 Image acquisition, quantification and statistical analysis

Confocal images were obtained using a ZEISS LSM 510 Meta Confocal Microscope with a Nikon 63x objective with sequential acquisition setting at 1024x1024 pixels resolution. Each image was a 'z' series projection of approximately 7 to 12 images taken at 0.75 µm depth intervals.

Transfected neurons were chosen randomly for quantification from two to ten coverslips from three to five independent experiments.

Spine number, fluorescence intensity and co-localisation measurements were performed using NeuronStudio and ImageJ (Jacop plugin for co-localisation). Statistical comparisons were performed with appropriate statistical test using GraphPad Prism 6 software. Data are expressed as mean ± SEM.

3.10 uPAINT

uPAINT experiments for GluA2 were performed as by Giannone and colleagues (Giannone *et al.*, 2010). Briefly, neurons transfected at DIV5 were put in an imaging chamber at DIV12 in Tyrode's buffer and mounted on an inverted microscope (model No. IX71; Olympus America, Melville, NY) equipped with a high 100X objective (NA . 1.4) and a charge-coupled device camera (Cascade 128; Roper Scientific, Princeton Instruments, Trenton, NJ). The setup used was the one available at the Bordeaux Imaging Center, part of the France-BioImaging national infrastructure.

Once a transfected neuron was found the anti-GluA2 antibody coupled to Atto-647 (gift of Dr Choquet) was added directly to the medium and rapidly resuspended.

Registration of single phluorophores excited with a HeNe laser at tilted angle started when a sufficient number of molecules were visible and lasted for 4000 frames of 20ms (80s). Around four acquisitions per coverslip were collected.

For Neuroligin-1 experiments, Neuroligin-1-AP, BirA-ER and either Scrambled or ShRNA-TSPAN5 were transfected. Coverslips were mounted on the same instrument as above. In this case, the detection of surface molecule were possible adding monomeric Streptavidin coupled to Atto-594 (gift of Dr Thoumine) to the medium before starting the recording.

The trajectories were analysed with a custom-made program (MATLAB, The MathWorks, Natick, MA) developed by Dr Sibarita through Metamorph.

For both proteins, only the trajectories that last for more than 8 frames were considered for analysis. The optical resolution of this acquisition was 50 nm. For each molecule, the diffusion coefficient D was calculated and then plotted versus the percentage of molecule for

each D. Mean global diffusion coefficient was used for statistical comparison.

3.11 Yeast Two-Hybrid Screening

For Yeast two-hybrid experiments, a fragment corresponding to the TSPAN5 C-terminal tail (aa 254-268) was cloned in frame with the GAL4 DNA-binding domain (pGBKT7 vector), and used as bait to screen a human adult brain cDNA library (Clonetech, Mate and Plate Library). Positives clones (3+) grew on plates containing X-α-GAL and Aureobasidin A (QDO/X/A plates) and expressed all four integrated reporter genes: HIS3, ADE2, AUR1C and MEL1 under the control of three distinct Gal4-responsive promoters. cDNA plasmids from positive clones were recovered via DH5a *Escherichia coli* (*E.coli*) transformation and plated on ampicillin plates and sequenced.

4. Results

4.1 TSPAN5 localisation in brain suggests two distinct functions

As so little is known about TSPAN5 in the brain we started evaluating its presence in different brain areas. We dissected adult mouse and rat brain separating hippocampus, cortex and cerebellum. By western blot, we were able to show that the protein is expressed broadly in the brain of both animals as it is present in all the three areas examined and in rat cultured hippocampal neurons (Figure 12 panel A). Moreover, the protein is present in its monomeric form (around 38 KDalton) and in aggregates of different molecular weights.

We then wanted to analyse further the distribution of the protein inside the neurons and we thus performed immunofluorescence staining in cultured hippocampal neurons following the maturation of the cultures. We observed that the protein is present during all the phases of neuron maturation in culture being present already at DIV1 and still expressed at DIV18 when the neurons are generally considered mature (Barnes & Polleux, 2009).

A closer look at the staining suggested that the protein is broadly distributed in immature neurons (until DIV12) with a pattern that is suggestive of membrane localisation (Figure 12 panel B, orange arrow). In mature neurons, instead, TSPAN5 becomes enriched in what resembles the perinuclear endoplasmic reticulum and in protrusions along the dendrites suggestive of dendritic spines (Figure 12 panel D, orange arrow).

To confirm this hypothesis we performed BS3 crosslinking experiment at two developmental stages: DIV12 representative of immature neurons where synaptogenesis is starting and DIV18 when the neurons are mature and spiny (Figure 12 panel E).

These experiments use BS3, a chemical crosslinker that is non-permeable to the membrane; in this way the treatment on live cultured neurons crosslinks only surface protein and after western blot analysis, it is possible to distinguish in the same gel lane between surface and intracellular protein as they have different molecular weights.

We observed that the ratio of extracellularly facing versus total protein was higher at DIV12 compared to DIV18 suggesting that the protein changes its localisation during neuronal maturation being mainly present on the surface of immature neurons while in mature stages it partially redistributes to intracellular compartments (Figure 12 panel E) (Extracellular/Total ratio: TSPAN5 DIV12 0,859±0,02, DIV19 0,670±0,04; N=3; * p value = 0,0272, T-Test Mann-Whitney Post-hoc). Transferrin Receptor, used as control for surface proteins, maintains a stable configuration (Extracellular/Total ratio: TfR DIV12 0,474±0,19, DIV19 0,511±0,21; N=3; NS p value = 0,9043, T-Test Mann-Whitney Post-hoc). Tubulin was used as control of the non-permeability of BS3 and of homogeneity in protein amounts.

We further analysed the mature localisation of TSPAN5 by separating postsynaptic densities from adult rat brain. TSPAN5 appears to be present in synaptosomes and in postsynaptic

density where PSD-95 is strongly enriched (Figure 12 panel F). Tubulin was present in all fraction whereas Synaptophysin, a presynaptic marker, disappears in the last fractions corresponding to PSD.

Concordantly, we found that the protein co-localised with postsynaptic markers such as PSD-95 and GluA2 in staining on mature cultured hippocampal neurons while it did it very little with presynaptic markers such as Bassoon, VGLUT1 (Vesicular GLUtamate Transporter 1) and VGAT (Vesicular GAba Transporter) (Figure 12 panel G) (Percentage of TSPAN5 co-localising with: GluA2 85,0±10,2; PSD-95 87,4±4,2; Bassoon 28,0±8,8; VGLUT1 4,9±4,5; VGAT 7,5±5,7; N=10 for each condition).

4.2 TSPAN5 regulates the formation of dendritic spines

The presence of TSPAN5 on surface membranes of neuron dendrites at developmental stages when synaptogenesis peaks suggests that this protein could regulate the formation of dendritic spines.

To investigate this point we designed a shRNA to knockdown the expression of this protein. The shRNA was tested by producing a lentivirus carrying the sequence and a GFP as reporter of the infection and compared to its Scrambled. The lentivirus infects the neurons and promotes the insertion of the sequences in random positions in the genome ensuring a stable expression of both the shRNA and the GFP. As observed in Figure 13 panel A the lentivirus was able to knockdown efficiently the level of the protein when applied to cultures for around five days in vitro (Figure 13 panel A).

The Sh-TSPAN5 vector, or the one expressing its Scrambled sequence used as control, was transfected in neurons at DIV5, before synaptogenesis occurs, and the neurons were analysed at DIV20 when the majority of spines are physiologically formed.

Figure 13 panel B and relative quantification show that, analysing only neurons that express the GFP as reporter of transfection, the number of dendritic spines was almost reduced to zero in condition of TSPAN5 silencing (Figure 13 panel B) (Dendritic spines density normalized to 1 for Scrambled: Scrambled 1±0,04, Sh-TSPAN5 0,04±0,02; N=10; *** p value < 0,0001; T-Test, Mann-Whitney post-hoc).

In this experiment, dendritic spines were count using NeuronStudio software that automatically recognizes spines by their morphology visible thanks to cytosolic GFP expression.

To further confirm this surprisingly strong effect we performed an immunocytochemical staining of a presynaptic (Bassoon) and of a postsynaptic (GluA2) protein to quantify the number of co-localising points, representative of synapses. As figure 13 panel C shows, the result was similar to the previous analysis (Number of co-localising points normalized to 1 for Scrambled: Scrambled 1±0,08, Sh-TSPAN5 0,06±0,01; N=10; *** p value < 0,0001; T-Test

Mann-Whitney post-hoc).

We also observed that different postsynaptic markers were significantly reduced (PSD-95 and GluA2) whereas presynaptic Bassoon has also a trend in reduction but not statistically significant, again strengthening the hypothesis of a main postsynaptic role of this protein (Figure 13 panel D) (Number of puncta normalized to 1 for Scrambled; Bassoon: Scrambled 1±0,15, Sh-TSPAN5 0,4±0,24; N=10; NS; GluA2: Scrambled 1±0,11, Sh-TSPAN5 0,34±0,03; N=10; ** p value = 0,0061; PSD-95: Scrambled 1±0,25, Sh-TSPAN5 0,55±0,02; N=10; * p value=0,0286).

The clear effect observed in these experiments strongly prompted us to investigate whether TSPAN5 could have a role directly in synaptogenesis and particularly in dendritic spines formation thanks to its predominantly postsynaptic localisation.

4.2.1 TSPAN5 organizes TEMs that accommodate Neuroligin 1 and GluA2

Since TSPAN5, as all the other members of the tetraspanin superfamily, is able to form Tetraspanin Enriched Microdomains and since TSPAN5 staining at immature stages is suggestive of the presence of the protein in clusters on the surface membrane, we wondered whether TEMs formed by TSPAN5 on neurons surface could be responsible of dendritic spine formation.

Dendritic spine formation is a process that is still under debate but the most recent and accepted hypothesis is that upon contact between forming presynaptic bouton and growing postsynaptic filopodium the initial trans-synaptic interaction between Neurexin, on the presynapse, and Neuroligin 1, on the postsynaptic side, triggers the concentration of a variety of molecule on both sides.

The protein that is thought to first concentrate is indeed Neuroligin 1 on the postsynapse; it would interact with the occurring Neurexins on the presynapse strengthening the adhesion between the two compartments.

After that, PSD-95 localises in what will become the Postsynaptic Density and will stabilize all the receptors (AMPA-Rs, NMDARs…) and accessory proteins necessary for the correct functioning of mature synapses. A more detailed explanation of the process is provided in Chapter 2 Introduction, paragraph 2.2.

We thus decide to investigate whether TSPAN5 TEMs could be the membrane scaffold that allows the stabilization of surface membrane proteins before the complete PSD complex is assembled.

To verify this hypothesis we took advantage of the solubilisation characteristics and high cholesterol content of TEMs.

The lysis with standard RIPA buffer, depleted of SDS, partially solubilizes Tetraspanins while lysis with a buffer containing digitonin 1% will lead to the precipitation of all cholesterol-

enriched membranes such as TEMs. The centrifugation at 10000g for 20 minutes allows to separate soluble and insoluble component.

As shown in Figure 14 panel A TSPAN5 is partially, if not almost completely, solubilized by RIPA as shown by the presence of the protein in the surnatant fraction and only very little in the pellet fraction. The opposite situation was obtained with digitonin; only very little amount of the protein was present in the solubilized fraction while the vast majority was in pellet. We decided to look first for Neuroligin 1 and secondly for GluA2/3, as representative of membrane proteins that concentrate in a second phase in the forming postsynapse. We observed that both proteins behaved in the same way as TSPAN5 while Transferrin receptor, used as a membrane protein not specifically concentrated in synapses, and Tubulin, used as cytosolic protein, had a much broader distribution in the different fractions (Figure 14 panel A; N=3).

4.2.2 TSPAN5 stabilize Neuroligin-1 and GluA2 at the forming postsynapse

To confirm that TSPAN5 TEMs concentrate Neuroligin 1 and AMPA-Rs we decided to verify whether the silencing of TSPAN5 could modify the solubility of these two proteins applying digitonin lysis as in the previous experiment.

As expected by our hypothesis, the distribution of Neuroligin 1 and GluA2/3 was changed in favour of solubilized compartment upon knockdown of TSPAN5 suggesting that the membrane domains formed by TSPAN5 accommodate these proteins important for dendritic spine formation (Figure 14 panel B and C) (Supernatant/Pellet ratio normalized to 1 for Scrambled, GluA2/3: Scrambled 1±0,05, Sh-TSPAN5 0,65±0,10; N=5; * p value=0,013; Neuroligin 1: Scrambled 1±0,01, Sh-TSPAN5 0,75±0,10; N=4; * p value=0,048). Transferrin receptor and Tubulin, instead were not affected by TSPAN5 silencing (Figure 14 panel B and C) (Supernatant/Pellet ratio normalized to 1 for Scrambled, TfR: Scrambled 1±0,04, Sh-TSPAN5 0,98±0,11; N=3; NS; Tubulin: Scrambled 1±0,03, Sh-TSPAN5 1,05±0,15; N=3; NS).

As control, we verified by BS3 crosslinking that the total and the surface/total ratio of both proteins were unchanged in TSPAN5 silencing condition confirming that the effects observed were only due to redistribution of the proteins and not to reduced expression or degradation (Figure 14 panel D) (Neuroligin 1: Total/tubulin normalized to 1 for Scrambled, Scrambled 1±0,11, Sh-TSPAN5 0,93±0,17; N=3; NS; Extracellular/Total ratio normalized to 1 for Scrambled, Scrambled 1±0,10, Sh-TSPAN5 0,96±0,13; N=3; NS; GluA2/3 Total/tubulin normalized to 1 for Scrambled, Scrambled 1±0,06, Sh-TSPAN5 1,11±0,07; N=3; NS; Extracellular/Total ratio normalized to 1 for Scrambled, Scrambled 1±0,22, Sh-TSPAN5 1,06±0,18; N=3; NS).

To strengthen this hypothesis we decided to use super-resolution microscopy in living neurons using a recently developed method named uPAINT (universal Point Accumulation

for Imaging in Nanoscale Topography).

This method allows the visualization of freely moving molecules on the surface membrane and the quantification of their degree of mobility. (Giannone *et al.*, 2013).

We analysed the mobility of endogenous GluA2 receptor in DIV12 rat hippocampal neuron transfected at DIV5 either with the Scrambled shRNA, the Sh-TSPAN5, the Sh-TSPAN5 plus a TSPAN5-RFP insensible to the Sh-TSPAN5 (Rescue) or the TSPAN5-RFP construct alone, applying an antibody against an extracellular epitope of the receptor coupled to a molecule of Atto-647 fluorophore (Figure 15 panel A).

Plotting the logarithm of diffusion coefficients (logD) versus the relative frequency of molecules moving with that coefficient, we obtained for GluA2 in Scrambled-shRNA two peaks, similarly to what observed by previous groups (Nair *et al.*, 2013), corresponding to immobilized receptors when the logD was inferior to -1,6 and to mobile receptors when the logD was above -1,6 (Figure 15 panel B). These two populations correspond respectively to receptors residing in synapses and thus immobilized, and to receptors that are free to move on the membrane. Comparing our results with the one previously published by other groups (Nair *et al.*, 2013) the balance was in favour of the mobile population of receptors in accordance with the fact that we analysed the mobility in younger neurons when the number of formed synapses is still much lower than in mature neurons.

When we analysed the mobility of GluA2 in neurons where TSPAN5 was knocked down we observed a shift of the curve towards higher diffusion coefficients corresponding to a higher mobility of the receptors (Figure 15 panel B). Concordantly with this, the mean global diffusion coefficient of GluA2 receptor was significantly increased (Figure 15 panel C) (Mean Diffusion Coefficient: Scrambled 0,013±0,002 N=22, Sh-TSPAN5 0,025±0,004 N=21; * $p < 0,05$; Rescue 0,008±0,001 N=18 ** $p < 0,001$ versus Sh-TSPAN5; Overexpression 0,007±0,001 N=18; ** $p < 0,001$ versus Sh-TSPAN5; One-Way ANOVA Tukey's Multiple Comparison) as well as the ratio between the mobile and the immobile population (Figure 15 panel D) (Mobile/Immobile Ratio: Scrambled 1,20±0,09 N=22, Sh-TSPAN5 1,6±0,12 N=21; * p value $< 0,05$; Rescue 1,04±0,08 N=18 ** p value $< 0,01$ versus Sh-TSPAN5; Overexpression 0,98±0,08 N=18 *** p value $< 0,001$ versus Sh-TSPAN5; One-Way ANOVA Tukey's Multiple Comparison).

When we co-expressed a shRNA insensitive TSPAN5-RFP together with the Sh-TSPAN5 we observed a reversal of the effect that was strong enough to produce an opposite variation on the mobility and mobile/immobile ratio. A similar effect was observed in neurons expressing the TSPAN5-RFP construct alone suggesting that the overexpression of TSPAN5, likely by increasing the number or the size of TSPAN5 formed TEMs, further stabilizes GluA2.

We then wanted to verify if Neuroligin 1 behaved the same in this condition. Unfortunately, no highly specific antibody directed to extracellular epitope of the protein are available and

we were forced to use an approach based on overexpressing a tagged version of Neuroligin 1 that presents an AP tag that is byotinilated by the bacterial enzyme BirA co-expressed in neurons (Chen *et al.*, 2005). Once the two proteins are co-expressed, and thus the overexpressed Neuroligin 1 is byotinilated on the surface, it is possible to use an Atto-594-coupled monomeric streptavidin to detect the protein and follow its mobility (Fig 15 panel E).
Compared to GluA2, Neuroligin 1 did not show a two populations behaviour while instead a single peak was detected. When TSPAN5 expression was silenced, the curve was once again shifted to higher diffusion coefficients (Figure 15 panel F) and the global diffusion coefficient was significantly higher than the one in Scrambled condition (Figure 15 panel G) (Mean Diffusion Coefficient: Scrambled 0,011±0,0009 N=18, Sh-TSPAN5 0,023±0,0048 N=18; * p value = 0,0192; T-Test Mann-Whitney post hoc).
This result suggests that also Neuroligin 1 can be trapped and stabilized inside TSPAN5 TEMs. Unfortunately, we weren't able to analyse the behaviour of Neuroligin 1 in Rescue and Overexpression as the RFP signal of TSPAN5-RFP construct would have masked and biased the detection of Atto-594 molecules. We are planning to implement the analysis of Neuroligin 1 mobility in these two conditions by using a bi-cistronic vector expressing either TSPAN5 with a GFP reporter alone or together with the Sh-TSPAN5.
All these results together strongly suggest that TSPAN5 assembles in membrane domains that are responsible of the formation, or stabilization, of dendritic spines in cultured rat hippocampal neurons.
Our findings propose that TEMs formed by TSPAN5 are able to stabilize Neuroligin 1 and GluA2 and to trap or at least slow down their free movements in the membrane increasing the likelihood of transynaptic interaction and intrasynaptic stabilization (Figure 16).

4.3 TSPAN5 regulates GluA2 recycling

4.3.1 TSPAN5 interacts with AP-4 and GluA2

As we hypothesized two different functions for TSPAN5 at different developmental stages, after having characterized the one during synaptogenesis, we wanted to deepen the role of this protein in mature neurons. As from our first experiments, we have found that this second activity was likely taking place in some membranous intracellular compartment, we decided to look for interactors on the intracellular side. We thus used the C-terminal tail of the protein to perform a yeast two-hybrid screening against an adult human brain cDNA library finding different interesting interactors. We decided to focus our attention on AP-4σ, a subunit of the Adaptor Protein Complex 4, an obligate tetrameric association of four proteins (AP-4ε, AP-4β, AP-4μ, AP-4σ) belonging to the family of Adaptor Proteins that also counts the more famous AP-1 and AP-2 all involved in vesicular trafficking (Figure 17 panel A).
Little is known on this protein and more details can be found in Introduction paragraph 2.3.

AP-4 is currently thought to traffic proteins cargo from the Trans Golgi Network to the endosomal compartment and it has been involved in sorting of AMPA-Rs to dendritic compartment through its binding with Stargazin, one of the TARP (Transmembrane AMPA receptor Regulatory Protein) very well known for its role in regulating stabilization, trafficking and dynamics of AMPA-Rs.

We were thus interested in understanding whether TSPAN5 could also be involved in AMPA-Rs trafficking and consequently in synapses functioning.

We reconfirmed the interaction of TSPAN5 with AP-4σ in immunoprecipitation experiments performed in lysates of rat hippocampi (Figure 17 panel B). We detected AP-4 σ and ε when we immunoprecipitated TSPAN5 with a specific antibody and we were also able to find TSPAN5 when we performed immunoprecipitation with anti AP-4σ or ε antibodies.

As it is already known that AP-4 complex is able to bind GluA2 through Stargazin we looked for this AMPA-R in immunoprecipitates of TSPAN5. We were able to detect GluA2 in immunoprecipitates for TSPAN5 in adult rat hippocampi lysates in HEPES-EDTA buffer, known to fluidify membrane, and after -80 freezing to destroy Tetraspanin Enriched Microdomains thus detecting only specific direct interactions (Figure 17 panel C).

4.3.2 Silencing of TSPAN5 reduces GluA2 and Stargazin

We were then interested in analysing if the knockdown of TSPAN5 could somehow influence GluA2 and Stargazin expression, especially on surface membrane considering the known role of Stargazin in trafficking and stabilization of AMPA-Rs.

We decided to perform BS3 crosslinking experiments in hippocampal neurons infected with a lentivirus carrying either the Scrambled-shRNA or the Sh-TSPAN5 and analysed via standard western blotting the expression on the surface of GluA2/3, Stargazin and GluA1 (Figure 18 panel A). Transferrin was analysed as control for surface proteins, tubulin for cytosolic proteins and GFP was used as control for efficient infection.

We found that GluA2 was strongly reduced both in total amount and in the surface/total ratio demonstrating an impairment of trafficking or stabilization of the protein on the surface with a likely consequent degradation of mis-localised receptors (Fig 18 panel B) (Total/Tubulin Ratio: GluA2/3, Scrambled 1±0,03, Sh-TSPAN5 0,43±0,01; N=4; *** p value < 0,001; Transferrin Receptor, Scrambled 1±0,1, Sh-TSPAN5 1,02±0,06; N=4; NS; Extracellular/Total Ratio: GluA2/3, Scrambled 1±0,01, Sh-TSPAN5 0,61±0,02; N=4; *** p value < 0,001; Transferrin Receptor, Scrambled 1±0,06, Sh-TSPAN5 1,02±0,06; N=4; NS; T-Test Mann-Whitney Post-Hoc).

However, GluA1 signal was not reduced, but instead showed an increase in the surface/total amount, probably due to an attempt of the cells to counteract the loss of GluA2 receptors (Total/Tubulin Ratio: GluA1, Scrambled 1±0,01, Sh-TSPAN5 1,32±0,02; N=3; * p value;

Extracellular/Total Ratio: GluA1, Scrambled 1±0,01, Sh-TSPAN5 1,4±0,11; N=3; * p value = 0,0278; T-Test Mann-Whitney Post-Hoc). We also detected a small decrease of Stargazin only on the surface with no total protein amount differences and this is in accordance with the role of Stargazin on all AMPA-Rs and not only on GluA2 subunit containing ones (Total/Tubulin Ratio: Stargazin, Scrambled 1±0,15, Sh-TSPAN5 1±0,10; N=4; NS; Extracellular/Total Ratio: Stargazin, Scrambled 1±0,06, Sh-TSPAN5 0,67±0,09; N=4; * p value=0,0285; T-Test Mann-Whitney Post-Hoc).

This result could represent that the mechanism observed is specific for GluA2 subunits of AMPA-Rs.

We were able to confirm the reduction of GluA2 on the surface also by surface immunostaining on hippocampal neurons transfected with Scrambled-shRNA or Sh-TSPAN5 (Figure 18 panel C) (Number of puncta/ area normalized to 1 for Scrambled; GluA2 Scrambled 1±0,12, Sh-TSPAN5 0,75±0,05; N=10; * p value = 0,033; T-Test Mann-Whitney post hoc).

As no good antibody for surface labelling were available for GluA1 we performed a standard immunocytochemistry protocol and reconfirmed the increase in GluA1 (Figure 18 panel C) (Number of puncta/ area normalized to 1 for Scrambled; GluA1 Scrambled 1±0,08, Sh-TSPAN5 1,59±0,17; N=10; ** p value = 0,0091; T-Test Mann-Whitney post hoc.

We also checked the effect of TSPAN5 silencing on dendritic spines number in mature neurons, as we have observed a fundamental role of TSPAN5 in its formation. We found a significant decrease in spine density but less pronounced than the one obtained when the protein was knocked down before the peak of synaptogenesis (Figure 18 panel D) (Spine Density normalized to 1 for Scrambled: Scrambled 1±0,05, Sh-TSPAN5 0,63±0,03; N=8; *** p value < 0,001; T-Test Mann-Whitney post-hoc). This reduction could be due to different effects: one it could be that the important role of TSPAN5 in spines formation would be affecting also the continuous renovation of dendritic spines that occurs also at mature stages; the second could be that the reduction in GluA2 is affecting spine stability. Unfortunately, we cannot exclude one or the other effect.

4.3.3 TSPAN5-induced GluA2 reduction is due to lysosomal degradation

We then wanted to deepen the mechanism through which GluA2 appears to be reduced. The most likely hypothesis would be that receptors that are somehow perturbed in their trafficking or in stabilization at synapses and thus retained intracellularly would then be degraded to avoid excessive accumulation.

Two are the main degradation pathways for proteins in the cells: the proteasome or the lysosomes. We thus decided to block these two pathways and look for GluA2. Using MG132, we blocked proteasome and then analysed GluA2/3 via immunostaining in neurons

transfected with Scrambled-shRNA or Sh-TSPAN5 (Figure 19 panel A and B). As appears by the quantification of total amount of GluA2/3, MG132 was able to increase receptors amount in Scrambled condition and in Sh-TSPAN5. However, the increase in this latter condition was not sufficient to reach the level of the treated Scrambled-shRNA suggesting that proteasome was not involved in this degradation (Mean Intensity normalized to 1 for Scrambled DMSO: Scrambled DMSO 1±0,07 N=6, Scrambled MG132 1,63±0,03 N=6, Sh-TSPAN5 DMSO 0,66±0,11 N=6, Sh-TSPAN5 MG132 1,27±0,06 N=6; Scr DMSO vs Scr MG132 *** p value < 0,001; Scr DMSO vs Sh-TSPAN5 DMSO * p value < 0,05; Sh-TSPAN5 DMSO vs Sh-TSPAN5 MG132 *** p value < 0,001; Scr MG132 vs Sh-TSPAN5 MG132 * p value < 0,05; One way ANOVA Holm-Sidak Multiple comparison). We thus decided to verify whether the lysosomal pathway could be responsible of this degradation.

In fact, as GluA2 is a transmembrane protein it will always reside in vesicles for each step of the trafficking making lysosomal degradation to be more likely involved in this process.

As expected, the treatment with leupeptin, a specific lysosomal blocker, was able to rescue the levels of GluA2/3 in Sh-TSPAN5 almost completely to the levels reached by the treated control Scrambled-shRNA (Figure 19 panel C and D) (Mean Intensity normalized to 1 for Scrambled H20: Scrambled H20 1±0,04 N=6, Scrambled Leupeptin 1,39±0,09 N=6, Sh-TSPAN5 H20 0,69±0,03 N=6, Sh-TSPAN5 Leupeptin 1,30±0,06 N=6; Scr H2O vs Scr Leupeptin ** p value < 0,01; Scr H2O vs Sh-TSPAN5 H2O * p value < 0,05; Sh-TSPAN5 H2O vs Sh-TSPAN5 Leupeptin *** p value < 0,001; Scr Leupeptin vs Sh-TSPAN5 leupeptin NS; One way ANOVA Holm-Sidak Multiple comparison).

4.3.5 TSPAN5 is localised in recycling endosomes

We demonstrated that TSPAN5 silencing causes a reduction of GluA2 receptors on the surface inducing an increased degradation via lysosomes.

We have shown that in mature neurons there is an increase in the level of TSPAN5 in intracellular compartment and that this protein can interact with AP-4 complex that is known to be involved in intracellular vesicles trafficking.

All these results support the hypothesis that TSPAN5 can be involved in the stabilization or in the sorting of GluA2 receptors in some intracellular vesicles.

To understand further which of the numerous types of vesicles could be involved in these processes we decided to perform a vesicle fractionation from rat brain hippocampi and cortices. We prepared crude synaptosomes and lysed them with hypo-osmotic shock to release the intracellular content that was then loaded on top of a linear gradient of sucrose.

Equal volume fractions were then collected and protein content was precipitated with Sodium Deoxycholate and Trichloroacetic acid and analysed by standard western blotting.

The fractions were characterized using different proteins as markers: EEA1 (Early

Endosomes Associated protein 1) marks early endosomes, VGLUT1 synaptic vesicles, Transferrin Receptor and RAB11 mark the recycling endosomes. When we analysed TSPAN5 we found a distribution very similar to the one of Transferrin Receptor and RAB11 strongly suggesting that this protein could reside in recycling endosomes vesicles (Figure 20 panel A).

To confirm this result we transfected DIV14 hippocampal neurons with either RAB4-GFP, RAB7-GFP or RAB11-GFP, as markers respectively for early, late and recycling endosomes, and immunostained for TSPAN5 (Figure 20 panel B). We found high level of co-localisation with all the three GFP tagged RABs as expected by the fact that TSPAN5 is a membrane protein and would pass through all this vesicles for its localisation and processing. However, a significantly higher co-localisation was found between TSPAN5 and RAB11-GFP strengthening the hypothesis that this protein mainly resides in recycling endosomes in mature hippocampal neurons (Figure 20 panel C) (Mander's Co-localisation index; RAB-4 0,76±0,02 N=5; RAB-7 0,79±0,06 N=5; RAB11 0,94±0,01 N=5; RAB-11 vs RAB-4 * p value < 0,05; RAB-11 vs RAB-7 * p value < 0,05; One-Way ANOVA Tukey's multiple comparison).

To further validate these results we performed an immunoprecipitation on rat brain hippocampi and cortices, lysed in digitonin 1% to remove TEMs, and found that TSPAN5 and GluA2/3 were associated with MyosinVa, a motor protein known to be involved in the transport of recycling endosomes to membrane surface (Figure 20 panel D).

4.3.6 TSPAN5 regulates GluA2 recycling

Once assessed that TSPAN5 resides in recycling endosomes and as this organelles are important to maintain the correct amount of GluA2 receptors on the surface, we decided to verify whether TSPAN5 could have a role in this process.

We used an antibody feeding assay to measure the recycling of the receptor: an anti GluA2 antibody was given in living hippocampal neurons transfected either with Scrambled-shRNA, Sh-TSPAN5 or shRNA plus TSPAN5-GFP and we left 30 minutes to the antibody to be internalized. After that, the antibody that was bound to receptor that did not internalize was bound to a secondary antibody coupled to Alexa-488 in excess. 0 or 10 minutes were given to the internalized receptor-antibody complex to recycle back to the surface. A secondary antibody bound to Alexa-555 was given after fixation in non-permeabilizing condition followed by a third secondary antibody bound to DyeLight 649 in permeabilizing condition (Figure 21 panel A). The ratio between the Alexa-555 signal and the sum of Alexa-555 and DyeLight 649 signals gives the amount of extracellular receptor that, given the experimental design, would be only the recycled one.

In this condition, we observed that with the Scrambled-shRNA in 10 minutes there was a 17 percent increase in the surface signal that was completely lost in Sh-TSPAN5. This

demonstrates that the removal of TSPAN5 leads to an almost completely disruption of this process. The concomitant overexpression of an shRNA insensitive TSPAN5-GFP restored completely the effect guaranteeing that we were not observing an unspecific effect of the Sh-TSPAN5 (Figure 21 panel B) (Extracellular/Total Mean intensity normalized to 1 for Scrambled 0': Scrambled: 0' 1±0,05 N=18; 10' 1,17±0,03 N=18; Sh-TSPAN5: 0' 0,97±0,05 N=12; 10' 0,92±0,04 N=12; Rescue: 0' 1,08±0,03 N=12; 10' 1,14±0,03 N=12; Scrambled 0' vs 10' * p value < 0,05; Sh-TSPAN5 0' vs 10' NS; Scrambled 10' vs Sh-TSPAN5 10' ** p value < 0,01; Rescue 10' vs Sh-TSPAN5 10' * p value < 0,05; One-Way ANOVA Tukey's multiple comparison).

To assess that the effect observed was not biased by any impairment in the internalization of GluA2, we used an antibody feeding assay and verified that no differences were present in the internalization rate of GluA2 in TSPAN5 silencing condition (Figure 21 panel C and D) (Intracellular/Total Mean intensity normalized to 1 for Scrambled 0': Scrambled: 0' 1±0,02 N=5; 5' 1,09±0,01 N=5; 10' 1,05±0,03 N=5; Sh-TSPAN5: 0' 0,97±0,02 N=5; 5' 1,07±0,03 N=5; 10' 1,01±0,04 N=5; Scrambled 0' vs 5' * p value < 0,05; Scrambled 0' vs 10' NS; Sh-TSPAN5 0' vs 5' * p value < 0,05; Sh-TSPAN5 0' vs 10' NS; Scrambled 0' vs Sh-TSPAN5 0' NS; Scrambled 5' vs Sh-TSPAN5 5' NS; Scrambled 10' vs Sh-TSPAN5 10' NS; T-Test Mann-Whitney post hoc).

All these experiments suggest that TSPAN5 in mature neurons is able to regulate GluA2 recycling through the binding of AP-4 complex (Figure 22).

Figure 12

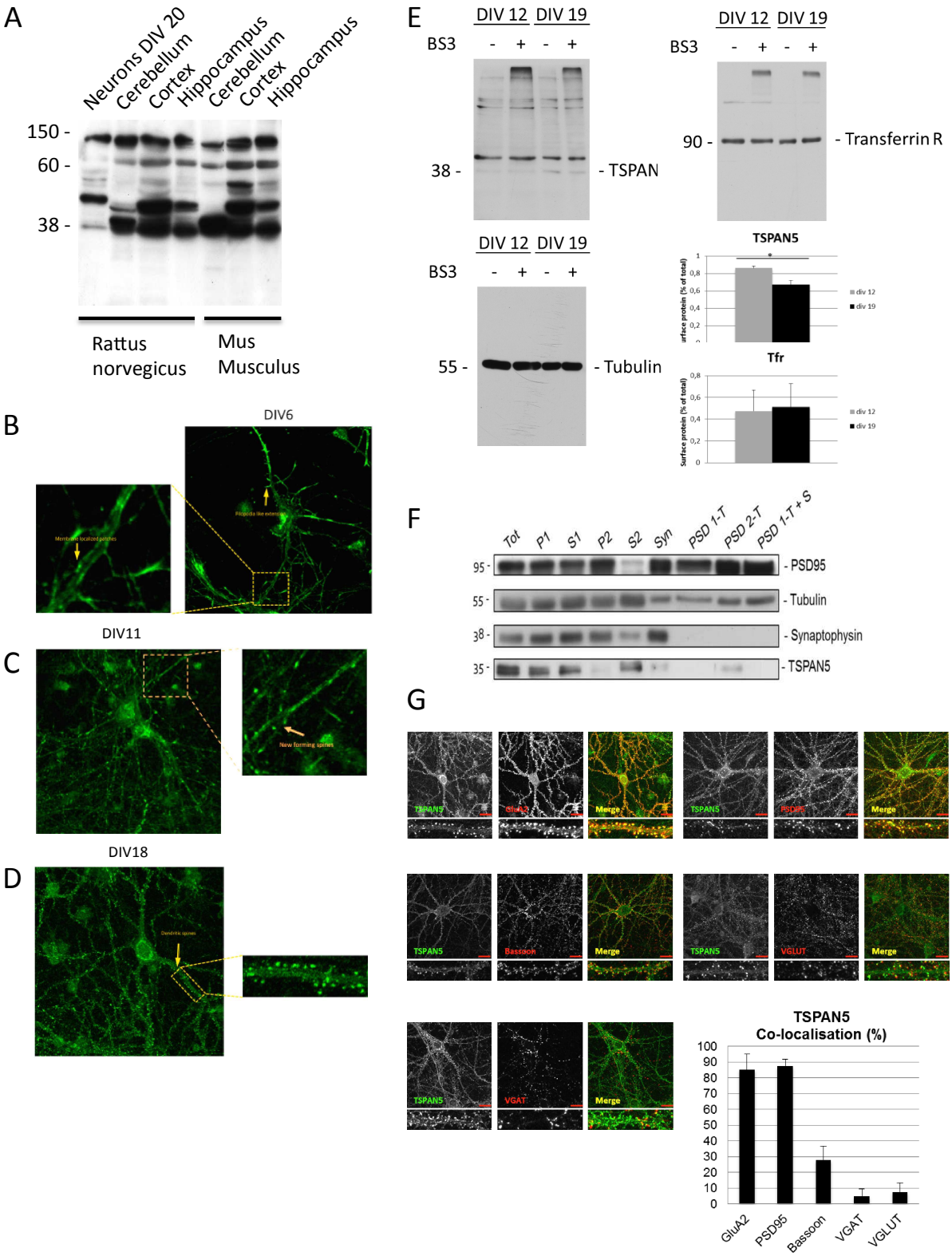

A
Neurons DIV 20
Cerebellum
Cortex
Hippocampus
Cerebellum
Cortex
Hippocampus
150 -
60 -
38 -
Rattus norvegicus
Mus Musculus
B
DIV6
C
DIV11
D
DIV18
E
DIV 12 DIV 19
BS3
- + - +
38 -
- TSPAN
90 -
- Transferrin R
55 -
- Tubulin
TSPAN5
Tfr
div 12
div 19
F
Tot
P1
S1
P2
S2
Syn
PSD 1-T
PSD 2-T
PSD 1-T + S
95 -
- PSD95
55 -
- Tubulin
38 -
- Synaptophysin
35 -
- TSPAN5
G
TSPAN5
GluA2
Merge
Bassoon
VGLUT
VGAT
TSPAN5
Co-localisation (%)
100
90
80
70
60
50
40
30
20
10
0
GluA2
PSD95
Bassoon
VGAT
VGLUT

Figure 12. TSPAN5 is expressed in mammalian brain and presents different localisation depending on neuronal developmental stage: A) Western blot showing TSPAN5 expression in brain lysates from Rattus Norvegicus and Mus Musculus. Cerebellum, Cortex and Hippocampus were analysed. The protein is present in its monomeric form (around 38 KDa) and in aggregates of different sizes. B, C, D) TSPAN5 immunofluorescence on cultured hippocampal neurons from DIV1 to DIV18. B) TSPAN5 at DIV6 is accumulated in filopodia extension (orange arrow, right panel) and in plasma membrane where it forms aggregates reminders of Tetraspanin Enriched Microdomain (orange arrow, left panel C). At DIV11 TSPAN5 starts to localise in forming dendritic spines (orange arrow, right panel D). In mature neurons (DIV18) TSPAN5 is almost completely concentrated in dendritic spines (orange arrow, left and right panel). E) BS3 crosslinking experiments on cultured hippocampal neurons at two different developmental stage. Western blots show TSPAN5, Transferrin Receptor and Tubulin. The quantification shows the ratio between extracellular and total signal and demonstrated that TSPAN5 is highly enriched on the surface in immature neurons while it tends to relocalise to intracellular compartment in mature neurons. F) Post Synaptic Density fractionation on adult rat brain hippocampi and cortices. Western blot shows the enrichment of PSD95 in the last fractions corresponding to the PSD whereas synaptophysin disappears, as it is a presynaptic component. TSPAN5 is present in the synaptosomes and in the PSD 2-T suggesting a postsynaptic localization. (Tot=Total homogenate, P1=fraction corresponding mainly to nuclei, S1=fraction corresponding to cytosol, P2=crude synaptosomes, S2= cytosolic membranes, Syn=purified synaptosomes, PSD-1T= postsynaptic density fraction extracted with Triton X-100, PSD-2T= PSD-1T re-extracted with Triton X 100, PSD-1T+S= PSD-1T extracted with sarcosyl). G) TSPAN5 immunofluorescence on DIV20 cultured hippocampal neurons together with GluA2 and PSD95 as postsynaptic markers, Bassoon as presynaptic marker and VGLUT and VGAT as presynaptic vesicular markers for excitatory and inhibitory synapses respectively. Scale bars are 20 µm. The graph shows the percentage of TSPAN5 colocalising with each of the analysed markers suggesting the preponderant postsynaptic localisation.

Figure 13

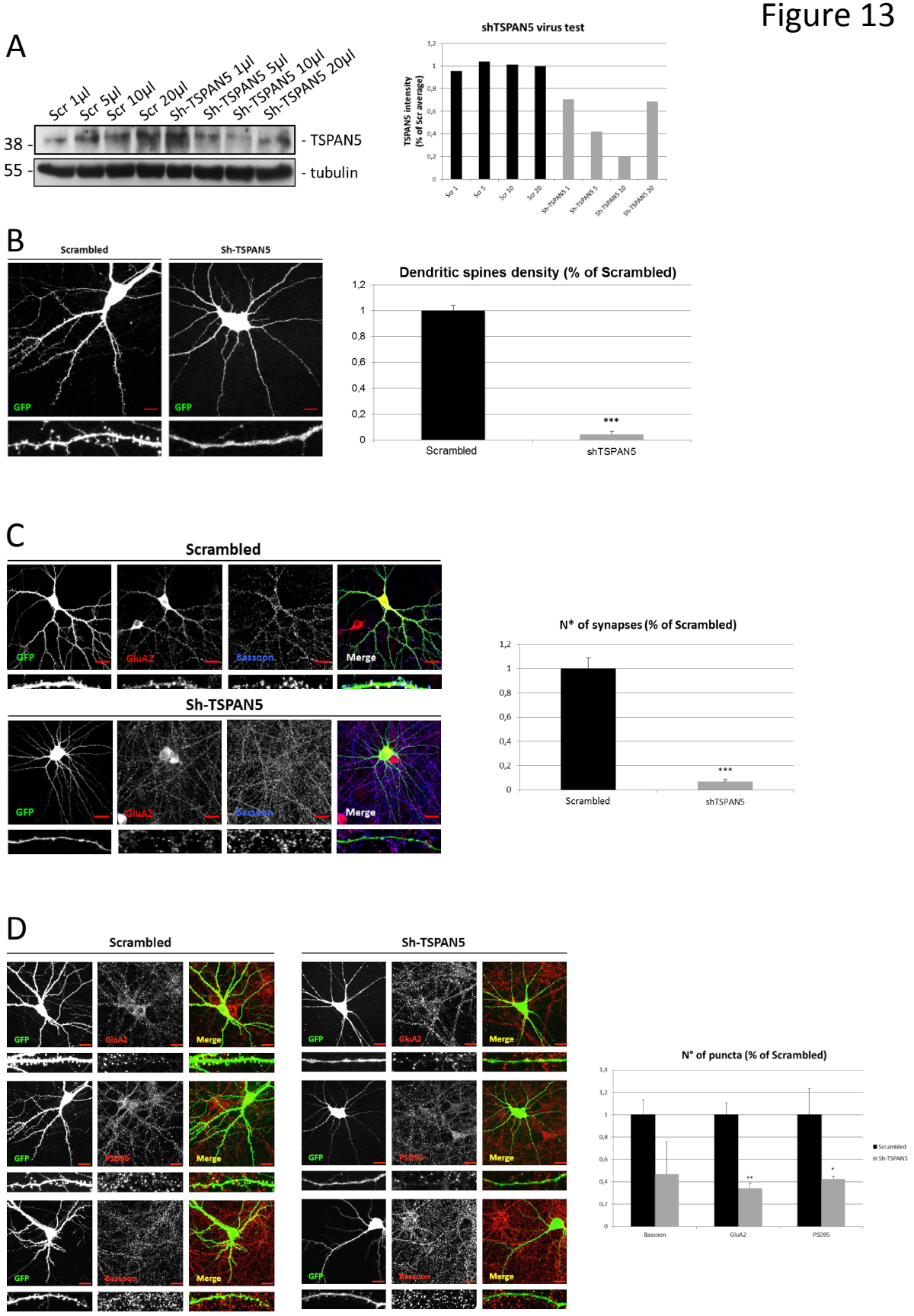

A
Scr 1µl
Scr 5µl
Scr 10µl
Scr 20µl
Sh-TSPAN5 1µl
Sh-TSPAN5 5µl
Sh-TSPAN5 10µl
Sh-TSPAN5 20µl
38 -
- TSPAN5
55 -
- tubulin
shTSPAN5 virus test
TSPAN5 intensity (% of Scr average)
Scr 1
Scr 5
Scr 10
Scr 20
Sh-TSPAN5 1
Sh-TSPAN5 5
Sh-TSPAN5 10
Sh-TSPAN5 20
B
Scrambled
Sh-TSPAN5
GFP
Dendritic spines density (% of Scrambled)
***
Scrambled
shTSPAN5
C
Scrambled
GFP
GluA2
Bassoon
Merge
Sh-TSPAN5
N* of synapses (% of Scrambled)
***
Scrambled
shTSPAN5
D
Scrambled
Sh-TSPAN5
GFP
GluA2
PSD95
Bassoon
Merge
N° of puncta (% of Scrambled)
Bassoon
GluA2
PSD95
Scrambled
Sh-TSPAN5

Figure 13. TSPAN5 silencing dramatically reduces the number of dendritic spines: A) Test for the Sh-TSPAN5 silencing. A lentivirus carrying the sequence was produced and cultured hippocampal neurons were infected at DIV5 with increasing amount of the virus and TSPAN5 was quantified by western blotting at DIV20. The graph shows that with a 10µl lentivirus suspension is possible to obtain an 80% reduction of the protein. B) Cultured hippocampal neurons were transfected either with the Sh-TSPAN5 or with its Scrambled ShRNA at DIV5 and analysed at DIV20 for dendritic spine density. The images and the graph show that TSPAN5 silenced neurons are almost completely deprived of dendritic spines. Scale bars are 20µm. C) Immunofluorescence analysis of GluA2 and Bassoon on neurons transfected as in panel B. The number of colocalising puncta between the two markers was taken as an indicator of the number of synapses. The images and the graph shows that also in this case TSPAN5 silencing almost completely removes synapses from transfected neurons. D) Immunofluorescence analysis for GluA2 and PSD95 as postsynaptic markers and for Bassoon as presynaptic marker in neurons transfected as in panel B and C. The images and the graph show that the removal of TSPAN5 significantly decreases the number of puncta for both GluA2 and PSD95 but not for Bassoon suggesting that the effect was mainly postsynaptic.

Figure 14

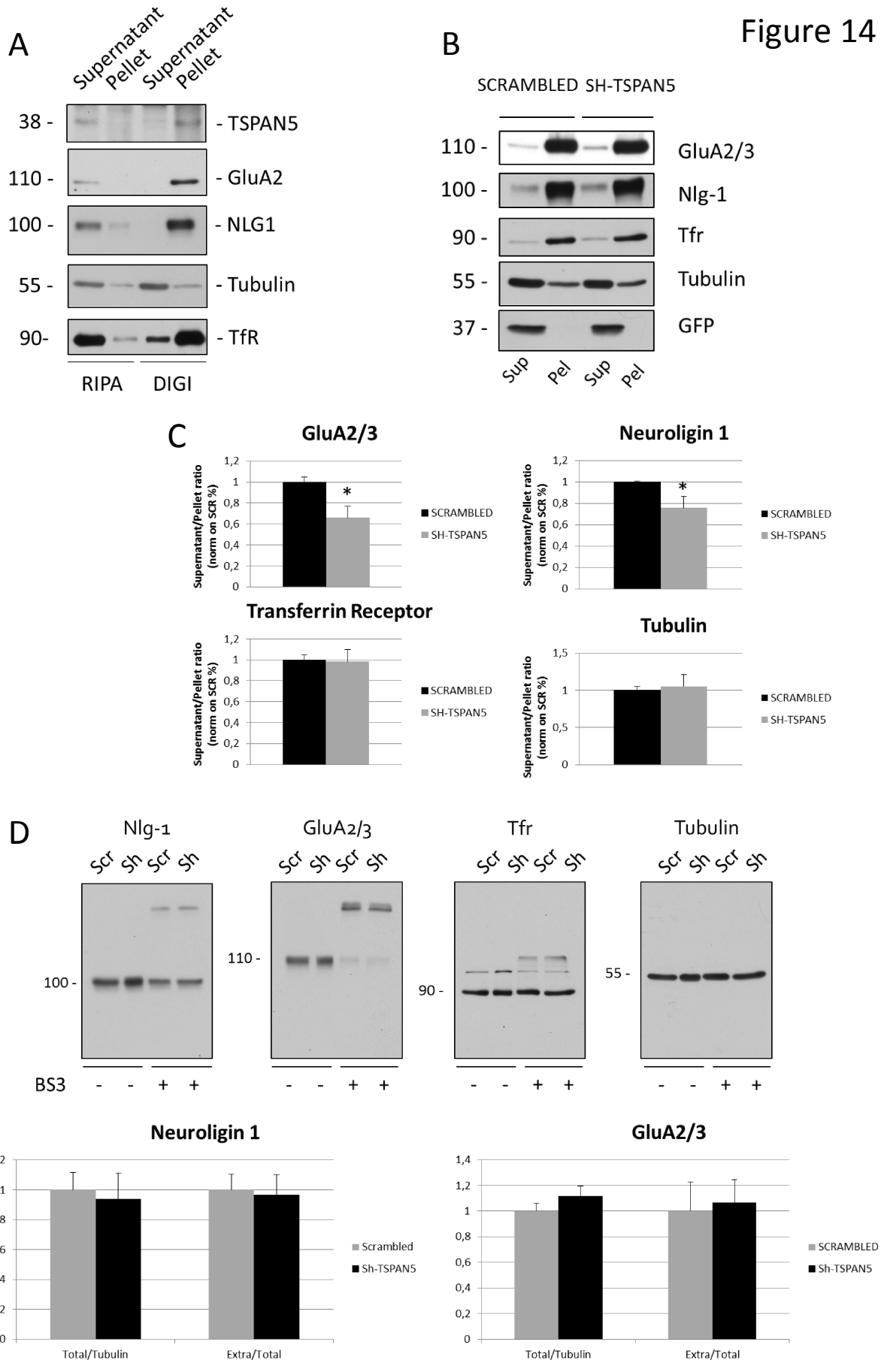

A
Supernatant
Pellet
Supernatant
Pellet
38 -
- TSPAN5
110 -
- GluA2
100 -
- NLG1
55 -
- Tubulin
90-
- TfR
RIPA
DIGI
B
SCRAMBLED SH-TSPAN5
110 -
GluA2/3
100 -
Nlg-1
90 -
Tfr
55 -
Tubulin
37 -
GFP
Sup
Pel
Sup
Pel
C
GluA2/3
Neuroligin 1
Transferrin Receptor
Tubulin
Supernatant/Pellet ratio (norm on SCR %)
SCRAMBLED
SH-TSPAN5
D
Nlg-1
GluA2/3
Tfr
Tubulin
Scr
Sh
100 -
110 -
90 -
55 -
BS3
Neuroligin 1
GluA2/3
Scrambled
Sh-TSPAN5
Total/Tubulin
Extra/Total

Figure 14. TSPAN5 TEMs accommodate Neuroligin 1 and GluA2: A) Neurons at DIV12 were lysed either in standard RIPA buffer or in a buffer with Digitonin 1% and then centrifuged. The western blot showed that TSPAN5 is solubilized in RIPA while it becomes completely insoluble in Digitonin 1% due to the cholesterol enriched nature of TEMs. Neuroligin 1 and GluA2 follow the same behaviour suggesting their presence in TEMs. Conversely, Transferrin Receptor and Tubulin have a much broader distribution. B, C) Hippocampal neurons infected either with Scrambled or Sh-TSPAN5 carrying lentivirus and lysed in digitonin 1%. Western blots and graph show that TSPAN5 silencing induces increased solubility of Neuroligin 1 and GluA2/3 demonstrating that they are relocalised to lighter membranes. Transferrin receptor and tubulin were not affected. GFP serves as control of infection. D) BS3 crosslinking experiments on cultured hippocampal neurons at DIV12 infected either with Scrambled or Sh-TSPAN5 lentivirus at DIV5. Western blots and their relative quantifications demonstrated that neither Neuroligin 1 nor GluA2/3 amount as total or extracellular/total ratio are changed upon TSPAN5 silencing.

Figure 15

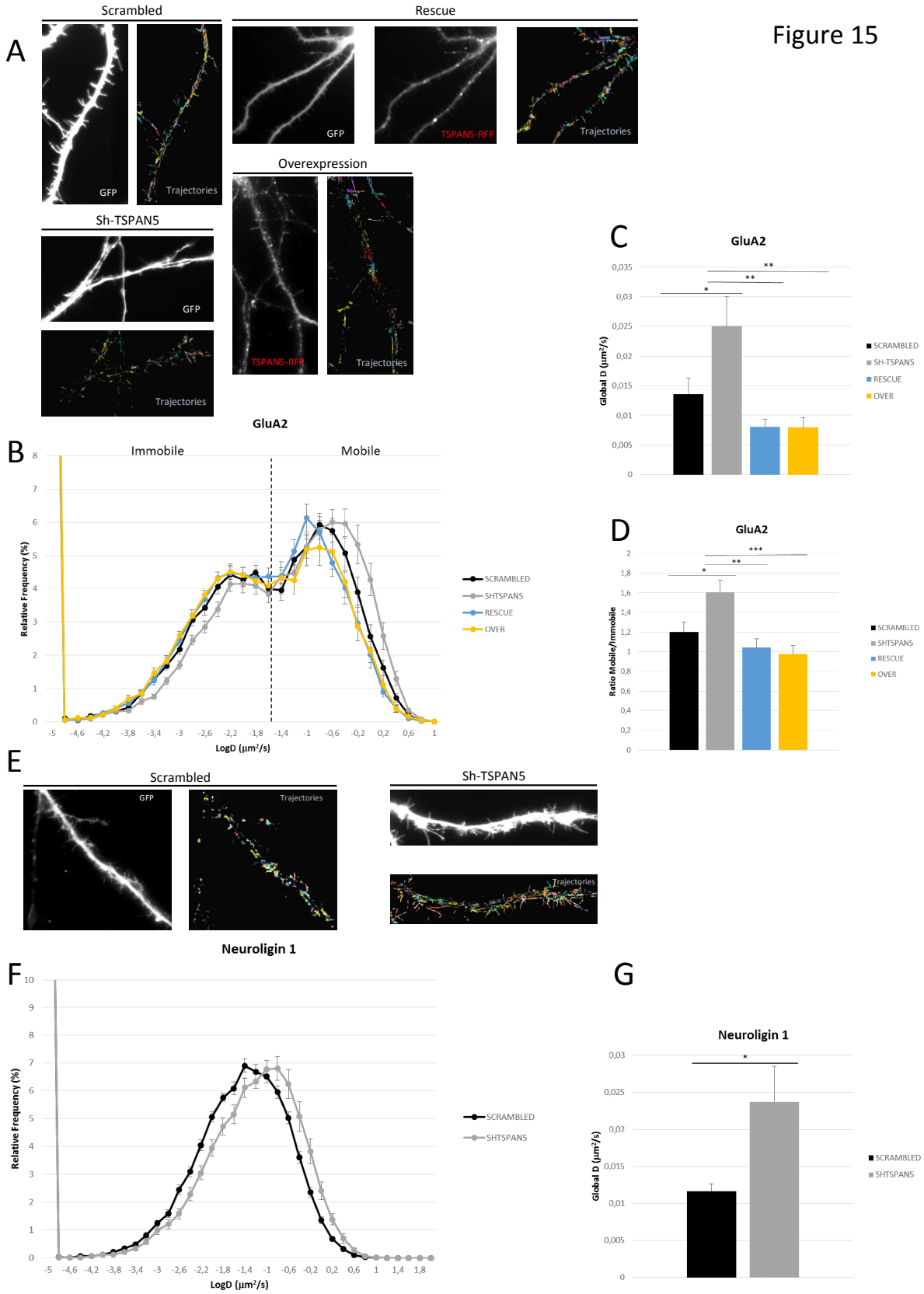

A
Scrambled
GFP
Trajectories
Rescue
GFP
TSPAN5-RFP
Trajectories
Overexpression
TSPAN5-RFP
Trajectories
Sh-TSPAN5
GFP
Trajectories
B
GluA2
Immobile
Mobile
Relative Frequency (%)
LogD (µm²/s)
SCRAMBLED
SHTSPAN5
RESCUE
OVER
C
GluA2
Global D (µm²/s)
SCRAMBLED
SH-TSPAN5
RESCUE
OVER
D
GluA2
Ratio Mobile/Immobile
SCRAMBLED
SHTSPAN5
RESCUE
OVER
E
Scrambled
GFP
Trajectories
Sh-TSPAN5
Trajectories
Neuroligin 1
F
Relative Frequency (%)
LogD (µm²/s)
SCRAMBLED
SHTSPAN5
G
Neuroligin 1
Global D (µm²/s)
SCRAMBLED
SHTSPAN5

Figure 15. TSPAN5 regulates GluA2 and Neuroligin 1 lateral mobility. A) Widefield images of DIV12 cultured hippocampal neurons transfected at DIV5 with either Scrambled-shRNA, Sh-TSPAN5, Sh-TSPAN5 plus TSPAN5-RFP (Rescue) or TSPAN5-RFP (Overexpression) and relative representations of GluA2 trajectories obtained by super resolution imaging of anti-GluA2 antibodies coupled to Atto-647. B) Plot showing the logarithm of the diffusion coefficient of GluA2 and the relative frequency for the condition described in panel A. The black dotted line at logD -1.6 represents the separation between immobilized (<1.6) and mobile receptors (> 1.6). C) Quantification of the mean diffusion coefficient of GluA2 for the condition described in panel A showing the increased mobility of the receptor upon TSPAN5 silencing and its decrease upon TSPAN5 rescue and overexpression. D) Quantification of the Mobile/Immobile ratio of GluA2 showing the shift in favour of mobile receptor in absence of TSPAN5 and of immobile receptor upon TSPNA5 rescue and overexpression. E) Widefield images of DIV12 cultured neurons transfected at DIV5 with Neuroligin 1-AP and BirA-ER and either Scrambled-shRNA or Sh-TSPAN5 and relative representation of Neuroligin 1 trajectories obtained by super resolution imaging of monomeric Streptavidin coupled to Atto-594. F) Plot showing the distribution of the logarithm of diffusion coefficient of Neuroligin 1 in the condition described in panel E. G) Quantification of the mean diffusion coefficient of Neuroligin 1 for the condition described in panel E showing the increased mobility of Neuroligin 1 in absence of TSPAN5.

Figure 16

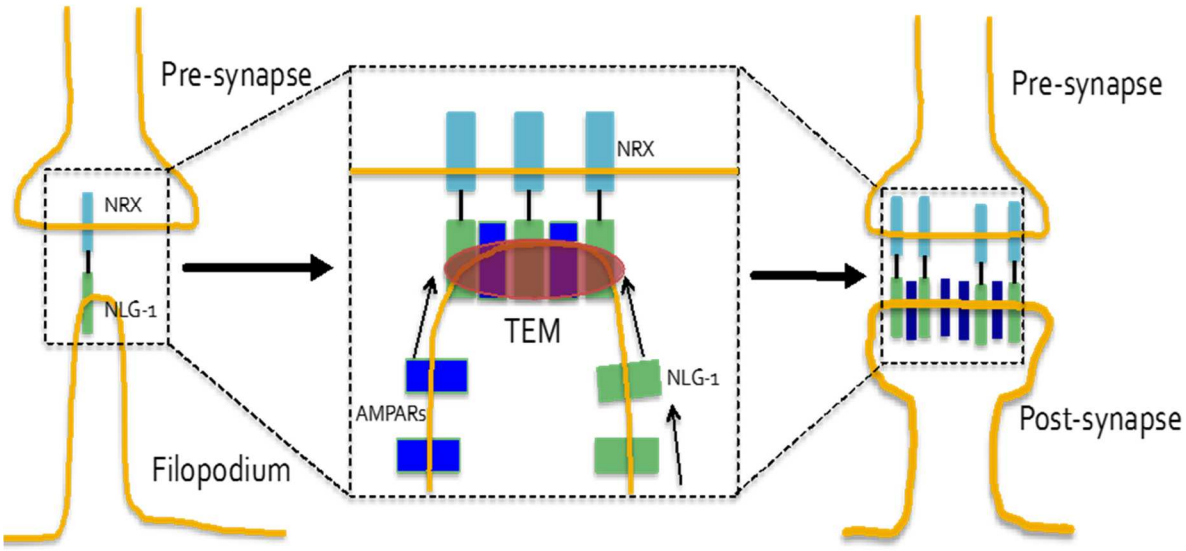

Pre-synapse
NRX
NLG-1
Filopodium
NRX
TEM
AMPARs
NLG-1
Pre-synapse
Post-synapse

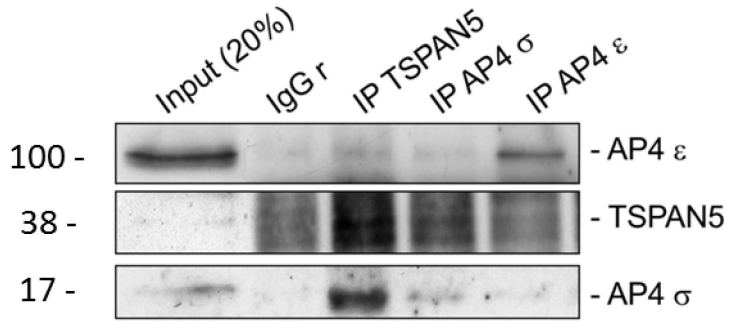
Figure 16. TSPAN5 regulates dendritic spine formation promoting the clusterization of Neuroligin 1 and GluA2 receptor. Schematic model representing the hypothesis of our work. The first signal of dendritic spine formation occurs upon contact between a dendritic filopodium and a forming presynapse through the binding of Neuroligin 1 and Neurexin. TSPAN5 formed TEMs localizes in the site of this interaction and favours the clusterization of more Neuroligin 1 that can interact with the occurring Neurexins to strengthen the trans-synaptic adhesion. AMPA receptors are also accumulated in the same site to make the synapse functional.

Figure 17

A

TSPAN5	AP4σ
Full length	3+
C-terminal (aa 253-268)	3+

B



C

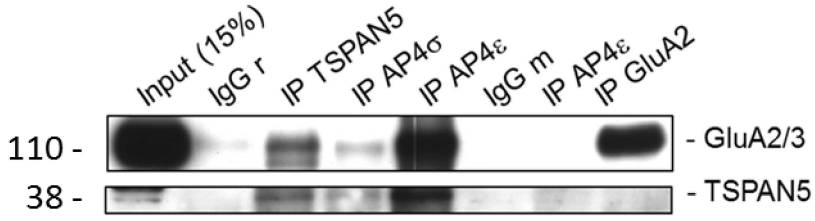


Figure 17. TSPAN5 interacts with AP-4 and GluA2. A) Table representing the results obtained in Yeast Two-Hybrid Screening. Both the full length and the C-terminal tail of TSPAN5 produces more than 3 positive clones with AP-4 σ subunit. B) Co-immunoprecipitation experiment on adult rat hippocampi and cortices lysed in RIPA buffer. The immunoprecipitation of TSPAN5 co-immunoprecipitates AP-4 σ and as the complex is obligated also AP-4 ε. Accordingly, both the immunoprecipitation of AP-4 σ and AP-4 ε co-immunoprecipitates TSPAN5. C) Co-immunoprecipitation experiment on adult rat hippocampi and cortices lysed in a buffer containing HEPES-EDTA and after -80℃ freezing. The immunoprecipitation of TSPAN5, AP-4 σ and AP-4 ε co-immunoprecipitate GluA2/3 receptor.

Figure 18

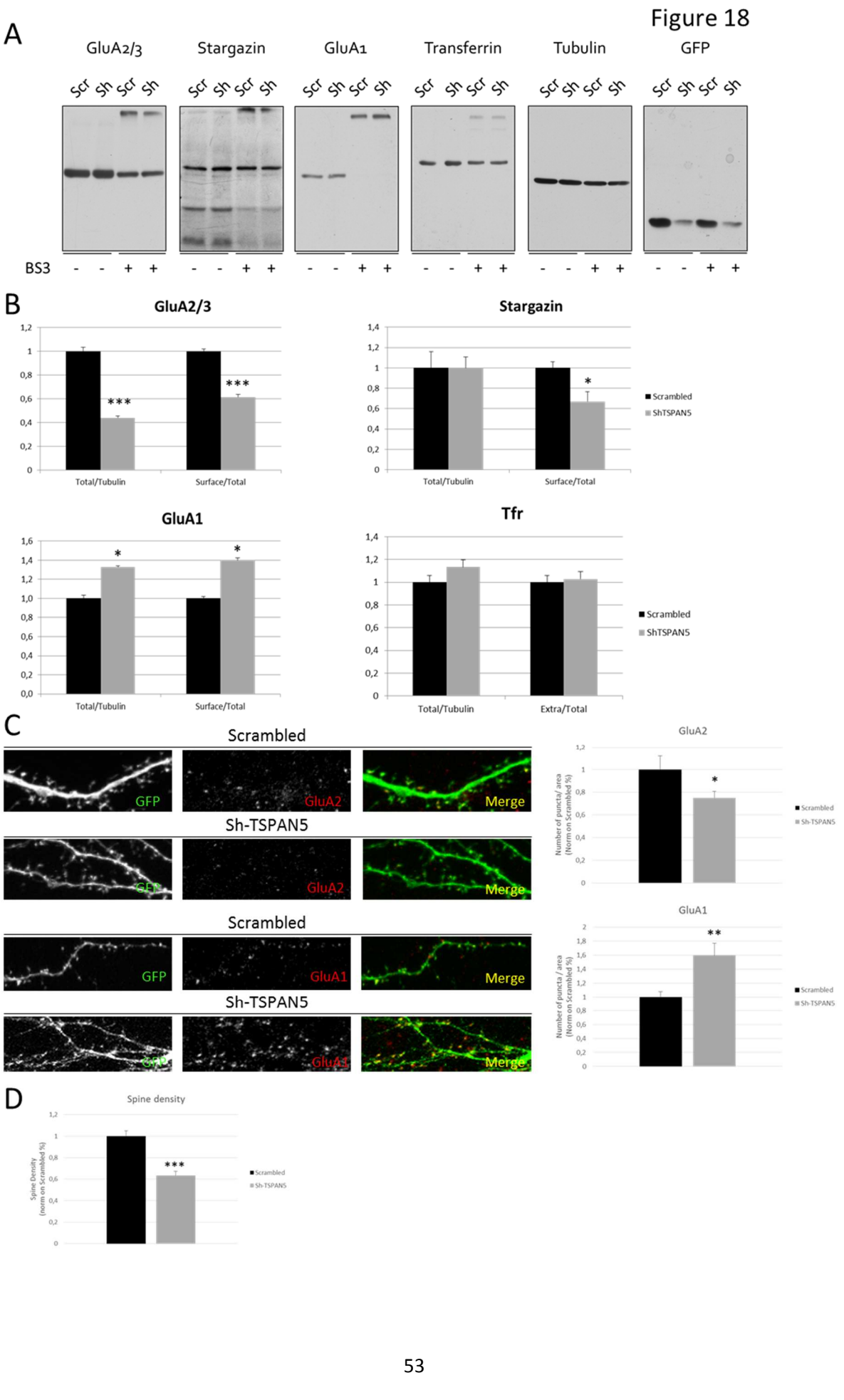

A
GluA2/3
Stargazin
GluA1
Transferrin
Tubulin
GFP
Scr Sh Scr Sh
BS3 - - + +
B
GluA2/3
Total/Tubulin
Surface/Total
Stargazin
Scrambled
ShTSPAN5
GluA1
Tfr
Extra/Total
C
Scrambled
Sh-TSPAN5
GFP
GluA2
GluA1
Merge
Number of puncta/ area (Norm on Scrambled %)
D
Spine density
Spine Density (norm on Scrambled %)

Figure 18. TSPAN5 silencing specifically decreases GluA2 AMPA receptor subunit. A) BS3 crosslinking experiment on DIV18 cultured hippocampal neurons infected at DIV12 with either a lentivirus carrying the Scrambled-shRNA or the Sh-TSPAN5. Western blots analysed GluA2/3, Stargazin and GluA1. Transferrin receptor was used as control for surface protein, tubulin for cytosolic and to normalize the amount of protein loaded and GFP was a control of infection. B) Relative quantifications show that TSPAN5 silencing strongly reduces GluA2/3 both in its total and surface amount whereas Stargazin was reduced only in the surface. GluA1 instead was increased in both total and surface amounts. Transferrin receptor was not affected. C) Immunostaining for surface GluA2 and total GluA1 receptor in DIV20 cultured hippocampal neurons transfected at DIV12 with either the Scrambled-shRNA or the Sh-TSPAN5. The images and the relative quantification show that the amount of GluA2 receptor on the surface was reduced upon TSPAN5 silencing. GluA1 signal instead was increased when TSPAN5 was removed. D) Quantification of dendritic spine density in DIV20 cultured hippocampal neurons transfected at DIV12 with either the Scrambled-shRNA or the Sh-TSPAN5 showing a significant reduction in the number of spines upon TSPAN5 silencing.

Figure 19

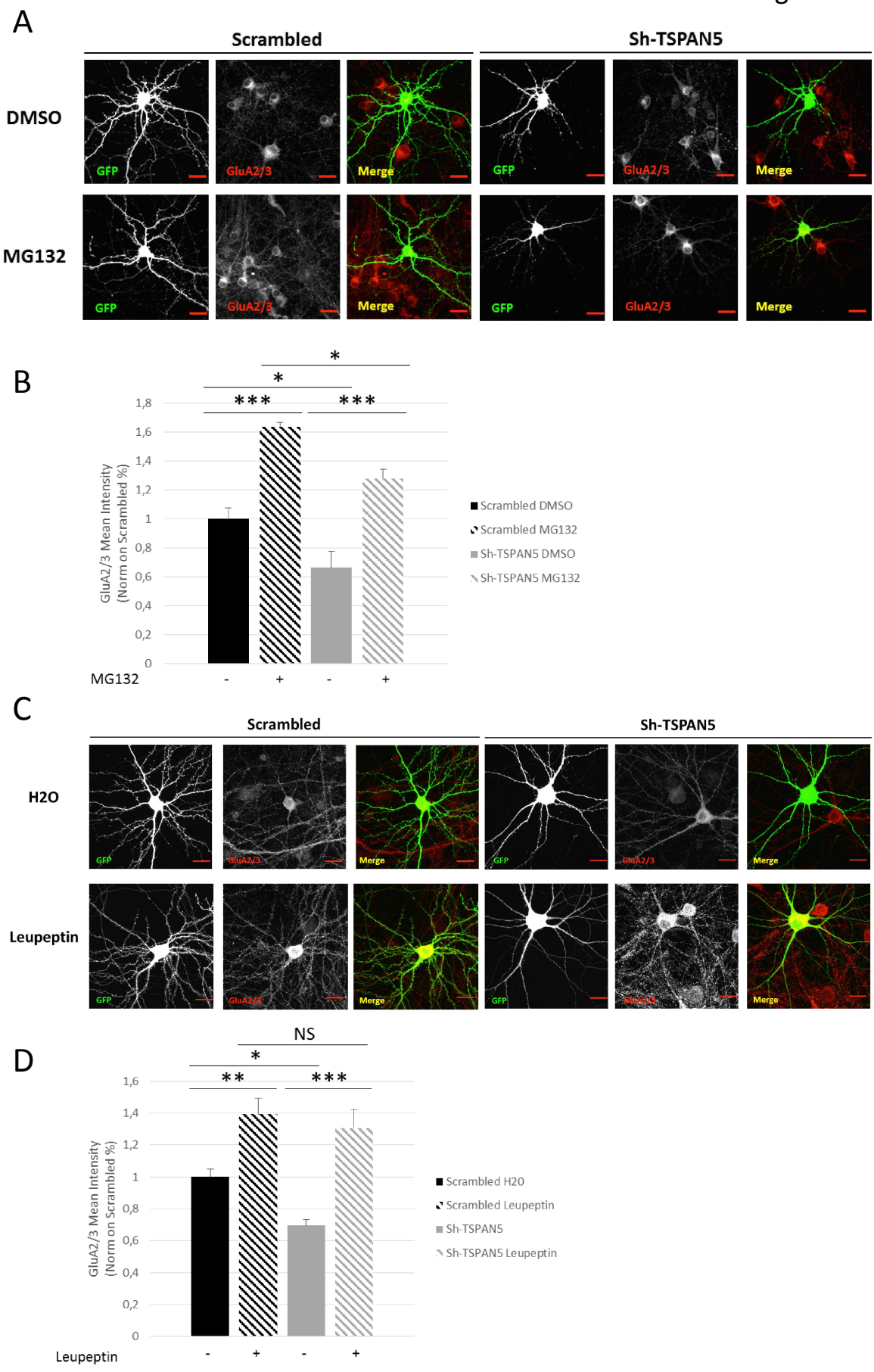
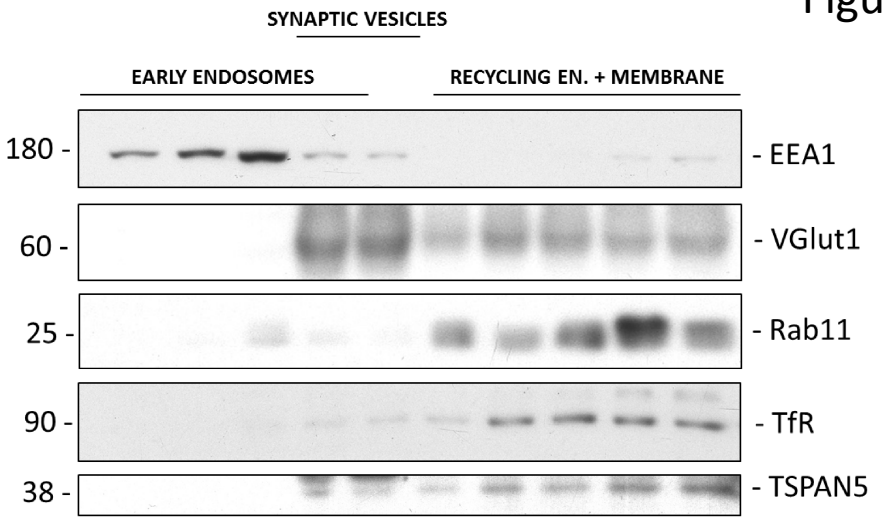

A
Scrambled
Sh-TSPAN5
DMSO
MG132
GFP
GluA2/3
Merge
B
*
*
***
***
GluA2/3 Mean Intensity (Norm on Scrambled %)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
MG132
-
+
-
+
Scrambled DMSO
Scrambled MG132
Sh-TSPAN5 DMSO
Sh-TSPAN5 MG132
C
Scrambled
Sh-TSPAN5
H2O
Leupeptin
GFP
GluA2/3
Merge
D
NS
*
**
***
GluA2/3 Mean Intensity (Norm on Scrambled %)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Leupeptin
-
+
-
+
Scrambled H20
Scrambled Leupeptin
Sh-TSPAN5
Sh-TSPAN5 Leupeptin

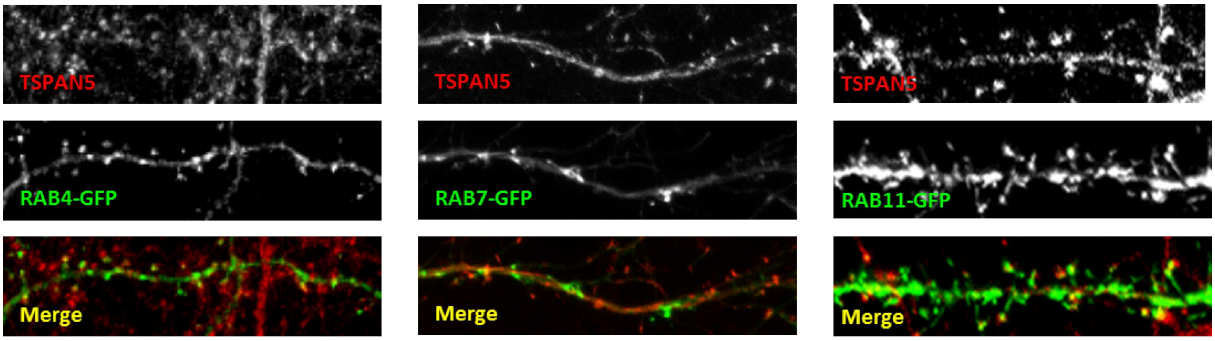
Figure 19. TSPAN5 silencing-induced GluA2 reduction is due to lysosomal degradation. A) Immunostaining for GluA2/3 on DIV20 cultured hippocampal neurons transfected at DIV12 with either Scrambled-shRNA or Sh-TSPAN5 and treated for 90 minutes with either DMSO or 10μM MG132 as proteasome inhibitor. Scale bars are 20 μm B) Relative quantification showing that MG132 increases the amount of GluA2/3 in both Scrambled and Sh-TSPAN5 condition. However, the increase in latter condition was not sufficient to reach the Scrambled MG132 level. C) Immunostaining for GluA2/3 on DIV20 cultured hippocampal neurons transfected at DIV12 with either Scrambled-shRNA or Sh-TSPAN5 and treated for 90 minutes with either H20 or 100μM leupetin as lysosomes inhibitor. Scale bars are 20 μm D) Relative quantification showing that the amount of GluA2/3 was increased by leupeptin treatment in both Scrambled and Sh-TSPAN5 and that this increase reaches similar levels demonstrating that lysosomal degradation is responsible of the observed reduction of GluA2.

Figure 20

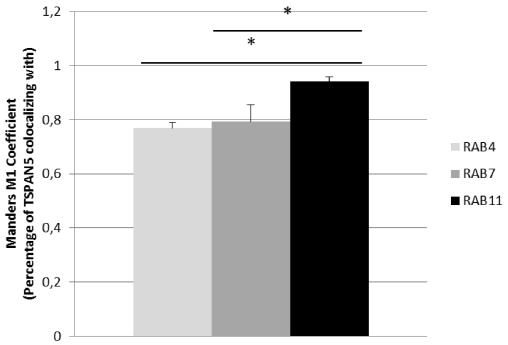
A



B



C



D

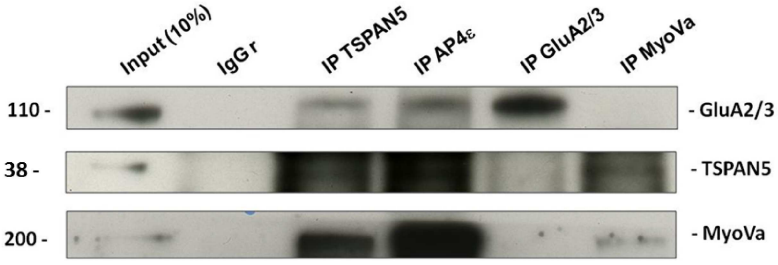
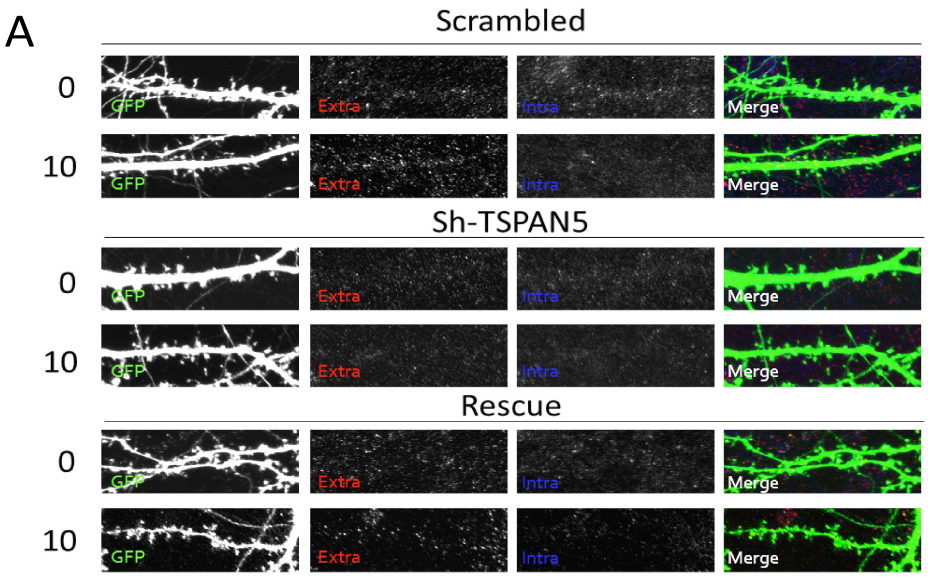
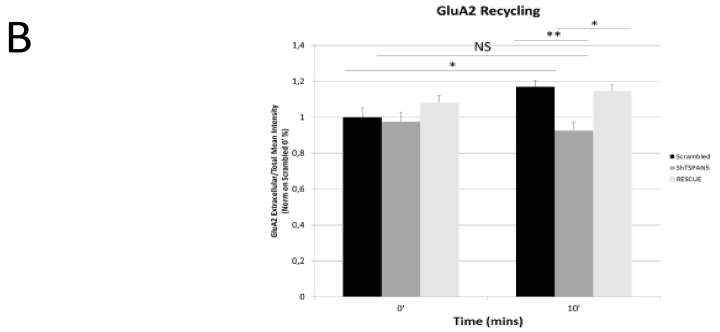
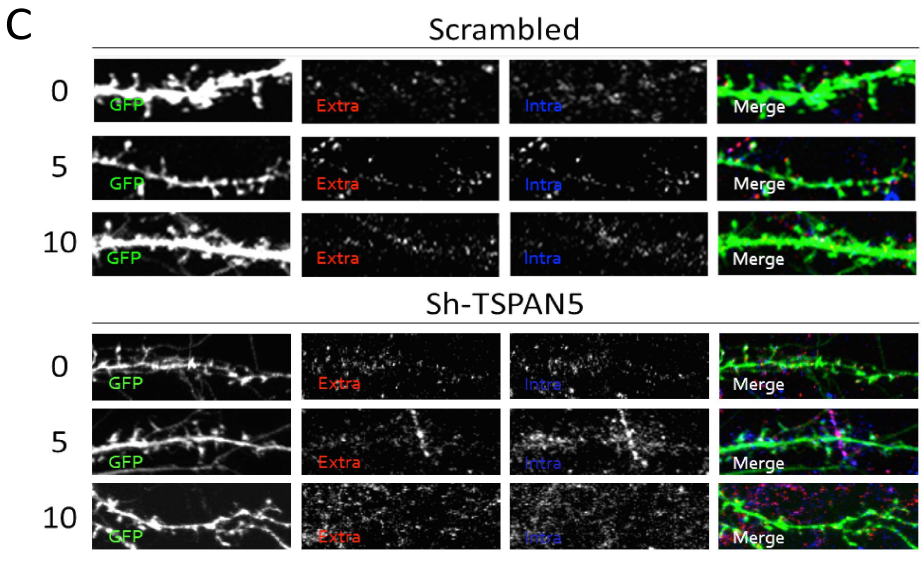


Figure 20. TSPAN5 is localised in recycling endosomes. A) Vesicles fractionation from adult rat hippocampi and cortices. The ten fractions obtained were analysed via western blotting for different markers: EEA1 for early endosomes, VGlut1 for synaptic vesicles, RAB11 and Transferrin Receptor for recycling endosomes. It is possible to observe that TSPAN5 co-fractionates with RAB11 and Transferrin Receptor suggesting its presence in recycling endosomes. B) TSPAN5 immunostaining in DIV18 cultured hippocampal neurons transfected at DIV14 with either RAB4-GFP, RAB7-GFP or RAB11-GFP as markers for early endosomes, late endosomes and recycling endosomes respectively. C) Quantification of the colocalisation of TSPAN5 with RAB4-GFP, RAB7-GFP and RAB11-GFP measured as Mander's coefficient. It is possible to appreciate that TSPAN5 has a high degree of colocalisation with all the three markers but that the coefficient is significantly higher with RAB11-GFP suggesting TSPAN5 localisation in recycling endosomes. D) Co-immunoprecipitation experiment on adult rat hippocampi and cortices lysed in Digitonin 1% buffer. The immunoprecipitation of both TSPAN5 and AP-4 ε co-immunoprecipitate MyosinVa, an actin motor known to transport AMPA-Rs on recycling endosomes to the plasma membrane.

Figure 21


A
Scrambled
0
10
GFP
Extra
Intra
Merge
Sh-TSPAN5
Rescue


B
GluA2 Recycling
NS
*
**
*
Time (mins)
0'
10'
Scrambled
SHTSPAN5
RESCUE


C
Scrambled
0
5
10
GFP
Extra
Intra
Merge
Sh-TSPAN5

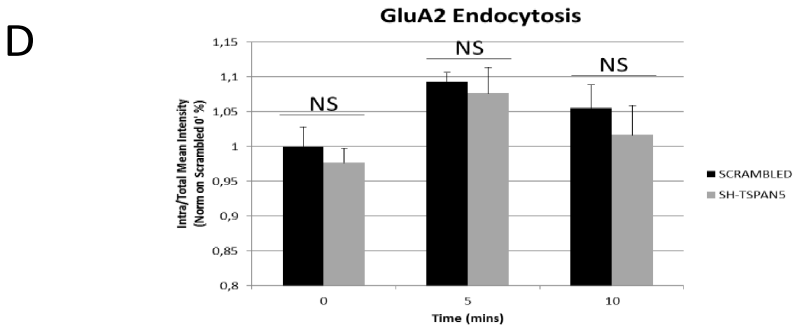

D
GluA2 Endocytosis
NS
NS
NS
Intra/Total Mean Intensity (Norm on Scrambled 0' %)
Time (mins)
SCRAMBLED
SH-TSPAN5

Figure 21. TSPAN5 regulates GluA2 recycling without affecting internalization. A) Antibody feeding recycling assay for GluA2 on DIV20 cultured hippocampal neurons transfected at DIV12 with either the Scrambled-shRNA, Sh-TSPAN5 or Sh-TSPAN5 plus TSPAN5-GFP (Rescue). The anti-GluA2 antibody was allowed to bind the receptor and internalize for 30 minutes and then allowed to recycle for 0 or 10 minutes. The use of non-permeabilizing condition allows to visualize the surface and thus recycled receptor (Extra) whereas the permeabilizing condition shows the intracellularly retained GluA2 (Intra). B) Relative quantification of Extracellular/Total ratio for GluA2 intensity showed that at 0' the three condition are comparable whereas after 10 minutes both the Scrambled and the Rescue increased their amount while the Sh-TSPAN5 remains at levels similar to the basal. This result demonstrates a dramatic impairment of the recycling of GluA2 upon TSPAN5 silencing. C) Antibody feeding internalization assay for GluA2 on DIV20 cultured hippocampal neurons transfected at DIV12 with either the Scrambled-shRNA or Sh-TSPAN5. The anti-GluA2 antibody was allowed to internalize for 0, 5 or 10 minutes and then visualized in non permeabilizing condition for the extracellularly retained antibody (Extra) and in permeabilizing condition for the internalized one (Intra). D) Relative quantification of the Intracellular/Total ration of GluA2 intensity showing that no differences are present for internalization when TSPAN5 is silenced.

Figure 22

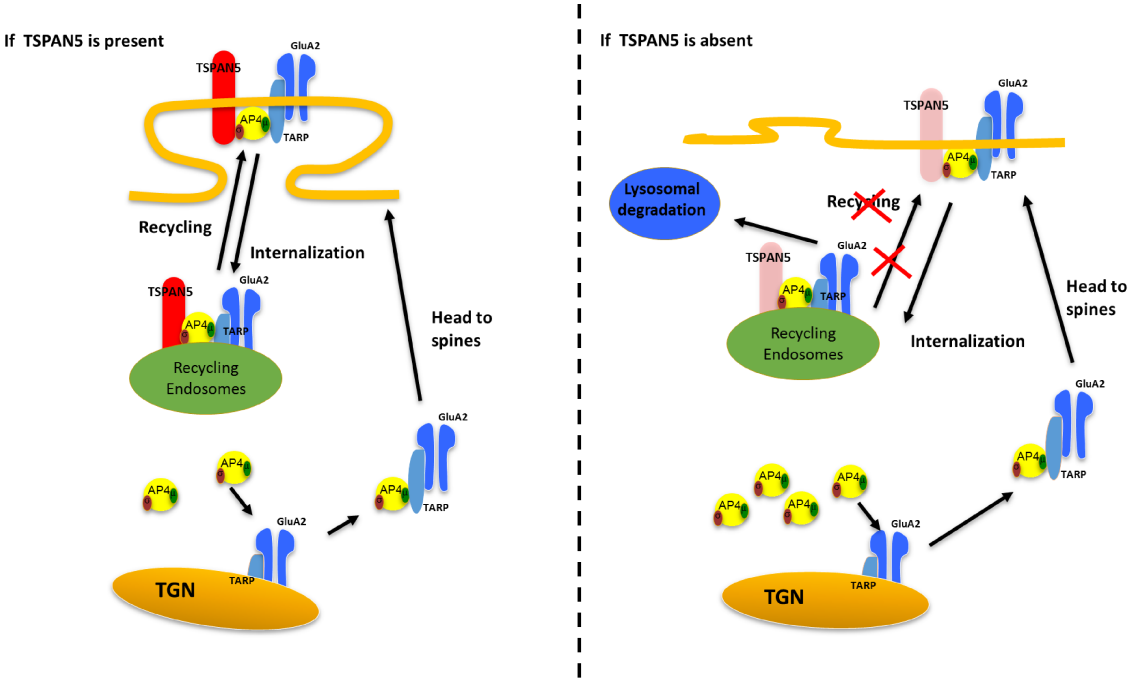

If TSPAN5 is present
GluA2
TSPAN5
AP4
TARP
Recycling
Internalization
Head to spines
Recycling Endosomes
TGN
If TSPAN5 is absent
Lysosomal degradation
Recycling
Internalization
Head to spines
Recycling Endosomes
TGN

Figure 22. TSPAN5 regulates GluA2 recycling. Schematic model of our hypothesis on TSPAN5 role in GluA2 trafficking. The left panel shows that, when TSPAN5 is present, GluA2 is trafficked to synapses thanks to TARPs (Stargazin) and AP-4. This complex continuously undergoes cycles of internalization and recycling with this latter event being promoted by TSPAN5 association through direct binding to AP-4. In the right panel, it is shown what we think happen upon TSPAN5 removal. The internalized receptor has no signals to be recycled and is thus directed to lysosome for degradation to avoid an excessive intracellular accumulation.

6. Discussion

In this work we have analysed extensively TSPAN5 expression and function in hippocampal neurons. Our hypothesis is that this protein has two distinct functions that have opposed importance at different developmental stages. In particular, we have found that TSPAN5 is important for the establishment and stabilization of dendritic spines, through the formation of Tetraspanin Enriched Microdomain; this process is of particular relevance during the maturation of neurons when synaptogenesis occurs (DIV10-12) but remains fundamental also in mature neurons to maintain the dynamicity of circuits. However, in mature neurons (after DIV18), and thus in formed and stable synapses, TSPAN5 appears to directly regulate AMPA-Rs recycling, a process necessary to maintain the correct amount of receptor on the surface both in basal and in stimulated conditions.

6.1 TSPAN5 has two distinct functions

We started our study by analysing the presence of the protein in brain, as two different works (Garcia-Frigola *et al.*, 2000) (Garcia-Frigola *et al.*, 2001) have shown the high level of expression of TSPAN5 mRNA in different mice brain areas. We were thus not surprised to find the expression of the protein in the three areas examined: hippocampus, cortex and cerebellum.

The detection of bands of molecular weight higher than the expected suggested the assembly of TSPAN5 in Tetraspanin Enriched Microdomain (Paragraph 2.1.2).

We then looked for intracellular localisation by means of immunocytochemistry on hippocampal neurons following the variation occurring during maturation from DIV1 to DIV18. Interestingly, the protein was present in all the phases of maturation starting from the very first day of plating. The major differences were observed between the DIV before and after 14-15. In fact, before this day the protein was broadly distributed with some enrichment in growing dendrites and with a pattern resembling plasma membrane proteins with areas of brighter signal suggestive of TEMs. Instead, after this DIV the protein was highly enriched in perinuclear endoplasmic reticulum and in dendritic spines. We then took advantage of a membrane impermeable BS3 crosslinker to verify that the protein was in fact more enriched on the surface in immature stages compared to mature neurons.

We were able to confirm the presence of TSPAN5 in dendritic spines in mature neurons by postsynaptic density fractionation and by co-localisation with postsynaptic markers such as PSD-95.

6.2 TSPAN5 is fundamental for dendritic spines formation

We designed an shRNA to further investigate the function of the protein. When we knocked down TSPAN5 from DIV5 to DIV20 we observed a dramatic reduction in the number of dendritic spines recognized both by morphology and by the co-localisation of pre (Bassoon) and postsynaptic (GluA2) markers. The reduction was so strong that an indirect effect on dendritic spines, as it is observed for many proteins that have functional role in synapses, was unlikely, prompting us to investigate a possible direct function of TSPAN5 in dendritic spines formation.

It is known from the literature that tetraspanins have the peculiarity of forming membrane domains known as TEMs that have the primary function of facilitating the concentration of different proteins (Maecker *et al.*, 1997). As one of the first step of dendritic spine formation is the clusterization of Neuroligin 1 on the postsynapse to stabilize the trans-synaptic interaction with Neurexins (Barrow *et al.*, 2009), it was logical for us to investigate whether TSPAN5 TEMs could be the molecular platforms where this clusterization occurs.

We first wanted to observe if Neuroligin 1 and GluA2/3 were present in TSPAN5 TEMs.We decided to analyse also AMPA-R as representative of proteins that assemble later on the forming postsynapse. To address this point we took advantage of the solubility properties of TEMs. In fact, these domains, as they are highly enriched in cholesterol compared to the rest of the plasma membrane, are partially solubilized with strong detergent such as Triton X-100 and become almost completely insoluble in buffer containing digitonin that directly binds cholesterol as described in paragraph 2.1.2. In this way, we showed that in young neurons TSPAN5 was almost completely present in surnatant in RIPA (RadioImmunoPrecipitation Assay) buffer lysed neurons whereas it accumulates in the pellet in digitonin lysed neurons. Neuroligin 1 and GluA2/3 behaved similarly whereas Tubulin and Transferrin Receptor, used as controls, did not.

This assay unfortunately is based only on lipidic composition of the membranes and thus lacks of specificity; the digitonin binding of cholesterol-enriched domain would result in precipitation of other domains that present these characteristics such as lipid rafts that have been found in synapses (Hering *et al*., 2003).

We then decided to observe whether the removal of TSPAN5, and thus of its TEMs, could alter the membrane distribution and the clusterization of Neuroligin 1 and GluA2/3. We thus infected the neurons with the shRNA for TSPAN5 at DIV5, before synaptogenesis starts, and analysed the solubility in digitonin of the two proteins at DIV12 when the peak of synaptogenesis is occurring. As expected, the distribution changed in favour of the supernatant soluble fraction suggesting that, in absence of TSPAN5, Neuroligin 1 and GluA2/3 were shifted to lighter membranes. Concordantly, Transferrin Receptor and Tubulin did not change their solubility properties. We also performed BS3 crosslinking experiments

and observed that the total and surface level of the two proteins were unaffected excluding the possibility of trafficking defects. This result also suggest that the second function of TSPAN5 on AMPA-Rs trafficking is not tacking place at this developmental stage and further confirm the separation of the two TSPAN5 roles.

As it is known that both Neuroligin 1 and AMPA-Rs are highly mobile in the membrane through lateral diffusion but becomes immobilized at forming synapses due to trapping mechanisms (Heine *et al.*, 2008) (Barrow *et al.*, 2009) (Mondin *et al.*, 2011), we wanted to investigate if the diffusion of these two proteins was affected by TSPAN5 levels of expression and thus by TEMs.

We used a recently developed technique called uPAINT to follow these movements in super-resolution. We observed that both Neuroligin 1 and GluA2 increased their mobility upon TSPAN5 knockdown suggesting the removal of the trapping trigger. Conversely, TSPAN5 overexpression was sufficient to reduce the mobility of GluA2; unfortunately, we were not able to perform the same experiment for Neuroligin 1.

Considering that it has been demonstrated that Neuroligin 1 is able to trap AMPA-Rs through PSD-95 (Mondin *et al*., 2011) we cannot exclude that the effect we observed on GluA2 was secondary to the one on Neuroligin 1.

All these results strongly suggest that TSPAN5 is able to organize Tetraspanin Enriched Microdomains in young neurons and that these domains accommodate Neuroligin 1, the first protein that clusterizes in the site of forming postsynapses, and GluA2, one fundamental receptor of excitatory synapses. To date it is still unclear how Neuroligin 1 can accumulate in the site of dendritic spine formation. Despite more experiments are needed to further validate our hypothesis, it seems very likely that TEMs are able to trap Neuroligin 1 and GluA2 promoting their accumulation and slowing down their lateral diffusion. This would increase the likelihood for Neuroligin 1 to bind trans-synaptically neurexins and, once PSD-95 is recruited, for AMPA-Rs to be anchored and stabilized thus enhancing the functionality of the synapse. The dramatic loss of dendritic spines in neurons depleted of TSPAN5 suggests that this role of TSPAN5 is of high importance for these processes. These results are supported by the fact that removal of Neuroligin 1 or AMPA receptors both results in reduction of dendritic spine density (Chih *et al*., 2005) (Passafaro *et al*., 2003).

Unfortunately, in our work it is lacking a direct evidence that TSPAN5 TEMs are formed in the site where the postsynapse will assemble. This is mainly due to technical problems, as no antibodies with high specificity directed against surface epitope of TSPAN5 are available and as the probability of observing the formation of a synapse in random dendritic branches is prohibitively low. To circumvent these problems we are planning experiments using an overexpressed TSPAN5 that will have an AP-tag, as we used for Neuroligin 1 in uPAINT, to follow its mobility on the surface of neurons with monomeric streptavidin coupled to

fluorophores. We will use this approach in conditions that enhance synapses formation such as the use of Neurexin coated beads or plates (Graf *et al.*, 2004) (Czondor *et al.*, 2013) or using glutamate uncaging to induce the development of a dendritic spine in a specific position (Kwon & Sabatini, 2011).

We also cannot exclude that other tetraspanins could be involved in similar or in the same process as for long time it has been suggested that TEMs accommodate different tetraspanins. However, a recent paper that analysed TEMs with super-resolution technique (Zuidscherwoude *et al.*, 2015) demonstrated that different tetraspanins form separate domains that could then interact without fusion events. This supports the idea that TSPAN5 alone can organize TEMs fundamental for dendritic spines formation. Another hint that fit our hypothesis is that the size of TEMs is, on average, similar to the one of PSD with divergence depending on the techniques used to observe the domains but always ranging at least in the same order of magnitude (150-500 nm of diameter) (Nydegger *et al.*, 2006) (Zuidscherwoude *et al.*, 2015).

We have shown in the introduction that alterations in the number of dendritic spines, both reduced or increased, are usually associated with neuropsychiatric disorders such as intellectual disability and autism spectrum disorders (Fiala et al., 2002).

There are in literature many examples of tetraspanin specific monoclonal antibodies directed against extracellular epitopes that could act disrupting or enhancing the formation of TEMs depending on the tetraspanin analysed (Hemler, 2008). As these proteins are exposed to the extracellular solution, they are relatively easy to reach in "*in vivo"* condition and they could thus be used as target for therapy. The use of a monoclonal antibody, still missing on the market, against extracellular epitope of TSPAN5, once assessed if having pro or anti aggregation effect on TEMs, could be useful to re-establish the correct number of synapses in intellectual disability or autism.

6.3 TSPAN5 regulates AMPA-Rs trafficking

The initial analysis of TSPAN5 distribution during development showed that the protein became enriched in intracellular compartment at mature stages compare to immature. We thus decided to investigate this possible second function by looking for intracellular interactors using a Yeast Two-Hybrid Screening with the intracellular C terminal tail of the protein. This domain shows the highest divergence with the other members of the family.

We identified AP-4σ, a subunit of the AP-4 complex. The absence on the sequence of TSPAN5 of motifs normally recognized by AP-4 (see paragraph 2.3) suggested us that this interaction was not only regulating the trafficking of TSPAN5 but could have other more important roles.

We reconfirmed the interaction in lysates from rat hippocampi in conditions in which TEMs were disrupted confirming that this binding was direct.

As we know from the literature that AP-4 is important for AMPA-Rs trafficking through the binding of Stargazin we investigated whether TSPAN5 could be also involved in this process.

We knocked down TSPAN5 expression in neurons at DIV14, when the peak of synaptogenesis is already occurred, to avoid the interference of the previously described function of TSPAN5.

When we analysed Stargazin and GluA2/3 levels in BS3 cross-linked neurons we found that Stargazin levels were unaffected as total amount with a little decrease in surface whereas GluA2/3 amounts was significantly decreased both in surface and in total level.

Instead, GluA1 was not reduced but surprisingly increased in the surface amount likely in an attempt of the neurons to counterbalance the loss of GluA2. This suggests the specificity of TSPAN5 function for GluA2/3 and that Stargazin level was probably less affected as this protein is important for all AMPA-Rs subunit traffic. These results are consistent with the literature as GluA2/3 receptors are constitutively trafficked whereas GluA1-GluA1 and GluA1-GluA2 are generally loaded in synapses upon stimulation (Correia *et al*., 2008).

We confirmed the reduction of GluA2 surface levels and the increase of GluA1 also via immunocytochemistry in transfected neurons.

As the reduction of GluA2 was pronounced also in the total amount of the protein it meant that the defect was probably causing an increased degradation. We treated TSPAN5 knocked down neurons with either proteasome or lysosomes inhibitor (MG132 and leupeptin respectively) and analysed the level of GluA2/3 via immunostaining. MG132 increased the amount of the protein but the level was still lower than the one observed in treated Scrambled-shRNA. On the other hand, leupeptin rescued almost completely the level of GluA2/3 demonstrating that, as expected from a membrane protein, the degradation observed was mainly occurring in lysosomes.

Different results suggested that TSPAN5 could have a role in sorting or trafficking of AMPA-Rs. First TSPAN5 is associated with AP-4 complex, known to be involved in vesicular trafficking; secondly, TSPAN5 was found in membranous intracellular compartments and last, GluA2 reduction occurring upon TSPAN5 knockdown was due to lysosomal degradation.

Consequently, TSPAN5 likely resides in some vesicular compartment and we thus wanted to investigate its specific localisation.

We first produced crude synaptosomes from rat hippocampi and then separated vesicles by hypo-osmotic shock and sucrose linear gradient.

We characterized the fraction obtained with different markers and found that TSPAN5 was migrating similarly to Transferrin Receptor and RAB11, both markers of recycling

endosomes. To confirm the result we transfected GFP-tagged RABs in neurons and analysed the co-localisation with TSPAN5. As expected from a transmembrane protein that cycles in and out the plasma membrane, TSPAN5 co-localised with RAB-4 (early endosomes), RAB-7 (late endosomes) and RAB11 (recycling endosomes) but the degree of co-localisation was significantly higher with the latter.

We also confirmed that TSPAN5 and GluA2/3 were associated in recycling endosomes as they co-immunoprecipitated together with MyosinVa, an actin-based motor protein known to transport recycling endosomes to the plasma membrane (Correia *et al.,* 2008).

At this point all the hints suggested that TSPAN5 could regulate the recycling of GluA2 receptor; to verify this hypothesis we used an antibody feeding assay and found that the levels of recycled receptor was similar to the starting point in TSPAN5 knockdown condition suggesting a dramatic defect in this process.

Moreover, the concomitant expression of Sh-TSPAN5 and of TSPAN5-GFP insensitive to the shRNA restored the recycling to the levels of Scrambled-shRNA confirming the specificity of the impairment observed.

We also checked, again with an antibody-feeding assay, that the internalization path was not affected in TSPAN5 knockdown condition to guarantee the validity of the recycling assay.

The very strong defect in recycling and the observation of the increased lysosomal degradation suggest that TSPAN5 act as a positive regulator of GluA2 recycling. Thus, in absence of TSPAN5, all the internalized receptor would get directly degraded dramatically decreasing its surface amount.

As we propose that the role of TSPAN5 in GluA2 recycling is directly occurring through AP-4 our results seem in apparent discordance with the one published by Matsuda and colleagues on the AP-4β knockout mouse (Matsuda *et al.*, 2008) but different hypotheses could be proposed to explain the difference.

Matsuda and colleagues suggest that AP-4 serves as a sorting machinery to direct AMPA-Rs and other proteins to the somato-dendritic domain avoiding the mis-localisation to the axons. Our results are in apparent contrast, as we did not observe defects in the axon while we found a reduction in GluA2 in the surface of dendrites, an effect that was absent in Matsuda's work. However, the authors cannot rule out if AP-4 has any direct effect on the formation or regulation of degradation organelles as they admit in the discussion. A function of AP-4 in lysosomes has been proposed by different works (Aguilar *et al.*, 2001) (Ruben *et al.*, 2001) (Raza *et al.*, 2015): AP-4, similarly to AP-3A (Matsuda *et al.*, 2013), could be involved in regulating the sorting of AMPA-Rs to lysosomes and TSPAN5 could instead block this sorting and promotes the recycling of the receptors.

A different hypothesis could be that AP-4 is directly involved in recycling of AMPA-Rs together with TSPAN5 and Stargazin. This hypothesis is supported by the fact that many

adaptors and regulatory proteins are involved in both sorting to the somato-dendritic compartment and in recycling of membrane proteins (Bonifacino & Traub, 2003) (Bonifacino, 2014). AP-4 could thus act on the two processes and the recycling function could have been underestimated in AP-4β KOs due to compensatory effect of other adaptors such as AP-1, which present numerous similarities to AP-4. In fact, also AP-1 was shown to bind Stargazin and, through it, AMPA-Rs in the same work (Matsuda *et al.*, 2013). Similar compensation is less likely to happen in our system that is based on acute removal of the protein instead of chronic effects of knockout models.

We thus thought that TSPAN5 is able to promote GluA2 recycling in mature synapses concurring in regulating the precise balance necessary to have functional receptors and that this activity occurs thanks to the binding of AP-4 complex and Stargazin.

7. Bibliography

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