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***Longins and the longin domain: pivotal  
elements in subcellular trafficking and  
neuronal differentiation***

**Direttore della Scuola:** Ch.mo Prof. Giuseppe Zanotti

**Coordinatore d'indirizzo:** Ch.mo Prof. Giorgio Valle

**Supervisore:** Ch.mo Prof. Francesco Filippini

**Dottoranda :** Lara Albania







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# ABBREVIATIONS

**AP:** Adaptor Protein  
**Arp:** Actin Related Protein  
**AS:** Alternative Splicing  
**CALCA:** CALCitonin-related polypeptide- $\alpha$   
**CAM:** Cell Adhesion Molecule  
**CCV:** Clathrin Coated Vesicles  
**CDC42:** Cell Division Cycle 42  
**CELF:** CUGBP- and ETR3-Llike Factor  
**CNS:** Central Nervous System  
**COL:** collagen  
**COPII:** COat Protein complex II  
**CT:** Carboxy-Terminal  
**ECM:** ExtraCellular Matrix  
**EE:** Early Endosome  
**EGFP:** Enhanced Green Fluorescent Pprotein  
**Ena/VASP:** ENAabled/VASolidator-Stimulated Phosphoprotein  
**ER:** Endoplasmic Reticulum  
**ERGIC:** ER-Golgi Intermediate Compartment  
**ESE:** Exonic Splicing Enhancer  
**ESS:** exonic splicing silencer  
**F-actin:** Filamentous Actin  
**FAK:** Focal Adhesion Kinase  
**FA:** Focal Adhesion  
**FC:** Focal Contact  
**FN:** FibroNectin  
**GEF:** Guanine Exchange Factor  
**GPI:** GlycophosPhatidylInositol  
**HRB:** HIV-Rev Binding protein  
**IgCAM:** Immunoglobulin superfamily  
**ISE:** Intronic Splicing Enhancer  
**ISS:** Intronic Splicing Silencer  
**LD:** Longin Domain  
**LE:** Late Endosome  
**LeX:** LewisX  
**LN:** LamiNin

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**Lyso:** Lysosomes  
**MT1-MMP:** Membrane Type 1-Matrix Metalloproteinase Protein  
**NSF:** *N*-ethylmaleimide-Sensitive Factor  
**NT:** amino-Terminal  
**PDL:** Poly-D-Lysine  
**PM:** Plasma Membrane  
**PPV:** Plasmalemmal Precursor Vesicle  
**PTB:** Polypyrimidine-Tract Binding protein  
**RA:** all-*trans*-Retinoic Acid  
**RE:** Recycling Endosome  
**RFP:** Red Fluorescent Protein  
**SC:** SubCellular  
**SCL:** SubCellular Localization  
**SED1:** SpondyloEpiphyseal Dysplasia Tarda  
**Sema:** Semaphorin  
**SF1:** Splicing Factor 1  
**SG:** Secretory Granule  
**SH-SY5Y:** human dopaminergic neuroblastoma cells  
**SIBLINGS:** Small Integrin-Binding glycoproteins  
**SNAP:** SyNaptosomal-Associated Protein  
**SNARE:** Soluble *N*-ethylmaleimide-sensitive factor Attachment protein REceptor  
**snRNP:** small nuclear RiboNucleoProtein  
**SR:** Ser-Arg  
**SRP:** Signal Recognition Particle  
**ss:** splice site  
**SV:** Synaptic-like Vesicle  
**TF:** Transcription Factor  
**TGN:** Trans-Golgi Network  
**TIA1:** T cell-restricted Intracellular Antigen 1  
**TIS:** Translation Initiation Start  
**TI-VAMP:** Tetanus neurotoxin-Insensitive VAMP  
**TM:** TransMembrane  
**TRA2:** TRAnsformer 2  
**t-SNARE:** target membrane SNARE  
**VacA:** Vacuolating cytotoxin A  
**VAMP:** Vesicle-Associated Membrane Protein  
**v-SNARE:** vesicle SNARE  
**WASP/WAVE:** Wiscott-Aldrich syndrome



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## ABSTRACT

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are the most investigated trafficking proteins due to their role in directing the fusion complex. Among these, long VAMPs, or longins are characterized by N-terminal longin domain (LD), controlling both SNARE complex formation and subcellular localization (SCL) and are prototyped by VAMP7, Sec22b and Ykt6. The LD adopts a stable, closed conformation, but the contribution of this state and each different domain to sorting is still unclear. Human VAMP7, encoded by gene SYBL1, is involved in multiple cell pathways, including control of neurite outgrowth. Furthermore, alternative splicing (AS) of SYBL1 results in the production of two isoform subfamilies that retain an intriguingly domain architecture. Non-SNARE longin variants share the inhibitory LD, whereas non-longin SNARE variants share the SNARE motif. Since previous evidence suggests inhibitory functions for the LD construct and growth promoting activity for the  $\Delta$ -longin construct, these subfamilies are likely to play opposite functions. Therefore, mechanisms mediating neurogenesis are not clear, in particular the contribution of extracellular stimuli and different SNAREs. This work focused on the characterization of VAMP7 LD and its isoforms in SCL and neuronal development. Expression analysis in different tissues and cell lines, real time RT-PCR and confocal microscopy analyses demonstrated that VAMP7 variants have different tissue specificities and SCL; furthermore, the LD-only isoform VAMP7i displays also a nuclear localization. Considering their variant domain combinations, these physiological splice variants were used as tools for studying targeting determinants in SCL. Moreover, recombinant fragments of the VAMP7a cytoplasmic region confirmed that individual domains are unable to determine sorting by alone, and open/closed conformational switch is not relevant to SCL in the absence of transmembrane region. Gain-of-function experiments on both neuroblastoma cells and primary neurons revealed that VAMP7 AS is able to regulate neurite outgrowth by balanced production of stimulatory (VAMP7dh) and inhibitory (VAMP7i) isoforms. These effects are also subjected to the substrate (Poly-D-Lysine or Laminin) in which neurons are cultured and to the co-expression of other VAMP7 isoforms or SNAREs (VAMP2), suggesting a fine regulatory mechanism mediated by VAMP7 AS. Additional investigation will be helpful in order to manipulate neurogenesis in cell therapy and clarify the role of tissue-specific variants in some neurological diseases. Further characterization of VAMP7 LD and isoforms can unravel novel molecular partners and mechanisms, helpful in some biotechnological applications.



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## SOMMARIO

Le proteine SNARE (Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) sono le più studiate nell'ambito del traffico subcellulare, dato il loro ruolo nella formazione del complesso di fusione delle membrane. In questa famiglia, le VAMP lunghe o longine sono caratterizzate da un dominio N-terminale denominato longin (LD), che ha una funzione sia nella formazione del complesso SNARE, sia nella localizzazione subcellulare delle proteine; le longine trovano inoltre un modello in VAMP7, Sec22b e Ykt6. Il LD adotta una conformazione chiusa che risulta stabile, ma non è ancora chiaro il contributo che tale conformazione e ogni differente dominio proteico portano alla determinazione della localizzazione stessa di tali proteine. VAMP7 umana, codificata dal gene SYBL1 è coinvolta in molteplici *pathway* subcellulari, compreso il controllo della crescita dei neuriti. Lo *splicing* alternativo nel locus di SYBL1 produce inoltre due famiglie di isoforme che mantengono un'interessante architettura di domini. Le longine non-SNARE condividono il dominio inibitorio LD, mentre le longine non-LD lo SNARE motif. Date le evidenze preliminari che conferiscono tale funzione inibitoria al costrutto artificiale LD e invece un'attività di promozione della crescita al costrutto non-LD, sembra plausibile che le due sottofamiglie rispecchino questi ruoli contrapposti. Tuttavia, i meccanismi coinvolti nella neuritogenesi non sono ancora completamente chiariti, soprattutto per quanto concerne il contributo degli stimoli extracellulari e delle diverse proteine SNARE. Questo lavoro di tesi si è incentrato sulla caratterizzazione del LD di VAMP7 e delle isoforme di *splicing* di questa proteina nell'ambito del loro ruolo sia nella localizzazione subcellulare, che nel differenziamento neuronale. Analisi di espressione in diversi tessuti e linee cellulari, dati quantitativi di *real time* RT-PCR e analisi di microscopia confocale hanno dimostrato come le varianti di VAMP7 presentino differenti tessuto-specificità e localizzazioni subcellulari; l'isoforma VAMP7i mostra inoltre una localizzazione anche nucleare. Considerando la loro diversa combinazione di domini, queste varianti di *splicing* fisiologiche sono state utilizzate come strumenti per lo studio dei determinanti di localizzazione. Per di più, frammenti ricombinanti della regione citosolica di VAMP7a hanno confermato che i singoli domini non sono in grado da soli di determinare la localizzazione della proteina e che il cambiamento conformazionale aperto/chiuso non è rilevante per la localizzazione subcellulare, in assenza della regione transmembrana. Esperimenti di *gain-of-function* su cellule di neuroblastoma e neuroni primari hanno mostrato l'esistenza di una regolazione

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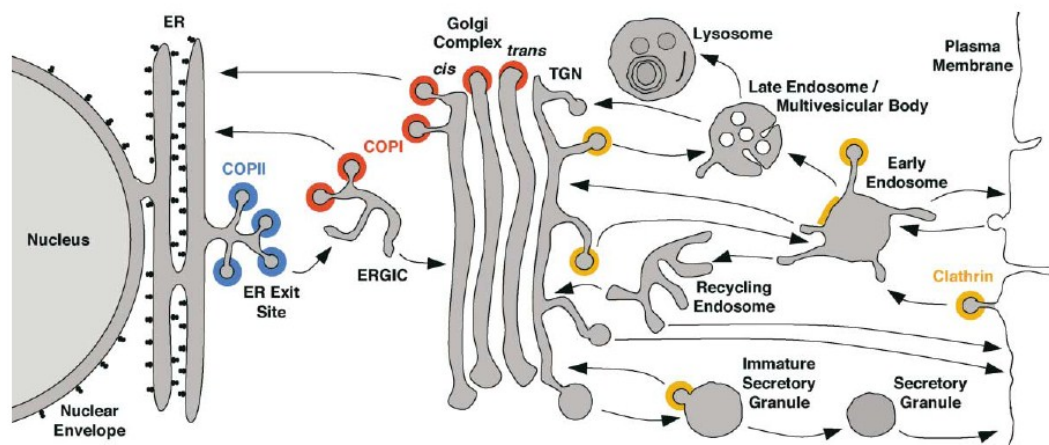
della crescita dei neuriti mediata dallo *splicing* alternativo di VAMP7, con la produzione di isoforme sia inibitorie (VAMP7i) che stimolatorie (VAMP7dh). Tali effetti dipendono anche dal substrato (Poli-D-Lisina o Laminina) in cui i neuroni sono cresciuti e dalla co-espressione con altre isoforme di VAMP7 o con altre SNARE (VAMP2), indicando la presenza di un meccanismo di regolazione fine da parte dello *splicing* alternativo di VAMP7. Ulteriori investigazioni potranno portare sia alla manipolazione della neuritogenesi per scopi di terapeutici, sia al chiarimento del ruolo che le specifiche varianti di *splicing* possono avere in alcune malattie neurologiche. La futura caratterizzazione del LD di VAMP7 e delle sue isoforme, ad esempio il ruolo di VAMP7i nel nucleo possono definire nuovi interattori e meccanismi molecolari, utili in alcune applicazioni biotecnologiche.

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# INTRODUCTION

## 1.1 Subcellular Trafficking

In the last decades, convincing evidence has demonstrated that diffusion is not the unique and most important process by which soluble proteins reach their target compartment and all the biochemical events occur. Indeed, eukaryotic cells are organized with an endomembrane system that consists of morphologically distinct organelles with dedicated functions in protein and lipid glycosylation, protein sorting, and degradation. These separate compartments communicate each other and with the plasma membrane through the exchange of vesicles that carry out lipids and proteins. Many subcellular trafficking routes are recognizable (Figure 1, for review see Bonifacino and Glick, 2004; Behnia and Munro, 2005; Brocker, 2010).



**Figure 1. Subcellular trafficking routes (from Bonifacino and Glick, 2004).**

The scheme depicts different compartments of the secretory, lysosomal/vacuolar, and endocytic pathways. Transport steps are indicated by arrows; colours indicate the locations of COPII (blue), COPI (red), and clathrin (orange). The coat protein complex II (COPII) vesicles accomplish the transport towards the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC), which functions as an interface between COPII and COPI-mediated transport and represents the first entity discriminating between anterograde and retrograde transport. Slow anterograde transport within the Golgi apparatus occurs by means of cisternae maturation, whereas retrograde cargo retrieval to the ER is mediated by COPI vesicles, and it is still under debate whether COPI vesicles mediate fast anterograde transport within the Golgi apparatus. The post-Golgi cargoes are sorted at the the trans-Golgi network (TGN). Then, vesicles can follow different trafficking routes in the secretory pathway (to and from the plasma membrane - PM), and the major organelles are essentially endosomes and lysosome; thus, it can be subdivided into constitutive secretion (vesicles are immediately delivered to the cell surface) or regulated secretion (vesicles are stored and then released, following specific signals). The former is ubiquitous, whereas the latter is present only within endocrine and neuroendocrine cells. Like in the COPII system, Clathrin Coated Vesicles (CCVs) biogenesis at the TGN and the PM involves sequential binding of a small GTPase, the Clathrin Adaptor Protein (AP) Complexes, and the heterohexameric coat protein Clathrin.

Correct sorting to a specific subcellular (SC) compartment is often crucial for proteins to reach their proper function. This is particularly important not only for normal, intracellular life but also when exogenous addition of proteins, drugs,

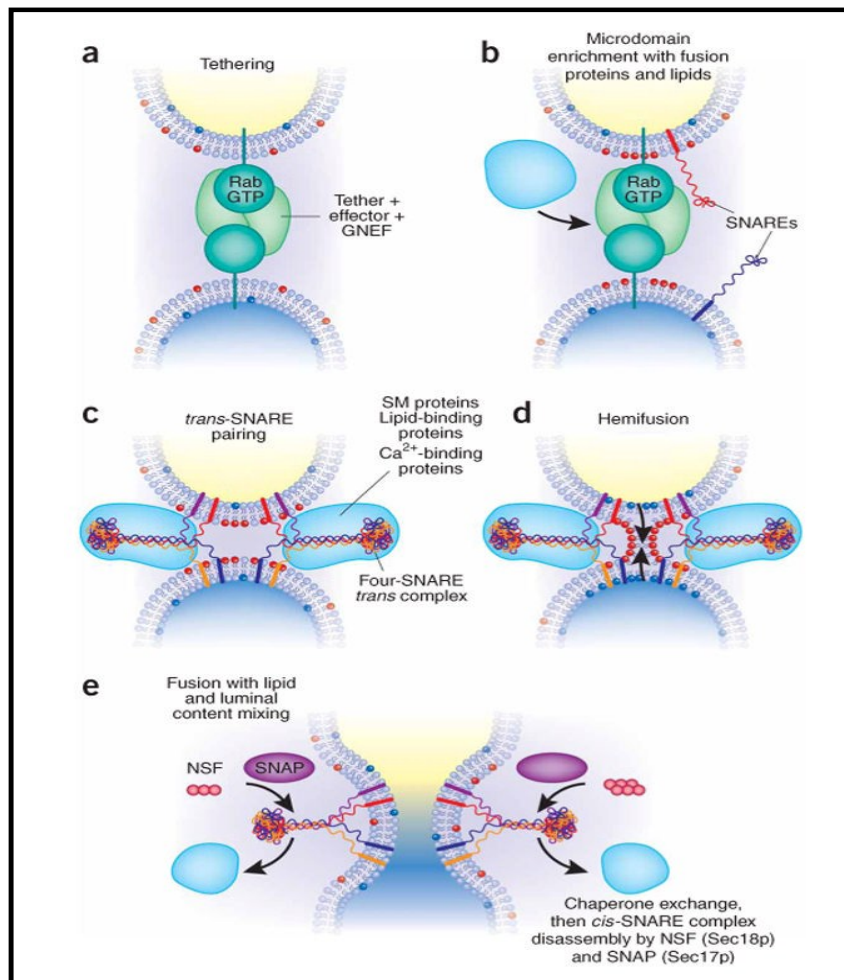
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molecular markers is required: indeed, improper SC targeting may result in ineffective or even negative results. Furthermore, many components of the trafficking machinery are absolutely necessary for cell survival. A number of diseases related to improper trafficking events are being discovered in the last years, and the molecular mechanisms underlying pathogenesis can be various (Albania *et al.*, submitted under revision).

### ***1.1.1 Membrane fusion and SNARE proteins***

Membrane fusion is an important process required in several functions of mammalian cells, including hormone and neurotransmitter release, membrane receptors recycling. It is considered the final and irreversible step of each SC trafficking route. Under normal homeostatic conditions, only the appropriate organelles fuse each other, suggesting that all steps are tightly regulated and balanced. Despite the enormous diversity in organelle size and shape, SC targeting is generally mediated by “trafficking machineries” consisting of multi-subunit protein complexes and by “sorting signals” which are present in the sorted molecules and are recognized by specific interactors. Some domains are conserved among all Eukaryotes, suggesting to play a central role in these mechanisms (for review, see Bonifacino and Glick, 2004; Behnia and Munro, 2005; Brocker *et al.*, 2010). The general, accepted model for SC trafficking retains that after cargo recruitment, new vesicles are generated by the deformation of a precursor membrane by coat proteins with the subsequent vesicle scission. In order vesicles are transported to the correct destination, they need to overcome the distance between organelles and must either bring along or acquire the machinery necessary for fusion. In most cells, this transport is mediated by cytoskeleton and its associate proteins. Once the final compartment is reached, vesicles fuse by the coordinated action of membrane-anchored Rab GTPases, Rab effectors (that bind active GTPases and stimulate GTP hydrolysis), tethering complexes that can consist of either long coiled-coil structures (functioning as a path that guides the vesicle) or (on the target membrane) of large hetero-oligomeric complexes and soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) (Wickner and Schekman, 2008). SNAREs play a central role in all the fusion events of secretory pathways, carrying out a conformationally controlled reaction cycle of high complexity (Figure 2). Similar to other intermediate involved in this cycle, *trans*-SNARE complexes are also regulated by other factors: for instance, Synaptotagmin 1, a  $\text{Ca}^{2+}$  sensing transmembrane protein is essential for fast,  $\text{Ca}^{2+}$  triggered fusion in neuronal exocytosis (Hong, 2005); NSF

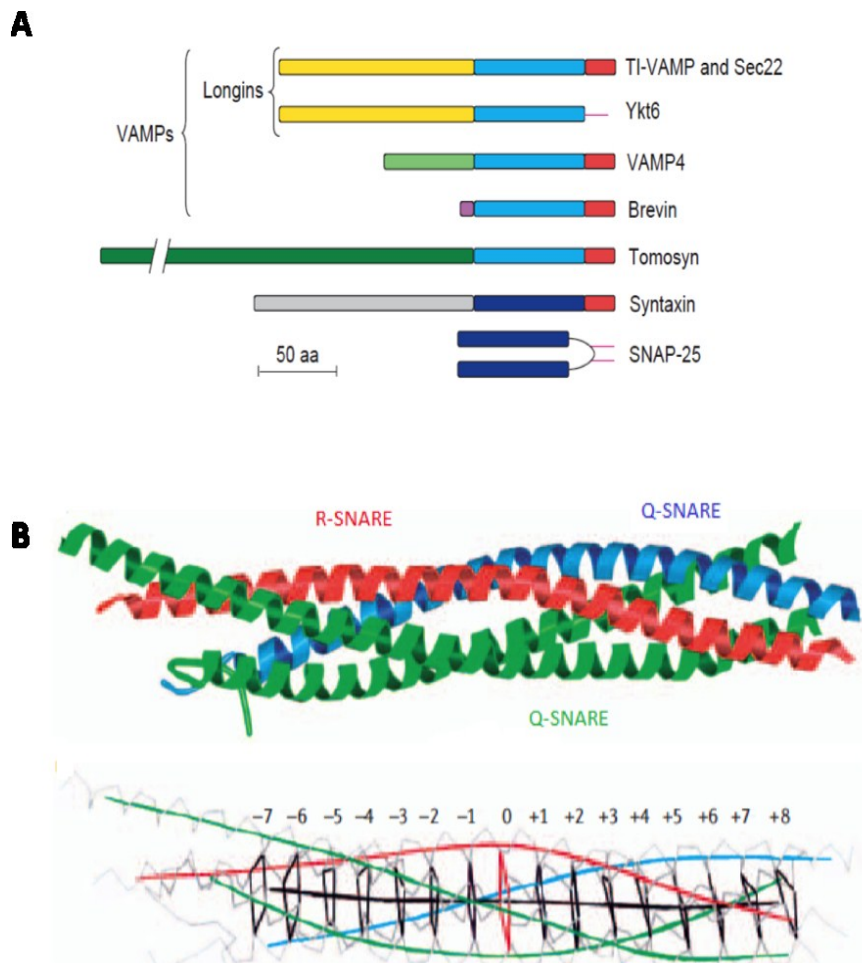
activity guarantees that free, uncomplexed SNAREs are constantly regenerated. Despite this free status, several proteins have been shown to bind to specific SNAREs, for accomplishing their recruitment on vesicles, sorting, entering in the helix bundle, and recycling. Therefore, this regulation is not only mediated by proteins that bind to SNAREs, but also by a panoply of protein kinases and phosphatases, and by other signaling proteins that can control SNAREs either directly or indirectly (Jahn and Scheller, 2006).



**Figure 2. Mechanism of membrane fusion (from Wickner and Schekman, 2008).**

Organelle-specific Rabs and Rab-effectors mediate tethering (a) and the assembly of fusion-competent microdomains (b), which achieve efficient fusion without lysis. After enrichment in microdomains, SNAREs located in the opposite membranes assemble with each other (c), forming a *trans*-SNARE complex that include additional bound factors such as Sec1/Munc 18-1 proteins, complexin and synaptotagmin (at synapses). This is able to drive bilayer distortion, triggering hemifusion (d) and then completion of fusion (e) by using the free energy that is released during the formation of a four helix bundle. After fusion, SNARE complexes exhibit a *cis*-configuration, in which all of the SNARE motifs reside together in the fused membrane. Disassembly of the *cis*-complex that is very stable requires a considerable amount of energy, provided by ATP hydrolysis: this process is carried out by the AAA-ATPase protein NSF, which binds indirectly the *cis*-complex through the  $\alpha$ -Synaptosomal-Associated Protein (SNAPs) (Hong, 2005).

SNARE proteins form a superfamily of small proteins with 25 members in *Saccharomyces cerevisiae*, 36 members in *Homo sapiens* and 54 members in *Arabidopsis thaliana*. They can significantly vary in sequence length (e.g. from <100 amino acids of some synaptobrevins to >1100 residues among tomosyns) because of their modular domain architecture (Figure 3A). The well-known SNARE motif - an  $\alpha$ -helical coiled-coil domain of approximately 60-70 residues long - is characterized by a hydrophobic heptad register interrupted at the so called “zero” layer by either a conserved R or Q residue (Fasshauer *et al.*, 1998) (Figure 3B).



**Figure 3. SNARE classification.**

A) Domain architecture of SNARE proteins, with the SNARE motif (pale blue) and a NT/CT region (from Rossi *et al.*, 2004). Despite this simple structure, SNAREs have important exceptions. Many SNAREs have independently folded domains that are positioned NT to the SNARE motif and may vary between the subgroups; on the contrary, brevins completely miss this region. Another subset of SNAREs lacks TM regions, but most of them contain hydrophobic post-translational modifications that mediate membrane anchorage. B) Skeleton representation of the SNARE complex and the zero layer (from Jahn and Scheller, 2006): SNARE motifs of Syntaxin 1 (red), SNAP25 (green) and Synaptobrevin II (blue), all oriented with their NT on the left. Based on the identity of the conserved residues at the “zero layer”, SNAREs are classified as R-SNARE or Q-SNARE. The Q-SNAREs can be further subdivided into Qa (syntaxin), Qb (SNAP-25 NT SNARE motif) or Qc (SNAP-25 CT SNARE motif) SNAREs. In order to accomplish the lipid bilayer fusion, one of each of the Qa-, Qb-, Qc- and R-SNARE are required. The Qa-, Qb-, Qc-SNAREs are clustered in nanodomains on the target membrane, constituting the acceptor complex, probably stabilized by other factors as Sec/Munc (SM) proteins.



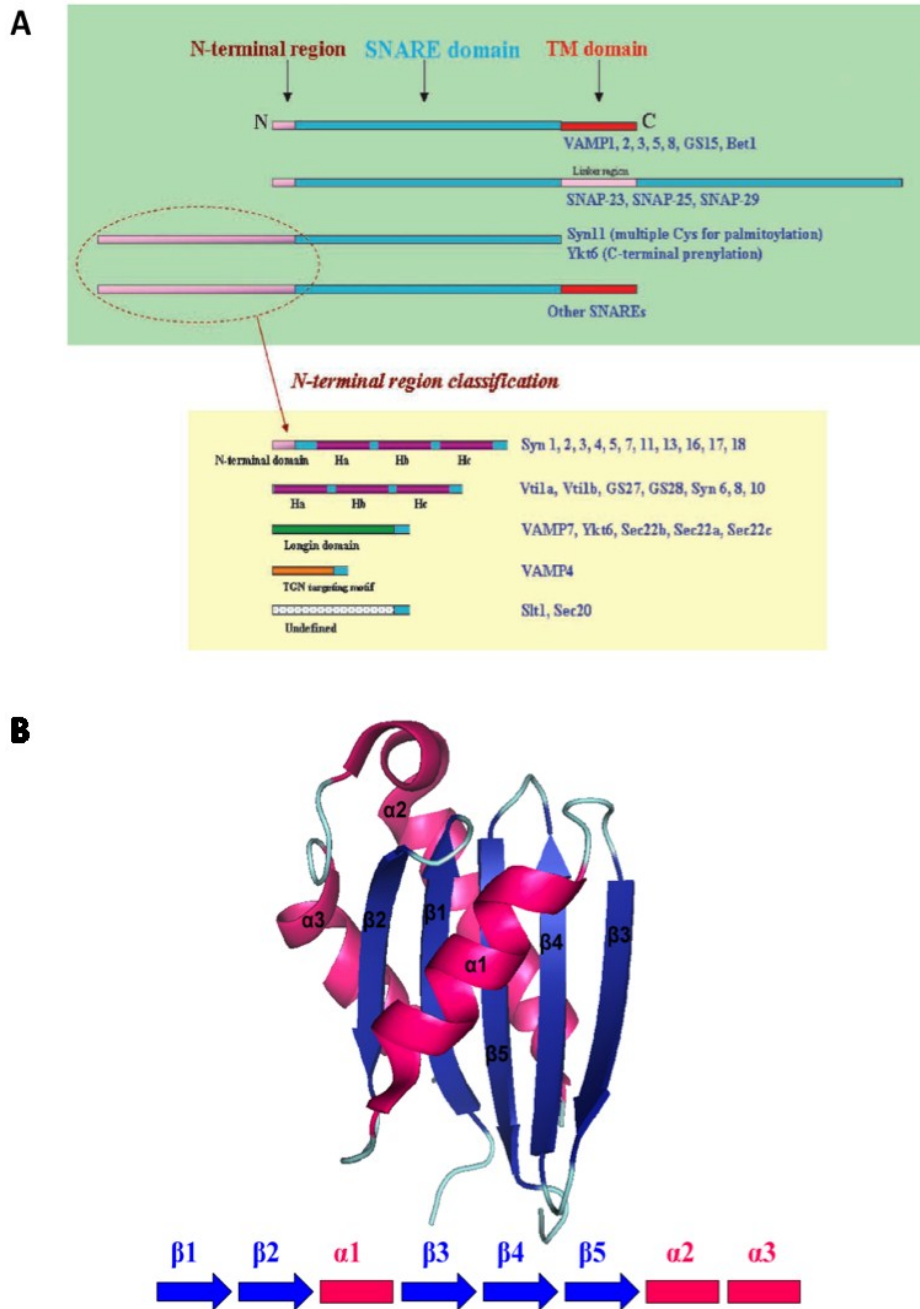
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For this reason, they are often referred to as R- or Q-SNAREs, overcoming the first functional classification based on their subcellular localization (SCL): the vesicle (v)-SNAREs or the target membrane (t)-SNAREs. The two classification methods are roughly superimposable, being R-SNARE usually placed on the vesicle in the heterotypic fusion. In addition to SNARE motifs, they can show further regions, such a carboxy-terminal (CT) transmembrane (TM) region or other motifs allowing post-translational addition of lipid anchors (as SNAP-25, Yk6) (Sudhof *et al.*, 2009) and a variable or conserved amino-terminal (NT) domain. The SNARE motif alone is sufficient for SNAREs to carry out the fusion of liposomes *in vitro*; however, its pairing cannot account for the high specificity observed in living cells. Moreover, although it is known that mutations in the SNARE motif can cause missorting, no defined sorting signals have been identified within this motif (Hong, 2005; Jahn and Scheller, 2006), suggesting that conformational changes might be involved in subcellular targeting.

Eukaryotic cells contain many SNAREs, with numerous members in each subfamily; when considering mammals, only nine v-SNARE are found (Ykt6, Sec22b, Synaptobrevin/Vesicle-Associated Membrane Protein (VAMP) 1 and 2, VAMPs 3-5, 7 and 8), with an high cell/tissue-specificity. Intriguingly, highly specialized organisms as human beings only use 36 SNARE proteins (Hong, 2005; Chaineau *et al.*, 2009). This might depend on specific regulatory mechanisms accomplished during gene expression. Although some SNAREs seem to function in only one intracellular fusion step and interact with only one set of partner SNAREs, others are less specialized, providing a healthy mix of robustness and flexibility for intracellular fusion reactions. For example, after fusion events, some SNAREs need to be recycled to their donor compartment, residing in the target membrane of organelles they mediate the fusion, as well as in the membranes of organelles being part of their recycling pathway. Thus, specific sorting mechanisms ensuring equipment of the appropriate set of SNARE proteins are required.

### ***1.1.2 SNARE NT domains, longins and longin-like proteins***

NT domains can regulate SNARE motif function, interaction with other proteins and SCL. Several different types have been identified (Figure 4A); in particular, short VAMPs or “brevins” are distinguishable for a short and variable NT domain, whereas long VAMP or “longins” share a 120-140 aminoacids long NT domain called the longin domain (LD) (Figure 4B).



**Figure 4. SNARE NT domains.**

A) The general structural frameworks of different SNAREs (from Hong, 2005). Unlike the conserved SNARE motifs, there are different types of independently folded NT domains. Qa-SNAREs, and some Qb- and Qc-SNAREs, have NT antiparallel three-helix bundles. These bundles can vary in length and are connected to the SNARE motif by a flexible linker. By contrast, the NT domains of many R-SNAREs have LDs that can be also found in proteins other than SNAREs. R-SNAREs that have LDs are conserved among all Eukaryotes, whereas the evolutionarily younger R-SNARE brevins lack a folded NT domain and have only a few amino acids beyond their SNARE motif (Jahn and Scheller, 2006). B) The LD shows a highly conserved globular fold based on a central beta flat sandwiched in between alpha helices, so that roughly two “sides” can be recognized. Intriguingly, both sides seem to mediate SCL since they bind different partners, possibly acting in cooperative fashion (Mancias and Goldberg, 2007). LDs able to target the protein to different SCLs show most sequence variation at the two “alpha sides” of the domain, i.e. local variation in surface epitopes seems to modulate SC targeting. The “alpha2-alpha3 side” of the LD seems to mediate binding to multi-subunit trafficking complexes as well as to single proteins, whereas the “alpha1 side” is involved in alternative intra- or intermolecular binding to alpha-helical domains, once again resulting in the regulation of SC targeting (Mancias and Goldberg, 2007; Pryor *et al.*, 2008).

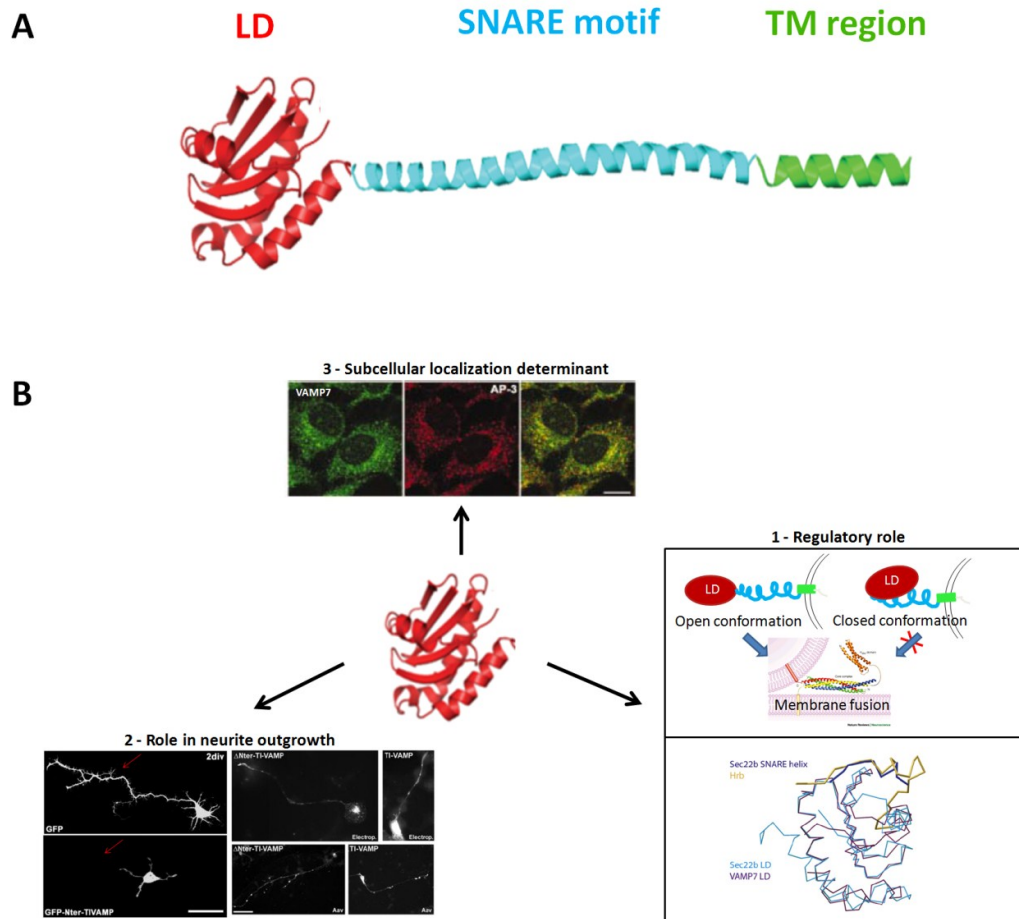
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Longins are the only R-SNAREs conserved in all Eukaryotes (Filippini *et al.*, 2001), whereas brevins are limited to bilateria and hence are absent from whole taxa (e.g. plants) (Rossi *et al.*, 2004). Longins group into three subfamilies, prototyped by Ykt6, Sec22b and VAMP7 (also called Tetanus neurotoxin-Insensitive (TI)-VAMP - Galli *et al.*, 1998) (Figure 5A).

The functional role of such NT domains has been elucidated only in few cases; for instance, the three helix bundle NT domain of syntaxins (H<sub>abc</sub> domain) is able to fold back onto the SNARE motif to form a four-helix bundle, giving a closed conformational state. This conformation is required for the syntaxin to bind Munc18-1, which in turn stabilizes the syntaxin closed conformation, thus inhibiting the SNARE complex formation and the membrane fusion (Hong, 2005). The LD of longins (Filippini *et al.*, 2001) was found to play multiple regulatory roles, as SC sorting and membrane fusion (reviewed in (Rossi *et al.*, 2004). In Sec22b, export from the ER is mediated by binding to Sec23/24, a process depending on a conformational epitope created by intramolecular LD-SNARE motif binding; this also results in preventing promiscuous, unspecific binding by sequestering the NT half of the SNARE motif (Mancias and Golberg, 2007). In addition to the SNARE longin Sec22b, the Sec22 family encompasses two further proteins named Sec22a and Sec22c, which share the LD but not the SNARE motif with longins. These proteins are suggested to be involved in trafficking, but their function remains still unclear (Tang *et al.*, 1998; Gonzalez *et al.*, 2001).

The LD targets Ykt6 to its SCL, likely by masking other localization signals (Hasegawa *et al.*, 2004; Wen *et al.*, 2010). Ykt6 does not share the TM region with other longins, undergoing substantially irreversible prenylation and reversible palmitoylation; this is a regulatory mechanism for its cycling between membrane and cytosol (Fukasawa *et al.*, 2004). In yeast, the LD of Ykt6p can regulate membrane fusion by inhibiting Ykt6p participation to the fusion bundle, by competitive intramolecular binding to the SNARE motif (Tochio *et al.*, 2001). VAMP7 LD also regulates membrane fusion (Figure 5B); furthermore, it is crucial to neurite outgrowth, as overexpression of a “deregulated” fragment missing the LD ( $\Delta$ -longin) increases neurite outgrowth, whereas reverse effect (outgrowth inhibition) is obtained when expressing the LD alone (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001). In addition, LDs serve as a dominant signal for SC targeting. For example, in animals LD targets VAMP7 to late endosomes by binding to the  $\delta$ -subunit of the AP-3 complex (Martinez-Arca *et al.*, 2003) and the HIV-Rev binding protein (HRB) (Chaineau *et al.*, 2008; Pryor *et al.*, 2008). Interaction with these binding proteins is likely regulated by VAMP7 open/closed-conformation, which is based on intramolecular LD-SNARE motif binding

(Vivona *et al.*, 2010). In plants, several VAMP7 proteins are targeted to their different SCLs by their LDs (Uemura *et al.*, 2005).



**Figure 5. VAMP7 and its LD.**

A) Domain architecture of longin VAMP7: NT LD (red), SNARE motif (pale blue), CT TM region (green). B) Roles of VAMP7 LD: 1) regulatory role by inhibition of the SNARE-complex when it is in closed conformation (Pryor *et al.*, 2008) and conformational epitope formation (Mancias and Golberg, 2007); 2) inhibition of neurite outgrowth by expressing LD fragment (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001); 3) role in SCL observed by missorting of VAMP7c isoform that misses LD (Martinez-Arca *et al.*, 2003; Rossi *et al.*, 2004).

Although the longin fold has been originally found in longins, an increasing number of single-domain proteins and multi-domain polypeptides, which are most often part of multi-subunit trafficking complexes share a conserved fold rather than an homologous sequence with the LD (De Franceschi *et al.*, manuscript in preparation). In *Homo sapiens*, example of such longin-like proteins are the  $\sigma$  and  $\mu$  subunits of the AP-1 and AP-2 complexes,  $\delta$  and  $\zeta$  subunits of COPI, TRAPP complex subunits 2 and 4, also called Sedlin and Synbindin. Longin-like proteins

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have been found to act also as scaffold proteins: this is the case for MP1 and p14 proteins. Finally, a longin-like fold is shown in the NT region of the Signal Recognition Particle (SRP) Receptor  $\alpha$  subunit (SR; the NT is called SRX). For some longin-like proteins, connections with different pathologies have been reported; e.g. mutations in Sedlin can be causative for SpondyloEpiphyseal Dysplasia Tarda (SED-T). The longin-like fold seems in turn to belong to a wider family of domains sharing mixed  $\alpha/\beta$  fold. In particular, also proteins from the DUF 254 family, profilin-like proteins and PAS and GAF domains are somehow to share a structural core similar to the longin fold (Gonzalez *et al.*, 2001; Kinch and Grishin, 2006).

A new subfamily of non-SNARE longins has been recently discovered and it is specific to plants; they are thus referred to as “Phytolongins”. Such proteins share NT and CT regions with longins, i.e. LD and TM region; in the central region instead, the SNARE motif is replaced by a specific “Phyl” region which is predicted to bind intramolecularly the LD, but not intermolecularly to other SNAREs (Vedovato *et al.*, 2009). Therefore, Phytolongins potentially share with longins integral membrane insertion and the open/closed conformational switch in the LD, which is crucial to tune SCL. Conversely, the function of the Phyl region has yet to be elucidated in order to understand Phytolongin involvement in the plant trafficking machinery.

### **1.1.3 Human VAMP7**

#### ***1.1.3.1 Physiology, functions and correlated pathologies***

The human gene SYBL1 (synaptobrevin-like 1) is ubiquitously expressed and finely regulated at different layers. Even if located in the Xq/Yq pseudoautosomal region, SYBL1 is subject to X inactivation (D’Esposito *et al.*, 1996) and its allelic expression is controlled by multiple epigenetic mechanisms (Matarazzo *et al.*, 2002). SYBL1 gene homologues were identified in rat, mice, non human primates, and also in plants and flies. Human SYBL1 gene structure consists of eight exons (Figure 6A) and the corresponding protein VAMP7 is a 220 residues long membrane protein (25 kDa), formed by three distinct domains, as previously described for longins: the LD of 120 residues at the NT is followed by a SNARE motif of 66 residues. At the CT, a TM region of 20 residues is followed by a short topological domain (11 residues). Considering the heptad register at the zero layer, VAMP7 shows the highly conserved RG-SNARE of longins, VAMP4 and some brevins (e.g., VAMP5, VAMP8), in contrast to the RD motif seen in

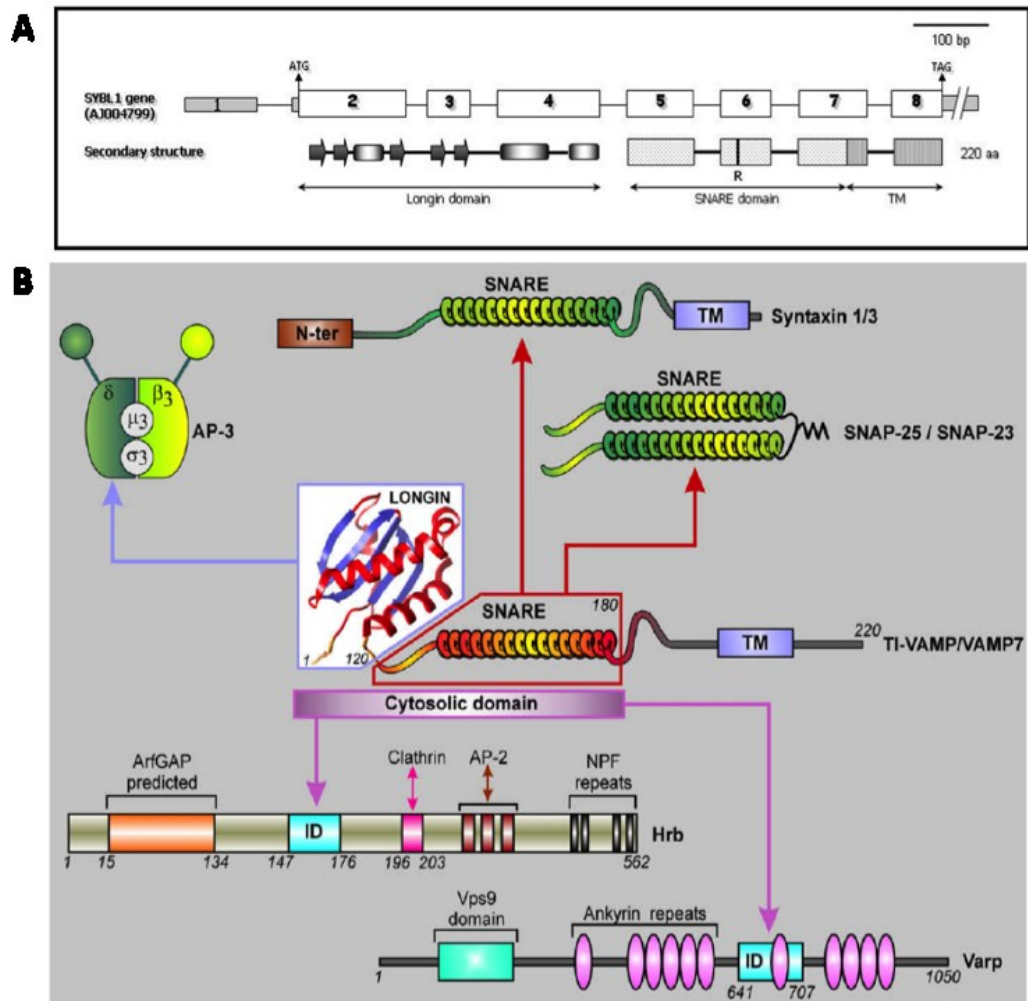
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synaptobrevin/cellubrevin v-SNAREs, e.g. VAMP1/2/3 (Rossi *et al.*, 2004). VAMP7 is also called TI-VAMP due to its insensitiveness to cleavage by clostridial neurotoxins, such as tetanus toxin and other isotypes of botulinum (B, D, F and G) neurotoxins.

A lot of proofs highlight the ubiquitous role of VAMP7, considering its different binding partners and cargoes (extensively reviewed in Chaineau *et al.*, 2009). It interacts with several t-SNAREs (depending upon the cell/tissue type) and proteins involved in its regulation (Figure 6B). Furthermore, VAMP7 trafficking is responsible for different type of cargoes that explain its function in several cell types: the Cell Adhesion Molecule (CAM) L1 in neurons, Membrane Type 1-Matrix Metalloproteinase Protein (MT1-MMP) in metastatic cells, the fucosylated carbohydrate LewisX (LeX) in the Central Nervous System, the tetraspanin CD82 (also known as KAI-1) in metastatic cells. It clear that all these trafficking activities require defined routes to be followed.

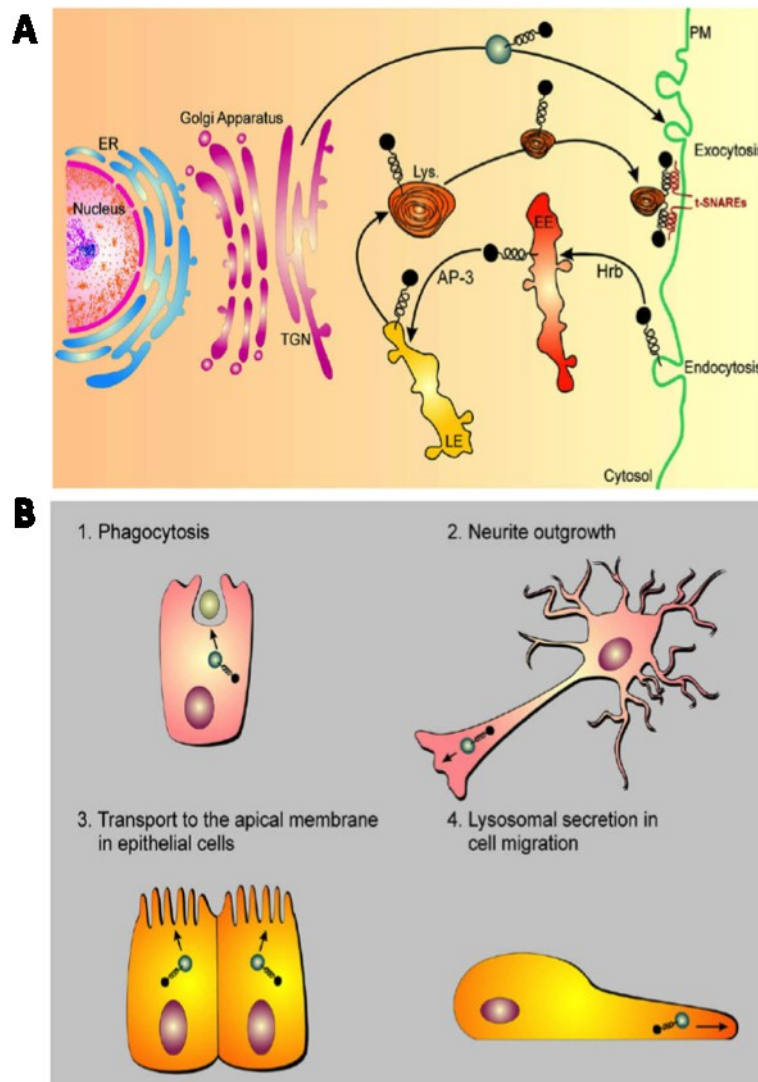
VAMP7 plays a pivotal role in the recycling pathway between late endosomes and lysosomes (Figure 7A). It is also involved in constitutive-like exocytosis, during neurite elongation (see neuritogenesis section) and post-synaptic receptor expression at the PM of mature neurons. VAMP7 may also associate with a variety of other SNARE partners to function in multiple membrane traffic steps which may in part be cell type-specific, identifying both basal and specialized functions (Figure 7B). It has been found in macrophages, dendritic cells and polymorphonuclear neutrophils, where actin polymerization is the driving force for morphological changes during phagocytosis or secretion. VAMP7-expressing vesicles are also released during cytotoxic granule exocytosis in NK cells (Krzewski *et al.*, 2011). Other experiments reported a role during cell division. An unconventional route for the trafficking of Kv4/KChIP1 is also mediated by VAMP7 in neurons and cardiac myocytes (Flowerdew and Bourgoyne, 2009). Recently, a contribution in the release of both ATP and cathepsin B from glial cells was also discovered (Verderio *et al.*, 2011); furthermore, VAMP7-Vti1a complex transports the Major Myelin Proteolipid Protein in neurons (Feldmann *et al.*, 2011). An interesting, new role was assessed in mediating homotypic fusion in autophagosome precursor maturation (Moreau *et al.*, 2011).

Since its involvement in several mechanisms, VAMP7 is likely to play a critical role in different pathologies. It delivers MT1-MMP to invadopodia, in invasive tumor cells (Steffen *et al.*, 2008). VAMP7 is also suggested to be the predominant v-SNARE involved in granule-derived mediator release from eosinophils and neutrophils, the principal cause of inflammatory responses in diseases such as asthma and chronic obstructive pulmonary (Logan *et al.*, 2006).



**Figure 6. VAMP7 and its binding proteins.**

A) Genome structure of SYBL1 (adapted from Vacca, Albania *et al.*, 2011). Top: exon-intron structure of the human gene SYBL1 (AJ004799). The mRNA originated from the SYBL1 gene is 2660 nucleotides long and it is composed by 8 exons: exon 1 (85 nt), exon 2 (155 nt), exon 3 (58 nt), exon 4 (138 nt), exon 5 (91 nt), exon 6 (68 nt), exon 7 (93 nt) and exon 8 (1887 nt). Exon 1 is all included in the 5' UnTranslated Region (UTR); exon 2 harbors a cryptic splice site, by which it can be divided into exon 2' (90 nt, containing the canonical start site) and exon 2'' (65 nt); within exon 8 the canonical stop codon and the poly A tag are located. Intriguingly, the borders between the protein domains roughly correspond to the limits between exons: the LD is coded by exons 2, 3 and 4; the SNARE motif by exons 5, 6 and the first part of 7; the TM region by the remaining part of exon 7 and by exon 8. The 100 bp reference bar only refers to exons; introns are not to scale. Exons are numbered: coding regions are white and noncoding ones are grey; start and stop codons are indicated. Bottom: domain architecture of the encoded protein VAMP7 (P51809), showing the LD, the SNARE motif (the black vertical bar indicates the conserved arginine of the polar layer) and TM region with intravesicular tail. B) VAMP7 binding proteins (from Chaineau *et al.*, 2009). As a v-SNARE, via its SNARE motif, VAMP7 interacts with syntaxin 1 and SNAP-25 in neurons, with syntaxin 4 and SNAP-23 in fibroblasts. Stable complexes are formed with syntaxin 4 and SNAP-23 at the PM of human mastocytes, with syntaxin 3 in rat mastocytes, and with syntaxin 3 and SNAP-23 at the apical side of the epithelial Caco2 cells. VAMP7 also interacts with the endosomal t-SNARE complex composed of syntaxin 7, syntaxin 8 and vti1b in fibroblasts and brain extracts. Furthermore, the calcium-sensor protein synaptotagmin VII likely interacts with the resulting SNARE complex in a calcium-dependent manner (not shown). Other important interactions for SC sorting are mediated by: the LD, in case of the  $\delta$  subunit of AP-3; the cytosolic domain, for Hrb and Varp, a Guanine Exchange Factor (GEF) for Rab21. An ArfGAP domain localized at the NT end of HRB has been predicted from *in silico* analysis (orange). In addition to its interacting domain with VAMP7 (ID), HRB also interacts with clathrin (pink), AP-2 (brown) and Eps15 via its NPF repeats (Asn-Pro-Phe, in gray). Varp regulates neurite outgrowth in association with VAMP7. Varp is also composed of a Vps9 domain (blue) that it is responsible for the GEF activity of the protein for Rab21 and of 11 ankyrin repeats (pink).



**Figure 7. VAMP7 routes and functions (from Chaineau *et al.*, 2009).**

A) VAMP7 generally localizes in late endosomes (50%), early endosomes (5%) and lysosomes (5%); a widely localization was also found within the TGN region (30%). VAMP7 is involved in the transport from the Golgi to the cell surface (secretory pathway): it is required for insulin and osmotic shock in adipocytes and constitutive exocytosis of growth hormone; moreover, a lot of different cargoes require this route. During neuronal development, it is mainly expressed at the tip of the growth cone in immature neurons and in the somatodendritic compartments of mature neurons (pyramidal cells). After mediating exocytic events via pairing with its t-SNAREs at the PM, VAMP7 is endocytosed in a clathrin-dependent manner by interacting with HRB. In early endosomes, VAMP7 interacts with AP-3 reaching late endosomes and lysosomes. This binding is important for protein targeting to different compartments, depending upon cell types, which are late endosomes in epithelial cells, and synaptic vesicles in certain neuronal cells. Then, VAMP7 mediates lysosomal and granule secretion in polarized cells and fusion events in phagocytosis. VAMP7 was also found in the endoplasmic reticulum (ER) and cis-Golgi of enterocytes, whereas it is absent in ER of liver or kidney. It seems to mediate ER to Golgi traffic that contains syntaxin 5 and rBet1, but three distinct complexes have been identified. ER: endoplasmic reticulum, TGN: trans-Golgi Network, Lys: lysosome, EE: early endosome, LE: late endosome. B) VAMP7 is involved in different cellular functions, in dependence of the cell type. PM remodeling is required for phagocytosis to occur in macrophages (1) and during mitosis. VAMP7 also plays a role in neurite outgrowth in neurons and neuronal cell lines (2) and in apical transport in epithelial cells (3), where it forms SNARE complexes with its t-SNAREs partners syntaxin 3 and SNAP-23. Lysosomal secretion is particularly active in cell migration (4), where VAMP7 mediates fusion of autophagosomes with lysosomes and for the release of exosomes; it has also been involved in local inflammatory responses mediated by specialized cells such as mastocytes, basophils, eosinophils or neutrophils. Finally, VAMP7 was found to regulate exocytosis in human neutrophils and eosinophils, like perforine and granzyme B.



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Furthermore, VAMP7 directly participates to the vacuolating cytotoxin A (VacA)-induced vacuolation mechanism, by which *Helicobacter pylori* colonizes the gastric mucosa (Mashima *et al.*, 2008). SYBL1 expression is also altered in human pathologies characterized by DNA methylation derangement (Scarano *et al.*, 2005), such as ICF syndrome (Harsen *et al.*, 2000; Matarazzo *et al.*, 2007) and hyperhomocysteinemia (Ingrosso *et al.*, 2003). Although SYBL1 is ubiquitously expressed in human tissues, its function appears to be particularly interesting in neurons with a possible implication in neurodevelopmental disorders (Saito *et al.*, 2000; Müller *et al.*, 2002).

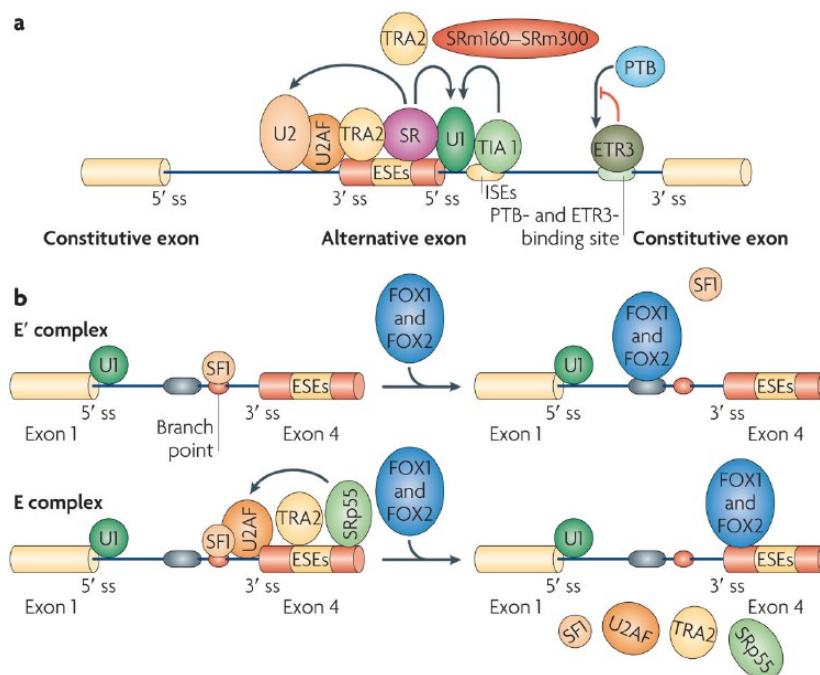
### 1.1.3.2 Novel regulatory mechanism at SYBL1 locus: alternative splicing

Like other highly regulated processes typical of eukaryotic cells, SC trafficking needs fine tuning of protein functions and structures. Alternative splicing (AS) is a crucial to both gene regulation and generation of proteomic diversity, by modulation of domain architecture and creation or deletion of sequence motifs. In metazoans, AS plays an important role in generating different protein products that function in diverse cellular processes, including cell growth, differentiation and death. (Stamm, 2002; Chen and Manley, 2009; Turner, 2011). From 50% to 74% of the human genes undergo AS, producing more than one polypeptide product (Stamm *et al.*, 2005; Stetefeld and Ruegg, 2005). AS affects range from subtle modulation to complete variation in protein function, due to the production of different splice variants: truncated proteins, inactive products by deletion or retention of entire domains or changing of signal peptides. As a consequence, missplicing events can cause several human diseases. There are four basic splicing patterns: exon skipping (cassette exons), alternative 5' or 3' splice site (cryptic splice sites), exon shuffling (mutually exclusive cassette exons) and intron retention; among them exon skipping is the most prominent. AS can be either constitutive or induced (e.g. by receptor stimulation, cellular stress, or differentiation during development) and it is mostly due to the activation of cryptic splice site as well as binding of suboptimal sites by components of the splicing machinery or splicing regulators (Stamm *et al.*, 2005; Fox-Walsh and Hertel, 2009).

The decision as to which exon is removed and which exon is included involves RNA sequence elements and protein regulators (Figure 8), but the exact mechanism is still unclear. Depending on the position and function of *cis*-regulatory elements, they are divided into four categories: exonic splicing

enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). Other mechanisms for AS regulation can be: i) secondary structures that mask splice sites or bind sites for splicing factors, ii) the inhibition and the activation of intron definition, iii) the recruitment of distinct splicing factors to the nascent pre-mRNA, resulting in the inclusion or exclusion of an alternative exon. Phosphorylation can also change the ability of splicing factors to interact with other proteins or mRNA substrates, leading to changes in splice site selection (Chen and Manley, 2009).

AS contributes to genomic diversity and tissue specificity, being controlled by differentially expressed splicing regulators and/or ubiquitously expressed splicing factors of different concentrations and/or activity. Among all human tissues, brain is the most functionally diverse tissue, as it has the highest occurrence of tissue-specific AS isoforms (Chen and Manley, 2009). Accordingly, several brain-specific factors have been identified, such as nPTB (Li *et al.*, 2007), NOVA1, NOVA2 (and Hu/Elav proteins). In addition, region- and cell type-specific expression of most of the >300 RNA-binding proteins examined was observed in proliferating and post-mitotic mouse brain cells (McKee *et al.*, 2005).



**Figure 8. Mechanism of splicing activation (adapted from Chen and Manley, 2009).**

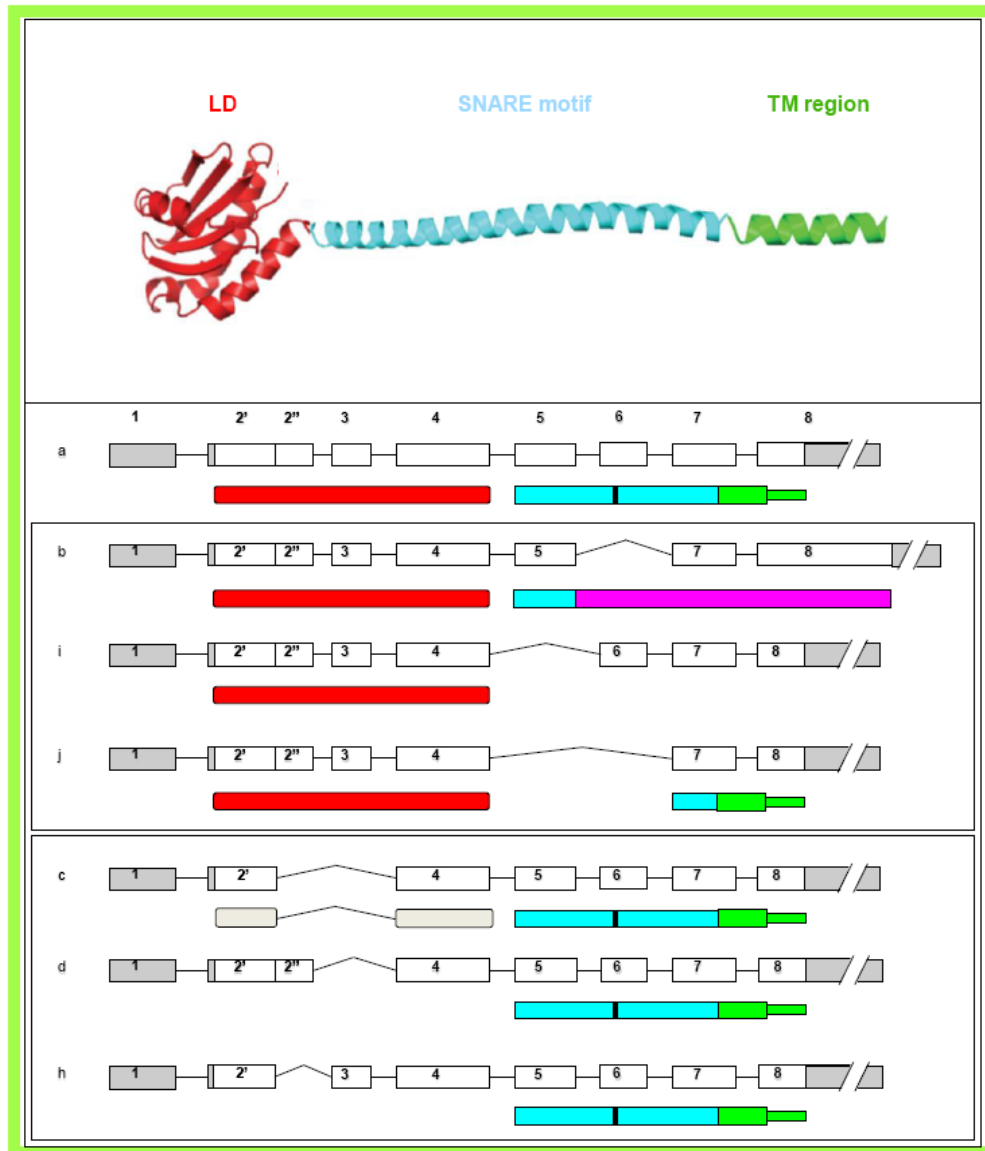
a) Ser-Arg (SR) proteins bind to ESEs to stimulate the binding of U2AF to the upstream 3' splice site (ss) or the binding of the U1 small nuclear ribonucleoprotein (snRNP) to the downstream 5' ss. SR proteins function with other splicing co-activators, such as transformer 2 (TRA2) and the SR-related nuclear matrix proteins SRm160-SRm300. T cell-restricted intracellular antigen 1 (TIA1) binds to U-rich sequences ISEs immediately downstream of 5' splice sites to facilitate U1 binding. CUGBP- and ETR3-like factor (CELF) proteins, such as ETR3, bind to similar sequences as polypyrimidine-tract binding protein (PTB), thereby activating splicing by competing with PTB. b) Fox1 and Fox2 inhibit the inclusion of calcitonin-related polypeptide- $\alpha$  (CALCA) exon 4 by blocking the binding of splicing factor 1 (SF1) to the branch point (top panel) and of TRA2 and SRp55 to ESEs (bottom panel), thereby inhibiting spliceosome assembly at two stages, the E' and E complexes. The arrow indicates that SRp55 and TRA2 promote binding of the U2AF complex.

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AS occurs in several trafficking-related genes, including SNARE genes, resulting in the production of isoforms with different SCL and trafficking properties. Developmentally regulated AS of SNAP-23 and SNAP-25 modulates interactions with accessory factors and SC localization (Shukla *et al.*, 2001; Nagy *et al.*, 2005). AS also regulates interaction properties in the large t-SNARE family of syntaxins, by varying their NT and/or CT regions (Nakayama *et al.*, 2004). In R-SNAREs, AS can finely tune vesicle subcellular targeting by varying the extreme CT region in splice variants of VAMP1 (Isenmann *et al.*, 1998; Berglund *et al.*, 1999), and tissue-specific AS of mammalian tomosyn is involved in the regulation of neuronal secretion (Yokoyama *et al.*, 1999; Groffen *et al.*, 2005). In most Eukaryotes, the involvement of VAMP7 in multiple trafficking events at different sub-cellular compartments is mediated by gene amplification and variation (Vedovato *et al.*, 2009). In mammals, VAMP7 is encoded by a single gene and yet it regulates multiple sub-cellular pathways (Chaineau *et al.*, 2009) hence, AS is a likely strategy to achieve such complexity in regulation.

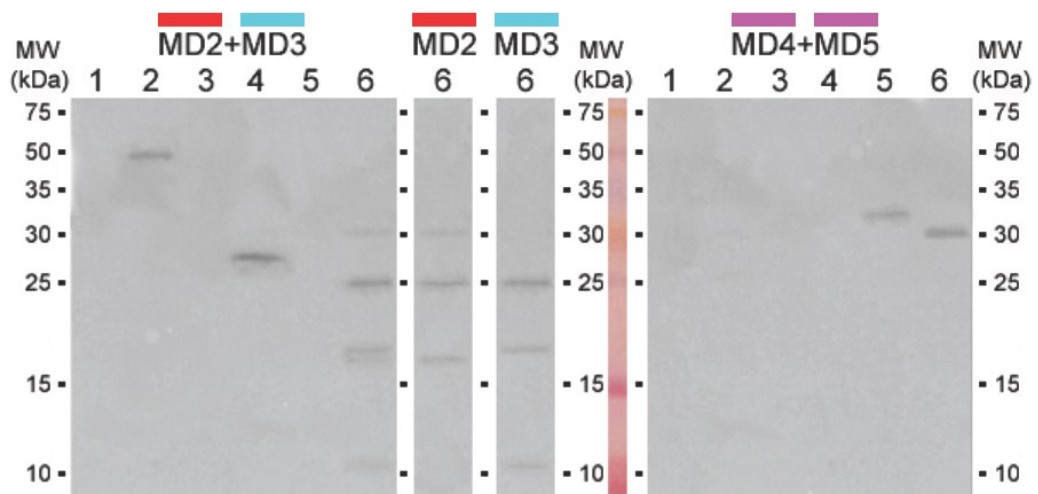
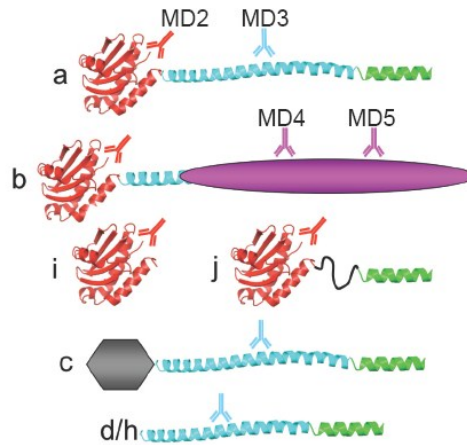
Interestingly, an AS mechanism regulating SYBL1 locus was already characterized: splice variant c is deleted from approximately one-third of its LD sequence (Rossi *et al.*, 2004) and it does not interact with the  $\delta$  subunit of AP-3 (Martinez-Arca *et al.*, 2003). Further investigation on the occurrence of AS at the SYBL1 gene (being part of this thesis work) reported the existence of several putative VAMP7 isoforms, produced by exon skipping (Vacca, Albania *et al.*, 2011) (Figure 9). They display interesting domain architectures that define two VAMP7 subfamilies: the non-LD and the non-SNARE isoforms. In particular, the novel VAMP7b CT sequence of unknown function partially disrupts the LD-binding motif on the SNARE motif, differently from the main isoform VAMP7a, in which the closed state is prominent (Vivona *et al.*, 2010).

In addition to AS mechanism, isoform d and h are subjected to alternative translation initiation. This is an as yet poorly characterized mechanism for producing several proteins from a single mRNA that is quite frequent in *Homo sapiens* (Kochetov, 2008). Depending on this mechanism, both isoform mRNAs produce the same polypeptide hence named “VAMP7d/h”. Although the existence of VAMP7 isoforms were demonstrated at protein level (Figure 10), further investigation were required for correlating AS at SYBL1 locus with expression levels in different cell lines and tissues (Vacca, Albania *et al.*, 2011) and moreover the physiological role of these isoforms.



**Figure 9. Putative VAMP7 splice variants (adapted from Vacca, Albania *et al.*, 2011).**

The main isoform, VAMP7a (220 aa) retains all 8 exons and the canonical start codon is within exon 2'. The central panel shows non-SNARE isoforms characterized by skipping of respectively: (i) exon 6 (VAMP7b), (ii) exon 5 (VAMP7i) and (iii) both exons (VAMP7j). VAMP7b is 40 amino acids longer than VAMP7a and it shares with the main isoform the LD and NT part of the SNARE motif. Then, a frameshift downstream of exon 5 results in substitution of both the CT half of the SNARE motif and the TM region by a novel CT region of 116 amino acids. On the other hand, VAMP7i basically consists of the LD alone: exons 1 to 4 encode for the first 114 residues of VAMP7, including all secondary structure elements needed to achieve a complete LD fold. Skipping of exon 5 then results in a frameshift that leads to the translation of just two additional residues, followed by a premature stop codon. As for VAMP7j, it is a kind of "membrane-anchored version" of VAMP7i: skipping of both exons 5 and 6 preserves the original frame in which the LD is followed by a short hinge region preceding the original TM and intravesicular tail regions, shared with the main isoform. In the bottom panel, "non-LD" isoforms - i.e. splice variants missing the LD are shown. They retain all the canonical exons from 4 to 8. VAMP7c was already described: the splicing event skips out 40 residues of the NT region (which thus cannot fold as a LD), but the mRNA retains the original reading frame, leading to the correct translation of the "synaptobrevin-like" part of the sequence. Translation of both VAMP7d and VAMP7h mRNAs would result in short peptides (59 and 45 aa, respectively) when starting from the canonical translation initiation start (TIS) of SYBL1, which is located in exon 2. However, a second, alternative TIS in exon 5 shows "optimal status" with respect to sequence context and distance from the upstream stop codons to reinitiate translation. Since the alternative TIS in exon 5 is common to "d" and "h" isoforms, both mRNAs are likely to produce the same polypeptide, hence named "VAMP7d/h". Intriguingly, VAMP7d/h, corresponding to last 94 residues of VAMP7a, lacks the LD and only consists only of the SNARE motif, TM region and intravesicular tail. Coding exons or their fragments are white; non coding ones are grey. Introns are not to scale. Domain architecture: LD, red; SNARE motif, cyan (the conserved arginine of the polar layer is indicated by a vertical bar); TM region and intravesicular tail, light green; NT region of VAMP7c, grey; unknown function region of VAMP7b, magenta.



**Figure 10. Expression of VAMP7 isoforms at protein level (from Vacca, Albania *et al.*, 2011).**

Immunoblotting with domain-specific anti-peptide antibodies. Each MD antibody shares the color with the recognized domains: MD2, LD, red; MD3, SNARE motif, cyan; MD4 and MD5, unknown function region of VAMP7b, magenta. Other represented regions are the NT region of VAMP7c (grey) and the TM region with intravesicular tail (light green). The two large blots share the same series of whole lysates from *E. coli* cells (lanes 1-5) or Jurkat cells (lane 6) and each blot was incubated with antibodies indicated on the top. Bacterial cells express empty control plasmids (lane 1, pGEX; lane 3, pET32H), or the following VAMP7 fragments: cytoplasmic of VAMP7a fused C-terminally to GST in pGEX (lane 2), LD of VAMP7a fused C-terminally to thioredoxin in pET32H (lane 4), C-ter region of VAMP7b fused C-terminally to thioredoxin in pET32H (lane 5). Molecular weight markers are reported. Anti-MD2 and anti-MD3 oAbs are specific to respectively the LD and the CT half of the SNARE motif: thus, they both recognize isoform "a" and "b". Anti-MD4 and anti-MD5 oAbs were designed instead to be specific only to the novel CT domain of the "b" isoform. The two bands in between 25 kDa and 15 kDa markers are compatible with expected MR of VAMP7c (~20 kDa) and VAMP7j (~19kDa) and the band slightly over the 10 kDa marker is likely to correspond to VAMP7d/h (~10.5 kDa). Absence of a band for VAMP7i is not surprising, given that this isoform shows low expression in Jurkat cells and it would be recognized only by the weakest antibody (MD2).

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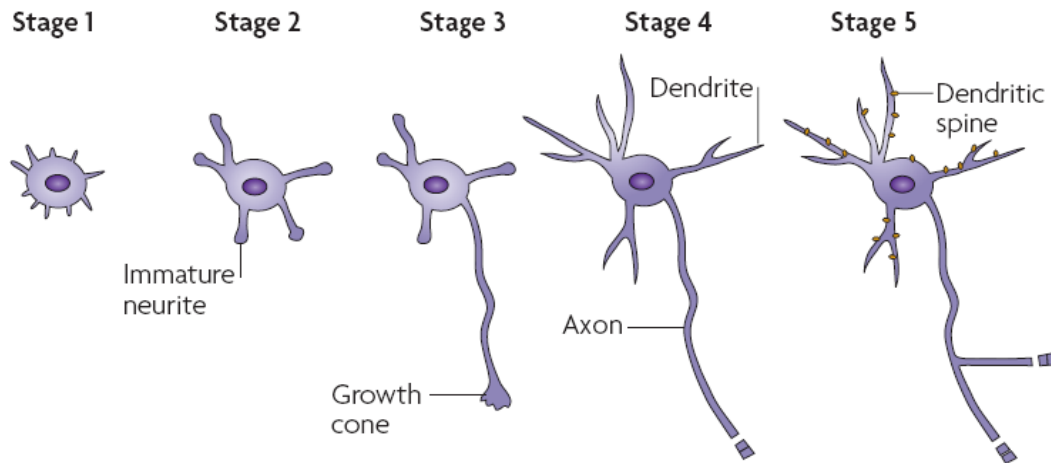
## 1.2 Neuritogenesis

### 1.2.1 Neurons and neuronal development: a general overview

The complexity of brain requires highly specialized cells and a fine regulation of all biological functions. Neurons are polarized cells involved into the synaptic transmission of several, different information required for this regulation. They employ various mechanisms to initiate, maintain and regulate their physiological function. In particular, the formation of neural circuits requires that proper connectivity is established between neurons during development. For this reason, neural precursors need to migrate and appropriately position their cell body for projecting their axon to synaptic target. Failure to achieve correct connectivity results in a dysfunctional nervous system. All neural circuits are defined by the structure of axons and dendrites, with single axons contacting and controlling the function of multiple targets, and individual dendrites integrating inputs from several sources. An early step in the establishment of connectivity involves the growth and guidance of axons. Axon growth is led by the growth cone, a specialized structure that sits at the tip of growing axons. Neuritogenesis and collateral branching (consisting in all processes by which post-mitotic neurons extend long primary axons towards targets to form appropriate connections) are crucial processes for correct wiring of the brain and for the generation of appropriate synaptic networks (Korobova and Svitkina, 2008). In few words, brain development relies on the extension, branching, and connection of axons and dendrites. Thus, deciphering the molecular mechanisms by which all these steps occur is critical for understanding brain ontogenesis, synaptic remodeling, and plasticity. The dynamic series of events characterizing neuronal development were first identified and grouped into five, different stages (Figure 11) on the basis of neuron morphology, in cultured embryonic cortical/hippocampal neurons (Dotti *et al.*, 1988).

Axon and dendrites are complex and regulated structures devoted to several functions. The initial segment of axon is critical for signal transduction at sites where the action potential starts. As a regulatory controller of SC trafficking (Horton and Ehlers, 2004; Allen and Chilton, 2009), axon also acts like a diffusion barrier for membranes and cytoplasm (Song *et al.*, 2009) that becomes competent only when the axon can establish synaptic contact (Dotti and Poo, 2003). A recent work showed that also axons from the central nervous system (CNS) are able to locally synthesize proteins (Taylor *et al.*, 2009) that are principally involved in establishment of the mature synapse (Kundel *et al.*, 2009). This mechanism is particularly active after axon injury (Taylor *et al.*, 2009; Yoo *et al.*, 2010). Nevertheless, the establishment of one axon is not the final destination

of all neurites. Indeed, during neuronal development, other neurites can change their fate and become an axon, if something happens to the others (transection).



**Figure 11. Changes in neuronal morphology during development (from Arimura and Kaibuchi, 2007).**

Five events characterize dynamic rearrangements in neuronal morphology of cortical/hippocampal neurons (Dotti *et al.*, 1988): i) after dissociation from brain, cultured neurons attach to the substrate and then form several thin extension of filamentous actin bundles, called filopodia (stage 1); ii) after several hours in culture, a number of immature neurites with the same length are found (stage 2); iii) after 12h in culture, one of these neurite starts growing, rapidly extending its surface area (stage 3). Neurite extension, as well as breaking of symmetry (polarization) is controlled by positive and negative feedback loops that are able to regulate actin filaments and microtubules, or influence vesicle transports and cargo delivery. The main site at which all these elements act is the neuronal growth cone, found at the tip of each neurite or axon; iv) after a week, these morphological and molecular changes are able to guide axon and dendrite formation (stage 4); v) at this point, dendrites start their maturation and specialization, being short and thick and establishing dendritic components for spine formation (stage 5). Spines are specialized compartments able to receive and elaborate the excitatory synaptic signaling. A mature synapse is finally formed and neurons are now able to form synaptic contacts.

This modulation explains the neuronal plasticity that is known to be retained in stage 4 hippocampal neurons (Goslin and Banker, 1989). Further evidence shows that CNS neurons have some intrinsic capacity for growth after axon injury (Campos *et al.*, 2008; Blesch and Tuszunsky, 2009; Meldolesi, 2011; Lau *et al.*, 2011). Interestingly, molecular mechanism supporting axon repair and growth clearly resemble mechanisms used in uninjured axon, requiring calcium, SNARE and cytoskeletal proteins. Furthermore, non neuronal cells utilize similar mechanisms for membrane repair, suggesting the existence of a common, conserved, basic program (Bloom and Morgan, 2011). Despite the well-known difference between dendrites and axons in both morphology and physiological roles, they share many of the cell body properties. Indeed, a fine cytoskeletal organization, with microtubules of mixed polarity (Burton, 1988; Baas *et al.*, 1989), ER and a Golgi apparatus are found. The ER extends into all dendrites, whereas Golgi is present only in a subset of them (Hanus and Elhers, 2008); lipids and proteins produced in the dendritic ER can be addresses directly to dendrites or back to the perinuclear Golgi network before secretion (Horton and Ehlers, 2003).

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Surface membrane trafficking is very active, including both endosome and endocytic/exocytic pathways (Horton and Ehlers, 2004; Chierregatti and Meldolesi, 2005).

Since establishing these morphological and physiological features is the final hallmark of all neurons, outgrowth occurs anywhere they can live in adequate conditions, *in vivo* and *in vitro*. Immortalized neurosecretory cell lines have been used for extensively studies among neurite outgrowth. Despite their many advantages, they considerably differ from neurons. Neither axon nor dendrites are detectable without induction treatment and in any case, their only undergo to a neuron-like differentiation (not complete differentiation), because they are able to establish axon commitment, but not synaptogenesis (Meldolesi *et al.*, 2011). For these reasons, primary cultures from rat/mouse cortical/hippocampal neurons are more fruitful. Anyway, in both cases, neurite outgrowth takes preferentially place at the growth cone (Pfenninger, 2009).

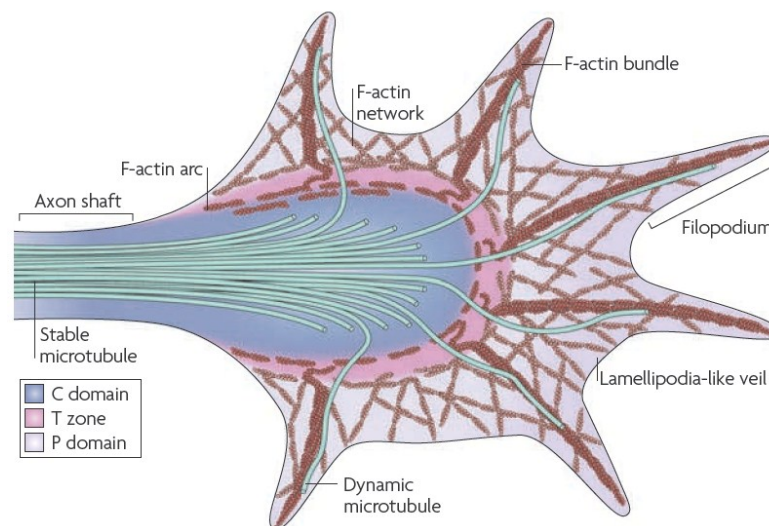
Growth, remodeling, maintenance and repair of axon and dendritic processes rely on both cytoskeleton and membrane trafficking. A great effort in coordinating protein sorting, vesicle budding, transport along the cytoskeleton and vesicle fusion is required (Dent *et al.*, 2007; Pfenninger, 2009; Tsaneva-Atanasova *et al.*, 2009; Gupton and Gertler, 2010). All these SC mechanisms are tightly regulated by members of Rho GTPase family. Furthermore, a linker between vesicles and actin cytoskeleton was identified in Synapsin: its simultaneous interaction with synaptic vesicles and actin positively regulates vesicle mobility (Bloom *et al.*, 2003). It can also bind all other elements of the cytoskeletal component implicated in growth cone and neurite dynamics (Bennet and Baines, 1992). Therefore, the complexity of such mechanisms and recent findings of their mutual interactions (Alberts *et al.*, 2003; Alberts *et al.*, 2006; Dent *et al.*, 2007; Gupton and Gertler, 2010) suggest that a delicate balance between great varieties of molecular elements is needed.

### ***1.2.2 Cytoskeletal components and the growth cone machinery***

Cells extend different PM protrusions at their leading edge that are characterized by involvement of filamentous actin (F-actin)-based structures, determined by the type of actin-associated proteins (Small *et al.*, 2002). The best described structures present in neurons are lamellipodia and filopodia: lamellipodia appear as thin, densely branched meshwork of F-actin behind the protruding cell edge, with fast-growing “barber” filament ends oriented toward the direction of protrusion and migration (Gupton and Gertler, 2007; Dent *et al.*, 2011). In mature neurons *in vivo*, they can extend long distances through the extracellular matrix (ECM),



pulling cells through the tissues (Friedl and Gilmour, 2009). Conversely, filopodia are bundled actin filaments that extend in a finger-like manner, exploring the cell surroundings (Dent *et al.*, 2007; Gupton and Gertler, 2007; Dent *et al.*, 2011). In early steps of neuritogenesis, the neuronal sphere is broken by localized budding (Da Silva and Dotti, 2002) or formation of segmented lamellipodia from an initial broad lamellipodium (Dehmelt and Halpain, 2004). Therefore, neurite initiation requires acting capping protein regulators for filopodia formation (Dent *et al.*, 2007). Since establishment of neuronal circuitry is the landmark in neuronal development, the growth cone present at the tip of axon and dendrites acts as a guide for reaching axon final targets with great precision, due to a particularly sensitivity to multiple sources of spatial information (Bentley and Toroian-Raymond, 1986; Dent *et al.*, 2003). Adhesive molecules and ECM provide defined surfaces to which growth cone receptor can adhere, acting as railways for the growing membrane, whereas diffusible chemotropic cues act as signs for its guidance (Lowery and Van Vactor, 2009). Growth cone shape and functions (protrusion and motility) are driven primarily by the cyclical polymerization and depolymerization of actin filaments that ensure the presence of its actin-and microtubule-rich structures (Figure 12).



**Figure 12. The structure of the growth cone (from Lowery and Van Vactor, 2009).**

The function of the growth cone is determined by its structure. Three different domains can be identified, on the basis of cytoskeletal distribution (Dent and Gertler, 2003): 1) a peripheral (P) domain containing filopodia, and lamellipodia-like veils; 2) a central (C) domain in which microtubules enter from the axon shaft (some of them also explore the P zone, but their spreading is limited by actin arcs), as well as various series of organelles and vesicles; 3) the transition (T) zone, in between these two domains. It is a hemircumferential ring formed by actin arcs, where microtubules are continuous with the axonal stalk (Horton and Ehlers, 2003; Pfenninger, 2009). During neuronal development, all these cytoskeletal components are engaged to drive forward and turn, in a continuous progression of the growth cone through three stages: protrusion, engorgement and consolidation. After binding of a substrate by growth cone receptors, intracellular signaling cascades are activated and a protrusion processes is started, where filopodia and lamellipodia-like veils extend the leading edge. During engorgement there is an advance of the C zone, with a reorientation of actin arcs following direction of the new site of growth and invasion of microtubules. Finally, myosin-II containing actin arcs compress the microtubules into the newly localized C domain and as a consequence, filopodia are not present any more in this region (consolidation).

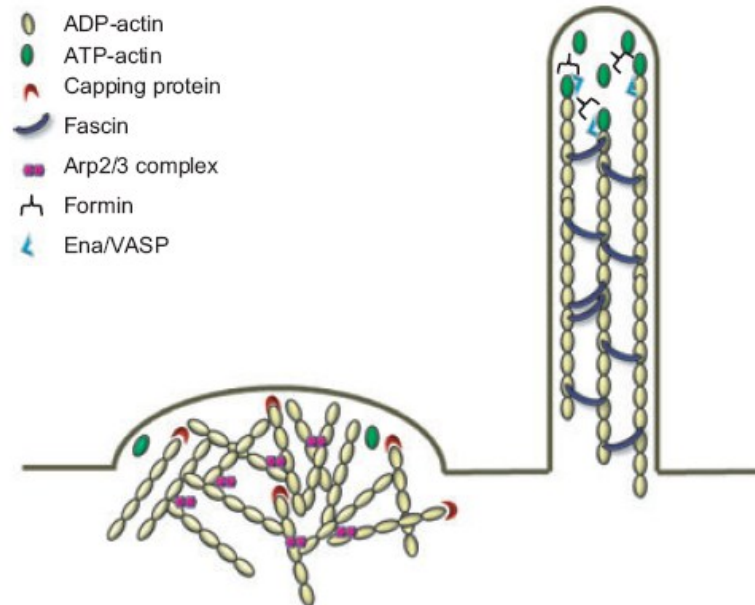
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Actin cytoskeleton remodeling is regulated by a complex repertoire of actin accessory proteins involved in polymerization, capping, severing, branching, bundling, contraction and adhesion to extracellular substrate (Figure 13) (Gupton and Gertler, 2007; Dent *et al.*, 2011). These multiple regulators include the actin nucleators actin related protein (Arp) 2/3 complex and the formins, and the F-actin polymerization factor enabled/vasodilator-stimulated phosphoprotein (Ena/VASP). Arp 2/3 complex is necessary for lamellipodium formation (Small, 1994) and filopodium initiation; its activation is accomplished primarily by Wiscott-Aldrich syndrome (WASP/WAVE) proteins. Actually, the role of this complex in axon outgrowth is difficult to completely define. It is likely that both the guidance cues Semaphorin (Sema) 3A and netrin utilize this actin assembly pathway. Conversely, Ena/VASP proteins are barbed-end binding proteins that promote assembly of long, sparsely branched actin filament networks and filopodial/lamellipodial protrusion; furthermore, these proteins have important roles in growth cone guidance downstream of both attractive and repulsive cues (e.g. netrin). Therefore, the precise nature of Ena/VASP function and its molecular mechanism remain controversial (Gupton and Gertler, 2007; Dent *et al.*, 2011). Some works suggest both its interaction with Arp2/3 during neurite outgrowth (Goldberg *et al.*, 2000; Gupton and Gertler, 2007), whereas evidence also promotes the idea of separate functions (Applewhite *et al.*, 2007; Gupton and Gertler, 2010). Furthermore, failure to form filopodia in Ena/VASP-deficient neurons induces a neurite initiation defect and blocks axon fiber tract formation in the cortex (Kwiatkowski *et al.*, 2007; Gupton and Gertler, 2010).

Two alternative models for actin polymerization have been suggested: the convergent elongation and *de novo* nucleation model (Gupton and Gertler, 2007). They could act at the same time, even responding to distinct sets of conditions, such as the abundance of specific cytoskeletal components, different signaling pathways, and diverse composition of the ECM. In the convergent elongation model, Arp2/3-seeded actin filaments are elongated by factors, such as Ena/VASP family proteins, that also protect them from capping, and are assembled into filopodial bundles by fascin (Ridley, 2011). On the contrary, in the *de novo* nucleation model, linear filament nucleators and elongators, such as formin and Ena/VASP family proteins, are assembled into submembranous complexes, promoting the processive elongation of parallel actin filaments, which are cross-linked into filopodial actin bundles (Mattila and Lappalainen, 2008; Menna *et al.*, 2011). Each actin regulator is controlled by several signaling molecules, usually including a Rho GTPase, membrane phospholipids, and protein phosphorylation.

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The balance of other actin-binding proteins also contributes to the length of actin filaments in the lamellipodium.



**Figure 13. Regulation of the equilibrium between lamellipodia and filopodia by actin-binding proteins (from Menna *et al.*, 2011).**

Filopodial initiation and extension require the coordinated activities of actin-capping and anti-capping proteins. They regulate the equilibrium between lamellipodia and filopodia, first maintaining the lamellipodial structure through the diverse action of capping and branching proteins, then promoting filopodia formation by their removal and the concomitant activity of actin-cross-linking (bundling) proteins. This step is required for filament elongation.

For example, Cofilin mediates the severing of existing cortical actin filaments, which generates new barbed ends and hence new filaments, to which the Arp2/3 complex can then bind and stimulate branching (van Rheenen *et al.*, 2009). Furthermore, several actin nucleators directly interact with microtubules, including the Diaphanous-related formin (mDia) proteins, Spire, and WHAMM (WAS protein homolog associated with actin, Golgi membranes, and microtubules). mDia proteins can also generate filopodia independently from N-WAS, the Arp2/3 complex, and lamellipodia (Takenawa and Suetsugu, 2007). Interestingly, expression of mDia2 or the motor protein myosin X can also rescue neuritegenesis in Ena/VASP-null neurons. Furthermore, neurons cultured on ECM component Laminin (LN) promotes the formation of filopodia-like actin-rich protrusions (Dent *et al.*, 2007) due to activation of VAMP7-mediated exocytosis driven by activation of Arp2/3 that rescue neuritegenesis (Gupton and Gertler, 2010). Thus, multiple mechanisms seem to drive filopodia formation, in relation to the expression profile of a particular cell type; similarly cell architecture may influence the mechanism used.

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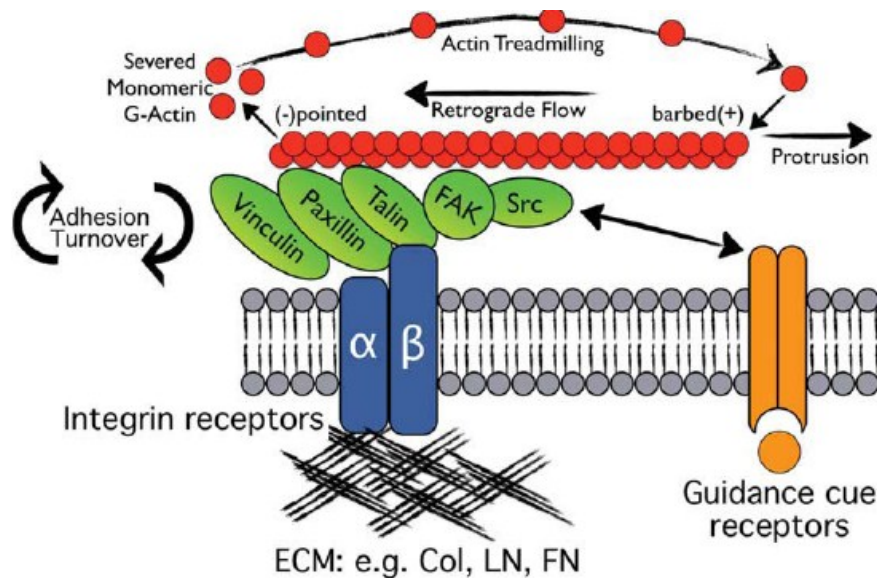
During development, filopodia emerging from growing axons, which are already characterized by the presence of actively recycling synaptic vesicles, are representative precursors of presynaptic boutons (Matteoli *et al.*, 2004). They seem to guide the coordinated growth of presynaptic and postsynaptic partners (Dailey and Smith, 1996; Ziv and Smith, 1996; Okabe *et al.*, 2001; Evers *et al.*, 2006). Conversely, dendritic filopodia may serve as precursors of new spines in the context of activity-dependent synaptogenesis (Knott and Holtmaat, 2008).

#### 1.2.2.1 Influence of extracellular matrix components on cytoskeletal remodeling during neurite outgrowth

ECM provides the physical microenvironment in which cells live, a substrate for their anchorage and a tissue scaffold. It consists of a complex assembly of many proteins and polysaccharides, forming an elaborate meshwork within tissues. The primary components are fibrous structural proteins (e.g. collagens, Col; LNs; fibronectin, FN; vitronectin and elastin), specialized proteins [e.g. growth factors, small matricellular proteins and small integrin-binding glycoproteins (SIBLINGS)] and proteoglycans, but the precise composition varies from tissue to tissue. Within a single tissue, the ECM is constantly being remodeled specially during embryonic development, when it is particularly active in transmitting environmental signals to cells and in guiding their migration for tissue morphogenesis. In the nervous system, neuron precursor cells must migrate, differentiate and establish proper connections with defined targets. Thus, ECM proteins are potent mediators of survival and axonal growth and guidance *in vivo* and *in vitro*, exhibiting either attractive or repellent guidance cues.

Primary neuron cultures require the choice of the best substrate, as it can profoundly affect cellular response (Geiger *et al.*, 2001; Myers *et al.*, 2011). Indeed, the growth cone expresses a variety of receptors at the cell surface and even at the tips of filopodia (O'Donnell *et al.*, 2009), which bind ECM ligands (Figure 14). ECM components, in particular LNs, are required for survival (Mitchell *et al.*, 2001; Chen *et al.*, 2009; Myers *et al.*, 2011), migration and differentiation of neurons in the developing nervous system, when their expression is both spatially and temporally regulated (Nurcombe, 1992) as well as in the remodeling of both normal and diseased adult brain (Rauch, 2004). LNs are heterotrimer with a characteristic cruciform structure, composed of a variety of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains (Timpl, 1996), of which  $\alpha 2$ ,  $\beta 1$ , and  $\gamma 1$  are highly expressed in the hippocampus (Hagg *et al.*, 1997). There are a number of LN isoforms that vary in tissue distribution and ability to promote neuritogenesis (Plantman *et al.*, 2008).

LN are particularly expressed in the cerebral cortex and play important roles in neuronal plasticity, degeneration and regeneration (Chen and Strickland, 2003; Yin *et al.*, 2003). Many integrin subunits are expressed at high levels during neuronal development, functioning in migration and axon extension on a variety of ECM proteins, in several CNS regions. LN is the primary ECM protein produced by meningeal fibroblasts (Miner and Yurchenco, 2004), first source of



**Figure 14. Schematic representation of key molecular components of the growth cone on the ECM (from Myers *et al.*, 2011).**

Receptors function as adhesive contacts with the surrounding environment, but also activate biochemical signals within growth cones. Together with cell adhesion molecules (CAMs), ECM proteins are involved in regulating direction and extension of neurites, along specific pathways (Kiryushko *et al.*, 2004), activating signals that positively or negatively influence axon outgrowth, and if locally generated, graded signals within growth cones promote axon turning. Adhesions are quite heterogeneous, but they share two common features: they are mediated by Integrins (a group of heterodimeric TM adhesion receptors) and interact with the actin cytoskeleton at the cell interior (Geiger *et al.*, 2001; Daley *et al.*, 2008). CAMs such as Integrins and CAMs of the immunoglobulin superfamily (IgCAMs) commonly follow a recycling pathway from the PM (Kamiguchi and Lemmon, 2000). One of those, L1-CAM seems to be particularly involved in neurite outgrowth and shows an interested linkage with VAMP7-mediated exocytosis (Alberts *et al.*, 2003; Alberts *et al.*, 2006). When integrin receptors bind to ECM elements (Col; LN; FN) (Buck and Horwitz, 1987), they become activated, leading to the assembly of multiple scaffolding proteins, such as talin, paxillin, and vinculin to the cytoplasmic tail; integrin receptor clustering directly active FAK and Src. An intracellular signaling cascade is then initiated by phosphorylation of downstream molecules that control cytoskeleton dynamic and promote microtubule assembly to enhance elongation of neuronal processes (Brakebusch and Fässler, 2003; Yoshimura *et al.*, 2005). In growth cones, integrin function is regulated not only by ligand binding, but it also appears to be regulated by receptor trafficking (Ezratty *et al.*, 2009). This has relevant importance, considering that directed insertion or retrieval of integrin receptors may locally amplify signals activated by gradients of guidance cues. These receptors connect cells to their environment at distinct contact points. Structures such as focal contacts (FCs) and focal adhesions (FAs) are highly complex and dynamic macromolecular assemblies that link the actin cytoskeleton to ECM proteins through integrin receptors. This mechanism can be strongly influenced by axon guidance factors (Mayers *et al.*, 2011) through binding and activation of FAK and Src. Cross-talk through FAK/Src signaling modulates adhesion assembly and turnover, as well as regulation of the actin cytoskeleton.

ECM in region lining the cortex. LN is one of the components of the pia basement membrane, whose integrity is assembled and maintained by most of cell adhesion molecules. In the cerebral cortex, LN of the pia basement membrane

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regulates neuronal migration through the radial glia scaffold, while neuronal LN regulates neuronal migration probably by promoting neurite extension. LNs produced by migrating neurons are hypothesized to be deposited around neurons, neuronal processes, and radial glial fibers. They induce extension of the leading process of migrating neurons toward the pia, and mediate radial glia-neuron interactions (Chen *et al.*, 2009). Although previous studies suggested that LN primarily functions in the pial basement membrane (Halfter *et al.*, 2002), evidence shows that LN also supports migration and process extension within the cortical plate. Cortical neurons are packed tightly and the cortex itself contains only small amounts of both LN and other type of ECM components; so cortical neurons are normally cultured without LN. Therefore, Chen and colleagues demonstrated that these neurons secrete their own substrate to support axon extension in an autocrine manner (Chen *et al.*, 2009).

Neuritogenesis is normally mediated by Ena/VASP protein and VAMP2-mediated exocytosis; anyway, in cortical neurons, another pathway for neurite elongation was found, driven by Arp2/3 and VAMP7-mediated exocytosis (Gupton and Gertler, 2010). In this case, LN is the extracellular signal for molecular switching: attachment to LN rendered Ena/VASP and VAMP2 dispensable. LN, mDia2 and Myosin X are capable of rescuing neuritogenesis in Ena/VASP null neurons (Dent *et al.*, 2007), but LN can specifically activate this second pathway (Gupton and Gertler, 2010). Furthermore, loss of LN in a large group (not all of them) of cortical neurons impairs neuronal morphogenesis and migration. This is probably due to differences in actin structures (architecture) formed by this pathway (Dent *et al.*, 2007.).

The processes of neuronal outgrowth and guidance have typically been studied in classic 2D cell culture systems that do not recapitulate topographical cues present in the *in vivo* ECM. A new study on advanced 3D nanopatterns coated by LN provided evidence for the presence of two distinct filopodia populations at the growth cone of neurons growing, suggesting new insights concerning the physiological sensing of geometric ECM cues (Jang *et al.*, 2010; Pertz, 2011).

#### *1.2.2.2 Diffusible chemotropic cues that drive the growth cone*

The role of the growth cone in precisely guiding neurites and axon at their final targets with is due to a particular sensitivity to multiple, attractive and repulsive sources of spatial information (Bentley and Toroian-Raymond, 1986; Dent *et al.*, 2003). These sources secrete guidance cues able to activate specific receptors, causing growth cone migration towards or away (Yoshikawa and Thomas, 2004).

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Depending on the receptor repertoire and on the signaling machinery expressed by a given neuron, these cues trigger multiple signaling cascades by modulating adhesion to the ECM and other cells. In response to guidance cues, asymmetric rearrangement of cytoskeleton (F-actin and microtubules accumulation) occurs in the growth cone, driving its migration in that direction (Zhou and Cohan, 2004; Menna *et al.*, 2011). At the same time, adhesive molecules and ECM provide defined surfaces to which growth cone receptors can adhere acting as railways for the growing membrane (Lowery and Van Vactor, 2009). As migrating axons encounter different environments, new signals may negatively regulate these steps to control morphological changes that occur during turning, branch formation, and synaptogenesis (Moore *et al.*, 2008). Key signaling pathways downstream of guidance receptors include the Rac GTPase and phosphoinositide 3-kinase signaling pathways (Ming *et al.*, 1999; Lundquist, 2003). Integration of all these signals can depend on the focal adhesion kinase (FAK), acting as a biosensor molecule (Chacón and Fazzari, 2011) that may link different, external stimuli to multiple intracellular pathways to control the maturation of adhesive structures, cell shape and motility (Liu *et al.*, 2004).

Asymmetry has also been observed in other processes, as exocytosis, calcium signaling, and protein translation, suggesting that they are active players in growth cone guidance (Gomez and Zheng, 2006; Tojima *et al.*, 2007; Hutchins and Kalil, 2008). Indeed, they can control receptor activation, clustering, removing from cell surface by recycling vesicles (Sann *et al.*, 2009) and modulate linkage with the cytoskeleton. In addition, signals generated by integrin receptors likely reciprocally influence responses to other axon guidance cues. This cross-talk between signals generated by adhesive integrin receptors and axon guidance cue receptors can provide great versatility for pathway selection by developing neurons.

Four conserved families of axon guidance cues, the netrin, Sema, ephrin, and Slit proteins, mediate guidance effects via receptors of either the Deleted in Colorectal Cancer (DCC) or UNC5, neuropilin/plexin, Eph, and Robo families, respectively (Nakamoto *et al.*, 2004). As previously described, some guidance cues can directly act on integrin receptors, whereas others indirectly influence integrin-associated adhesion complex proteins, in a continuous cross-talk between different stimuli. For example, neurotrophins (Staniszewska *et al.*, 2008), Wnt5a (Kawasaki *et al.*, 2007), and netrin (Yebra *et al.*, 2003) each can bind specific integrin heterodimers. A novel axon guidance molecule, draxin, shares no sequence homology with other known guidance molecules (Islam *et al.*, 2009); nevertheless, it can bind multiple netrin-1 receptors and its high-affinity interaction with DCC is

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required for the inhibitory effect on the cortical and olfactory bulb (OB) neurite outgrowth (Ahmed *et al.*, 2011).

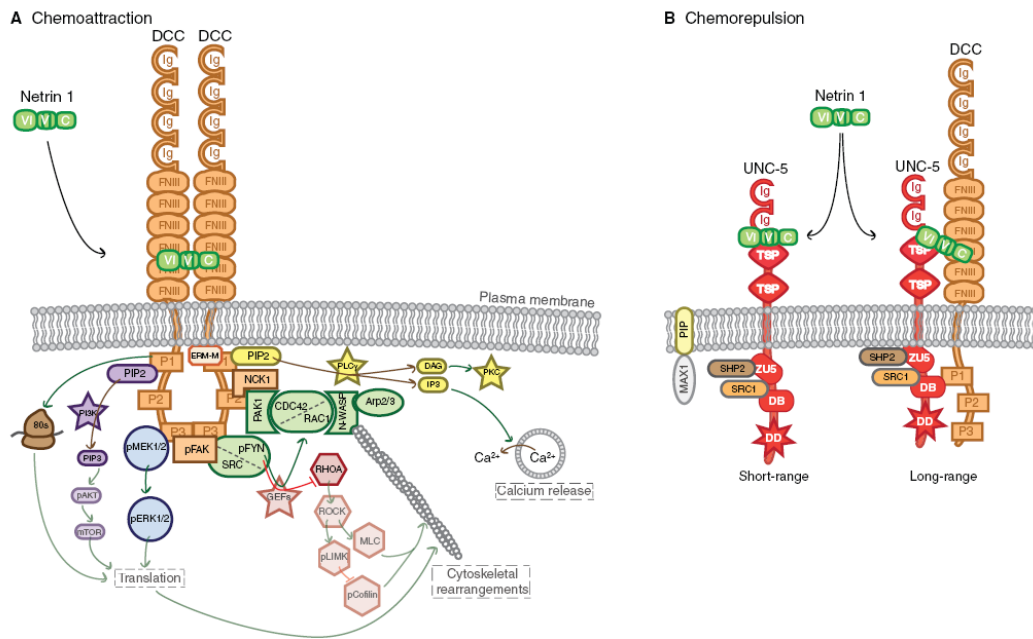
Netrins are the most characterized chemotropic cues that belong to the family of extracellular, LN-related proteins (Yurchenco and Wadsworth, 2004). All netrins are composed of ~600 amino acids and their NT sequences are homologous to domains VI and V found at the NTs of LNs. In mammals, three secreted netrins, netrin-1, 3 and 4, and two membrane-tethered glycosylphosphatidylinositol (GPI)-linked netrins, netrin G1 and G2, have been identified. Among them, netrin-1 is expressed in regions of both the developing and adult nervous systems, including the optic disc, forebrain, cerebellum and spinal cord (Livesey and Hunt, 1997). It functions not only in outgrowth and attraction (Métin *et al.*, 1997) but also in migration (Stanco *et al.*, 2009) and in branch formation (Dent *et al.*, 2004; Tang and Kalil, 2005) in cerebral cortical axons. Netrin-1 is also highly expressed in various tissues outside of the nervous system, including the developing heart, lung, pancreas, intestine and mammary gland (Shin *et al.*, 2007; Zhang and Cai, 2010). Its principal receptor in chemoattraction, DCC is a type I single pass TM protein that mediates netrin-1-dependent axon outgrowth (Figure 15). Direct and independent binding of DCC and integrin receptors also supports cell adhesion (Yebra *et al.*, 2003; Shekarabi *et al.*, 2005).

Furthermore, integrin signaling appears to modulate netrin function downstream of DCC receptors, directing the activation of FAK and Src (Höpker *et al.*, 1999). Other studies provided evidence of a netrin-1 signaling mediated by membrane depolarization (Bouchard *et al.*, 2008), where DCC is recruited to the PM via a mechanism that requires PKA, PI3-Kinase, and PKC pathways and this enhances axon growth with a mechanism that requires VAMP7-mediated exocytosis (Cotrufo *et al.*, 2011). Localized binding of netrin to its receptors can also activate translation and function to restrict new protein synthesis to specific subdomains of a cell or growth cone, acting as an anchor for the translation machinery to the PM (Tcherkezian *et al.*, 2010), thus spatially restricting protein synthesis (Sun *et al.*, 2011).

### **1.2.3 Membrane expansion and exocytosis**

PM is the largest neuron's organelle. *In vivo*, its generation occurs in four different moments: during *de novo* outgrowth of a neurite tipped by a growth cone; during axon elongation; during the formation of collateral sprout; during regeneration of a severed neurite (Pfenninger, 2009). Membrane expansion commonly requires SC trafficking of different type of vesicles and exocytosis (Figure 16).





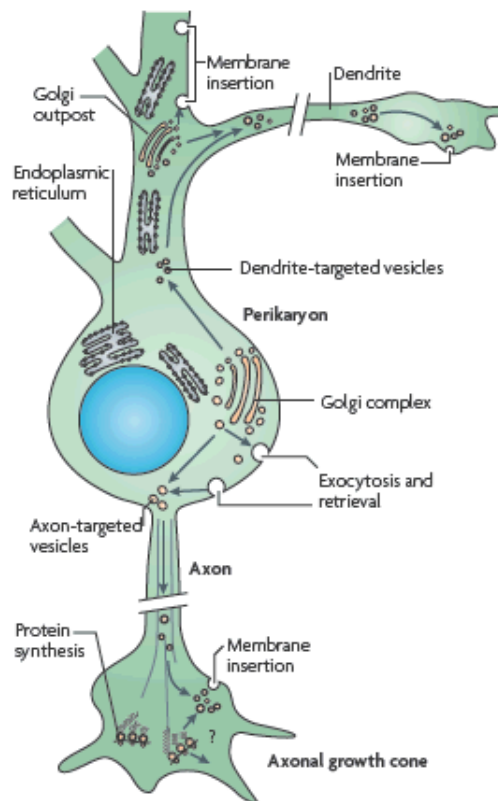
**Figure 15. Netrin signaling cascade (from Lai Wing Sun *et al.*, 2011).**

(A) During chemoattraction, netrin-1 binds DCC and triggers the homodimerisation via the cytoplasmic domain (Stein *et al.*, 2001; Matsumoto and Nagashima, 2010), for constitutively activating the bound to NCK1 and FAK. This initiates the recruitment of a number of intracellular signaling components. Recent studies have shown that netrin-1 can activate multiple downstream signal transduction molecules that regulate cytoskeletal dynamics and process extension, including Src and members of the Rho GTPase family, the release of  $Ca^{2+}$  stores, protein translation and, ultimately, the rearrangement of the actin cytoskeleton (Huber *et al.*, 2003; Lai Wing Sun *et al.*, 2011). (B) During chemorepulsion, netrin-1 signals activate UNC5/DCC heterodimers (Keleman and Dickson, 2001), which are thought to facilitate long-range responses by increasing the sensitivity to relatively low netrin concentrations, or UNC5 (Hong *et al.*, 1999) in the absence of DCC to mediate relatively short-range repellent responses. Signal transduction components illustrated as 'faded' are speculative and direct evidence for their involvement has not been obtained. 80s, eukaryotic ribosomes; Arp2/3, complex of the actin-related proteins ARP2 (ACTR2) and ARP3 (ACTR3); CDC42, cell division cycle 42; DAG, diacylglycerol; ERM-M, ezrin/radixin/moesin and merlin protein family; GEFs, guanine exchange factors; IP3, inositol 1,4,5-triphosphate; MAX1, motor axon guidance PH/MyTH4/FERM domain cytoplasmic protein; MLC, myosin light chain; mTOR, mammalian target of rapamycin; NWASP, neuronal Wiskott-Aldrich syndrome protein; NCK1, non-catalytic region of tyrosine kinase adaptor protein 1; pAKT, phosphorylated RACalpha serine/threonine protein kinase; pCofilin, phosphorylated cofilin; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; pFAK, phosphorylated focal adhesion kinase; pFYN, phosphorylated Src family kinase FYN; pLIMK, phosphorylated LIM domain kinase 1; pMEK1/2, phosphorylated mitogen-activated protein kinase kinase 1/2; PAK1, p21-activating kinase 1; PI3K, phosphatidylinositol-3 kinase; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol (4,5) bisphosphate; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PKC, protein kinase C; PLC $\gamma$ , phospholipase C $\gamma$ ; RAC1, ras-related C3 botulinum toxin substrate 1; RHOA, Ras homologue gene family member A; ROCK, RhoA kinase; SHP2, Src homology region 2 domain-containing phosphatase 2; SRC, tyrosine kinase sarcoma.

As extensively discussed in the previous section, general mechanisms underlying membrane traffic can be divided into four essential steps - that include vesicle budding, transport, tethering and fusion (Bonifacino and Glick, 2004) - and are regulated by Rab GTPases (Grosshans *et al.*, 2006) and by SNARE proteins (Jahn and Scheller, 2006). Transport of new components to the PM is crucial for the expression of newly synthesized membrane proteins (CAMs, growth factor, receptors), and all proteins and lipids necessary for membrane expansion, motility of neuronal precursors, neurite outgrowth, and target recognition. In addition, recycling of these proteins plays a role in controlling their density at the PM and

may be involved in vectorial membrane traffic during neurite outgrowth (Kamiguchi and Yoshihara, 2001).

Neuritogenesis and growth cone dynamics are dependent on a regulated phenomenon of SNARE-mediated exocytosis (Pfenninger et al, 2003; Laurino *et al.*, 2005) of cytoplasmic vesicles that are distinct from the canonical neurosecretory vesicles endowed with VAMP2 (Bonanomi *et al.*, 2006). Indeed, growth cone motility and responsiveness to extracellular cues is influenced by localized insertion of membrane components driven by asymmetrical exocytosis on the peripheral (P)-domain, at the site of the attractive  $Ca^{2+}$  signals (Sabo and McAllister, 2003; Alberts *et al.*, 2006; Tojima *et al.*, 2007; Pfenninger, 2009). An example is the DCC insertion for netrin-1 stimulation (Tojima *et al.*, 2007; Hutchins and Kalil, 2008). Generation of PM is also due to and necessary for ionic current propagation within mature neurons.



**Figure 16. Sites of synthesis and plasmalemma insertion of membrane components in a growing, polarized neuron (from Pfenninger, 2009).**

The perikaryon is the primary site of membrane synthesis, producing plasmalemmal precursor vesicles (PPVs) for export into the axon and dendrites. During axon outgrowth a selective membrane expansion occurs primarily at the growth cone. Therefore, an uniform insertion also occurs, followed by retrieval of vesicles from domains where they do not belong. Transcytosis (plasmalemmal insertion at the perikaryon and dendrites, followed by selective retrieval and targeting of endocytic vesicles to the axon) is another mechanism for membrane fusion. Despite the presence of a basic secretory pathway shared with other cell types, membrane insertion in neurons is more complex due to several membrane domains to which the different types of PPV have to be appropriately targeted. Furthermore, axons and dendrites independently regulate their membrane insertion.

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All SNAREs not directly involved in neurotransmitter exocytosis are either brain-enriched or have distinct neuron-specific functions, suggesting a peculiar role of subcellular trafficking in neurons. Syntaxins 12/13 regulates glutamate receptor recycling via its interaction with neuron-enriched endosomal protein of 21 kDa (NEEP21). Ykt6 is highly enriched in the cerebral cortex and hippocampus and is targeted to a novel compartment in neurons. Syntaxin 16 has a moderate expression level in many tissues, but is rather enriched in the brain (Wang and Tang, 2006). Various Q-SNAREs are involved in this process: SNAP23/25, Syntaxin 3 (Darios and Davletov, 2006), syntaxin 13 (Hirling *et al.*, 2000), syntaxin 6 (Kabayama *et al.*, 2008). Recently, VAMP4 was linked to another type of exocytic vesicle, enlargesome (Cocucci *et al.*, 2008), seen in at least three types of nerve cells. This organelle sustains a new, very rapid form of neurite outgrowth, induced by the activation of the small GTPase Rac1 (Takefuji *et al.*, 2007; Rachetti *et al.*, 2009). Similar regulated secretory-vesicle exocytosis also occurs in mature neurons, during neurotransmitter release (Pfenninger, 2009) or in dendrite physiology. Although axon protein trafficking seems to be more complex than dendrite one, they have similarities in the mechanism that regulate exocytosis and membrane expansion (Sann *et al.*, 2009). Recently, a study on Vti1a that is known to associate with VAMP4, syntaxin6 and syntaxin13 for early endosome regulation (Antonin *et al.*, 2000) revealed the presence of separated vesicle pools at the mature synapse (Ramirez *et al.*, 2012).

In addition to the secretory pathway, recycling pathways from the PM to late endosomes can regulate neurite outgrowth. Several studies have also revealed coordinate activity among endosomes, cytoskeleton and the exocytosis complex in membrane addition and neurite dynamics. (Sann *et al.*, 2009). Anyway, once an axon has reached the final target, its growth ends and the growth cone machinery is disassembled and replaced by a presynaptic terminal. Thus, further membrane insertion in the processes of a mature neuron occurs with a different mechanism (Pfenninger, 2009).

#### 1.2.3.1 SNARE VAMP7 and its relation to VAMP2 in neuronal development

The above described complex mechanisms explain the difficulty in having a complete vision of all proteins, complexes and pathways that occur in neurite outgrowth. This becomes quite clear when considering v-SNAREs responsible for exocytosis in early steps of neuronal developments. A great effort was made by a

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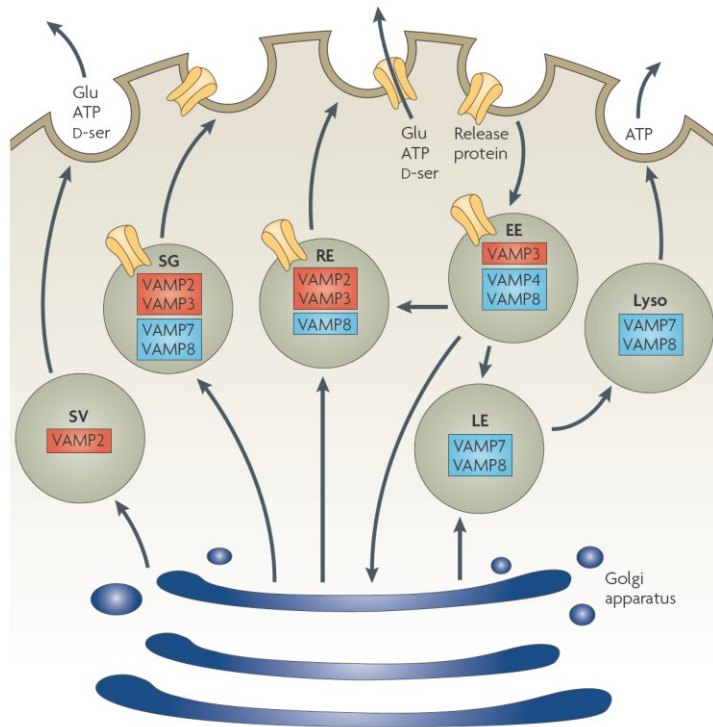
many researchers in order to understanding such mechanisms; therefore, a lot of points are controversial or completely missing.

VAMP7 is known to mediate the fusion of vesicle with both the apical and axonal plasma membranes (Galli *et al.*, 1998; Lafont *et al.*, 1999). Heterogeneous vesicles commonly characterized by the presence of VAMP7 are seen in the growth cone of both dendrites and axons. This actin-dependent accumulation of the VAMP7 compartment in the growth cone periphery seems to represent a mechanism to integrate actin- and membrane-dynamics to support directed growth (Alberts *et al.*, 2006; Pfenninger, 2009). During neurite outgrowth, VAMP7 displays an essential role for axonal elongation to full extent (Chaîneau *et al.*, 2009; Sato *et al.*, 2011), likely being the v-SNARE involved in exocytosis processes for PM addition. Indeed, two artificial constructs (LD and  $\Delta$ LD) were used as tools for studying VAMP7 role in neuronal development, showing respectively an inhibition and an increase of neurite outgrowth (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001). LD construct can also block exocytosis in developing neurons, confirming the importance of this mechanism for neuronal development. In addition, recent evidence indicates a well-defined neuritogenesis pathway for VAMP7-mediated exocytosis: cortical neurons lacking Ena/VASP protein are rescued in neurite growth when growing on LN or VAMP7 is overexpressed on Poly-D-Lysine (PDL) coating. LD construct display its inhibitory role on VAMP7 pathway (wild type neurons are insensitive to it when cultured on PDL that switch the neuritogenesis pathway) (Gupton and Gertler, 2010). A new VAMP7 partner, Varp (Vsp9 domain and ankyrin-repeat-containing protein) is a positive regulator of neurite outgrowth, controlling the endosomal trafficking of beta-integrins (Pellinen *et al.*, 2006). It is also a GEF for Rab21 (Zhang *et al.*, 2006), implicated in neurite elongation (Burgo *et al.*, 2009). Therefore, a recent work on VAMP7 knockout mice reported no particular differences between ko and wt brains (Sato *et al.*, 2011), highlighting the complexity of mechanisms involved in neuritogenesis.

Although the SNARE brevin VAMP2 is well known for being involved in neurotransmitter release (Schiavo *et al.*, 1992), recent evidence revealed a possible interesting role also in neurite outhgrowth. VAMP2 is usually considered the dominant v-SNARE for synaptic vesicle exocytosis, but VAMP7 was also seen to interact with the dominant t-SNAREs SNAP25 and Stx1 involved in transmitter release (Alberts *et al.*, 2003; Cotrufo *et al.*, 2011). It seems quite apparent that these two SNAREs act in different manner, stages and places during the entire neuron lifetime. Nevertheless, a precise role for them is still unclear. In general, it is known that VAMP7 is a less active but more controlled R-SNARE compared to

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VAMP2 and that the two proteins do not co-localize (Alberts *et al.*, 2006; Gupton and Gertler, 2010). Early data reported that costriatal neurotoxins cleaving VAMP2 had no effect on the development of neurons in culture (Grosse *et al.*, 1999), suggesting no implication of synaptobrevin 2 in this mechanism. In addition, both VAMP2 and SNAP25 knockout mice were found to develop a normal brain (Washbourne *et al.*, 2002). Alberts and colleagues defined the presence of a differential regulation in the compartmentalization of VAMP7 and VAMP2 vesicles, in immature neurons: VAMP2 silencing did not produce any change in neurite elongation; furthermore only VAMP7 was found in neuronal growth cones in an actin-dependent manner (Alberts *et al.*, 2003). Nevertheless, in the same year, Kimura and coworker found that overexpression of VAMP2 promotes neurite outgrowth (Kimura *et al.*, 2003). Other data showed the involvement of VAMP2 in growth cone guidance: Ca<sup>2+</sup>-induced attraction, but not repulsion, requires VAMP2-mediated exocytosis, in a mechanism involving the acute modification of molecular compositions and the continuous expansion of the plasmalemma in a spatially restricted manner (Tojima *et al.*, 2007). More recently, silencing experiments on both newborn mice neurons and PC12 cells again showed no implication of VAMP2 in neurite elongation (Burgo *et al.*, 2009). When considering netrin-1 chemoattraction during neurite protrusion, DCC receptor was found to associate specifically with the SNARE proteins Sytx1 and VAMP7, while VAMP2 is dispensable (Cotrufo *et al.*, 2011). Conversely, recent results seem to reveal a relationship between these two proteins, relating their action to the extracellular environment (Gupton and Gertler, 2010). All these experiments point out a complex scenario in which multiple stimuli (ECM component, guidance cues) can recruit different machinery for undergoing neuritogenesis; nevertheless components of these machineries can also interact to each other. The activation of these pathways may depend on the capacity and properties of distinct axonal guidance receptors to co-associate with different SNARE proteins. This mutual presence in neuron's life is apparent also in the mature synapse, where VAMP7 is specifically enriched in the resting pool vesicles, while VAMP2 in the recycling pool. Experiments performed with a  $\Delta$ LD construct showed an increase in the spontaneous releases of VAMP7 that affects also VAMP2 exocytosis, suggesting a specific presence in a subset of synaptic vesicles (Hua *et al.*, 2011). This was recently confirmed by similar experiments of Ramirez and coworkers (Ramirez *et al.*, 2012), suggesting that non canonical SNAREs represent an attractive possibility to mediate specific forms of neurotransmission (Figure 17).



**Figure 17. VAMPs in membrane trafficking (from Hamilton and Attwell, 2010).**

Different type of VAMPs can share similar pathways in membrane trafficking. The  $\text{Ca}^{2+}$ -dependent exocytosis of neurotransmitter containing small synaptic- like vesicles (SVs) and larger secretory granules (SGs) is TeNT- sensitive due to the presence of VAMP2 and/or VAMP3. However, VAMP3 is also present on early endosomes (EEs) and recycling endosomes (REs), and constitutive exocytosis of proteins from the RE to the plasma membrane is often  $\text{Ca}^{2+}$  modulated and mediated by VAMP2. Late endosomes (LEs) and lysosomes (Lyso) express TeNT- insensitive VAMPs, like VAMP7 (shown in blue). Proteins that mediate non- exocytotic transmitter release (shown as orange 'channels') can be inserted into the plasma membrane through the constitutive pathway via EEs and REs and possibly via the regulated exocytosis pathway through SGs. Recent data confirm that these molecular pathways need more investigation for being completely unraveled.

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## AIM OF THE PROJECT

Since the complexity of SC trafficking machinery of which SNAREs are important regulators and all the intricate mechanisms present in the developing brain, my work aims at elucidating the role of human VAMP7 and its LD in this scenario. In particular, it follows three subsequent steps:

- the characterization of VAMP7 splice variants, after the discovery of an AS mechanism that rules SYBL1 gene;
- the characterization of LD role as SC targeting determinant (or co-determinant) in relation to other VAMP7 domains and its open-closed conformation;
- the characterization of VAMP7 splice variants and their mechanisms during neuronal differentiation





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# METHODS

## **Human cell culture and neuronal differentiation**

The following human cell lines were used for VAMP7 isoform detection by using real time RT-PCR: SH-SY5Y (neuroblastoma); C63 (skin fibroblasts); HepG2 (hepatocellular carcinoma); HeLa (cervical carcinoma); Jurkat (T-cell leukaemia). All cells were grown at 37°C in a humidified atmosphere, 5% CO<sub>2</sub>. SH-SY5Y and Jurkat cells were grown in the following media (all reagents from Invitrogen), supplemented with Glutamax and 10% heat-inactivated fetal bovine serum: SH-SY5Y, 1:1 DMEM+Ham's F12 supplemented with gentamicin (50 µg/ml); Jurkat, RPMI supplemented with 10mM HEPES and PEN-STREP. C63, HepG2 and HeLa cells were kindly provided by colleagues from other laboratories.

Neuronal development of SH-SY5Y cells was induced by all-*trans* retinoic acid (RA) (Sigma-Aldrich) (Lopez-Carballo *et al.*, 2002; Merrill *et al.*, 2004) as follows: 6-4-2 10<sup>5</sup> cells were seeded on 24mm Poly-L-Lysine (1µg/ul) (Sigma Aldrich) + gelatine (0.005%) coated coverslips placed in 6-well plates and treated with 10 µM RA 24 h after seeding.

## **Primary mouse culture**

Cortex and hippocampus were isolated from day 16 (E16) black6 mouse embryos, following guidelines approved by the UCSF IACUC. They were dissociated in Dissection medium (DM) composed by Hanks' Balanced Salt Solution (HBSS) and HEPES, containing trypsin/EDTA (Gibco/Invitrogen) for 20 min at 37°C, washed in DM (Gibco BRL), triturated, resuspended in fresh Plating medium (Neurobasal medium containing 2% B27 supplement, 1% glutamine, 10% FBS), and prepared for transfection (electroporation) or seeded, according to the experimental procedure.

## **Gain-of-function experiments in neuronal cells**

Two protocols were used to follow up the effect of VAMP7 isoform overexpression on neuronal differentiation:

- Protocol A: SH-SY5Y cells are transfected by Lipofectamine<sup>TM</sup>2000 reagent; after 24h they are observed (time zero, untreated CTRL) and differentiation is normally induced by addition of RA; imaging (and/or harvesting for RNA extraction) occurs after 1, 3, 5 and 7 days from induction. Cortical/hippocampal

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neurons are transfected immediately after dissociation using an electroporation method with 10 µg DNA per at least 10<sup>6</sup> cells (mouse neuron kit; AmaxaBiosystems). After transfection, cells were plated in complete Serum Free Medium (Neurobasal medium containing 2% B27 supplement, 1% glutamine) at a density of 10<sup>6</sup> cells per 35 mm PDL/LN-coated glass bottom dish (MatTeK Corporation) for live cell imaging and 0.2 10<sup>6</sup> per 12 mm PDL/LN-coated glass coverslips (for immunofluorescence) in a 24-well plate. Transfected primary hippocampal cultures grown for 1-3 days or 1-2 weeks *in vitro* were prepared for immunostaining;

- Protocol B: RA induction of SH-SY5Y is performed first and cells are transfected only 24h before each different time point (i.e. at 0 or 2 or 4 or 6 days after RA induction), at which they are observed as reported above (same time points as in Protocol A). The same method is applied also at primary neurons in culture.

### **Molecular Biology**

The main isoform and splice variants of VAMP7 were tagged by fluorescent protein (either red, RFP, or enhanced green, EGFP) cloning the isoform cds in pRFP-C3, pEGFP-C3 or pEGFP-N1 (Invitrogen). VAMP7b has C-terminal tag, whereas VAMP7d/h, VAMP7i and VAMP7j have N-terminal tag. Most of the plasmids were already used in (Vacca, Albania *et al.*, 2011). In addition, VAMP7i and VAMP7dh sequences were subcloned in RFP-tag. VAMP7a cytosolic fragments (1-118, 1-150, 1-160, 1-180) were amplified by PCR from the VAMP7a cds and fused at C-terminally to GFP/RFP-tags.

### **Immunofluorescence**

SH-SY5Y cells were fixed after 1/3/5/7 days from differentiation, while transfected primary cortical/hippocampal neurons after 1-3 days or 1-2 weeks. Fixing solution was 4% paraformaldehyde, 4% sucrose in PBS; then, cells were permeabilized, and blocked in 5% BSA solution before immunostaining. Mouse anti-VAMP2 (1 µg/ml) and anti-neurofilament (2 µg/ml) mAbs (Sigma) were used for primary staining of neuroblastoma cells; Alexa fluor 488-555 (4 µg/ml) (Sigma) for secondary staining. Cortical/hippocampal neurons were immunostained for βIII tubulin with mouse anti-βIII tubulin (Promega), for actin with phalloidin (Invitrogen) at 1:1000 dilution. Alexa fluor 568/647-conjugated secondary antibodies (Sigma) were used at 1:200-500 dilution.

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### **RNA extraction and retrotranscription**

Total RNA from cell cultures was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. Aliquots (5 µg) of DNase I-treated total RNA were reverse transcribed using SuperScript<sup>®</sup> III RNase H- reverse transcriptase (Invitrogen). Total RNA from human tissues are from BD Clontech. Aliquots (1 µg) of total RNA from human tissues were reverse transcribed using QuantiTect<sup>®</sup> reverse transcription kit (Qiagen). All these procedures are described in Vacca, Albania *et al.*, 2011.

### **Real-time RT-PCR**

All experiments were performed as follows: RNA for each sample was extracted and reverse transcribed in three replicates; then, to minimize variation depending on experimental error, real-time experiments were performed in five replicates for each sample, resulting in 3x5=15 replicates. Real-time PCR experiments were performed using SYBR<sup>®</sup>-Green core reagent kit and AmpliTaq Gold DNA Polymerase (Applied Biosystem) in a final reaction volume of 25 µl. The thermal cycler (Rotor-Gene 3000 from Corbett Research) was set as follows: 9' at 95°C followed by 40 cycles consisting of 30" melting at 95°C + 30" annealing at 60°C + 35" extension at 72°C. The gene encoding human ribosomal protein S13 is the housekeeping control for normalization [48]. For quantitative data analysis, the Rotor Gene software (version 6.0.34) was used according to (Pfaffl, 2001; Marino *et al.*, 2003). The following F and R primers (SIGMA-Genosys) were used:

VAMP7a-F: 5'-CACTGATGATGATTTTGAACG-3';

VAMP7a-R: 5'-CTGAGCTACCAGATCTATGTTTCT-3';

VAMP7b-R: 5'-TTGAAGGTGACAGACTATGTTTC-3';

VAMP7c-F: 5'-CTTCCTGGAGGATTTTGAAC-3';

VAMP7d-F: 5'-CACATGGCAAGATTTTGAAC-3';

VAMP7h-F: 5'-GGAACTTCCTGGAGTTATTTG-3';

VAMP7h-R: 5'-TCTCCATCACTTTGTCTAGGC-3';

VAMP7i-F: 5'-GCAGATTCTGGCTAAGATACC-3';

VAMP7i-R: 5'-TACCAGATCAGCTGTGCAG-3';

VAMP7j-F: 5'-ACAGCTGTCTGTACCTTC-3';

VAMP7j-R: 5'-TCAGTATGTGAAAGGCTTGAG-3';

S13-F: 5'-TACAACTGGCCAAGAAGGG-3';

S13-R: 5'-GGTGAATCCGGCTCTCTATTAG-3'.

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Such primers were designed and combined in order all amplicons have (or almost) equal length (250 to 254 bps) hence quantitative comparison can be extended to different amplicons from all isoforms; primer combinations to amplify specific isoforms are: VAMP7a, VAMP7a-F + VAMP7a-R; VAMP7b, VAMP7a-F + VAMP7b-R; VAMP7c, VAMP7c-F + VAMP7a-R; VAMP7d, VAMP7d-F + VAMP7a-R; VAMP7h, VAMP7h-F + VAMP7h-R; VAMP7i, VAMP7i-F + VAMP7i-R; VAMP7j, VAMP7j-F + VAMP7j-R.

For following up the neuronal development, total RNA extraction from human cells and tissues was performed as reported (Vacca, Albania *et al.*, 2011). In addition to primers specific to VAMP7 isoforms, the following F and R primers (SIGMA-Genosys) were used:

TrkB-F1: 5'-TAGATCCTGAGAACATCACCG-3'

TrkB-F2: 5'-GAGATTGGAGCCTAACAGTGT-3'

TrkB-R: 5'-GGATCAGTTCAGACAAGTCAA-3'

### **Imaging**

HeLa cells were transiently co-transfected using Lipofectamine<sup>TM</sup>2000 protocol (Invitrogen) and data were collected after 24h. SH-SY5Y cells were transfected and differentiated as previously described. Live cell imaging was performed using TCS SP2 and TCS SP5 II confocal scanning microscopes (Leica, Heidelberg, Germany). Sequential excitation with 488 nm, 543 nm laser beams, 20X air, 63X and 100X oil Leica objectives and LAS AF Software were used. Images (size set to either 512x512 or 1024x1024 pixels) were assembled by using ImageJ software.

Transfected neurons were imaged after 1-3 days or 1-2 weeks *in vitro* (DIV). Immunofluorescence images were acquired on an inverted zdc2 microscope (Olympus) using a 20x 0.85 NA, 40x 1.3 NA uplanfluor oil objectives (Olympus). Time-lapse images were acquired on the same microscope modified to allow for through-the objective multispectral TIR-FM using a 100X 1.49 NA objective (Mattheyses *et al.*, 2010). Laser light was focused at the aperture plane and directed to the coverslip by a dichromatic mirror, and the laser angle was adjusted manually with a micrometer. Neurons were kept in Serum Free medium at 37°C and 5% CO<sub>2</sub> in an incubation chamber fitted for the microscope. Exocytic fusion events were imaged at 0.5 s intervals for 5min, only in the pHluorin channel using MetaMorph imaging software (Molecular Devices).

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## Data Analyses

Morphological stages were identified and quantified after 3 days in culture, as reported (Gupton and Gertler, 2010). Neurites were considered any narrow and consolidated extension proximal to cell body; stage 1 neurons lack neurites, stage 2 have one or more minor neurites, and stage 3 have one neurite at least twice as long as any other. Exocytosis analyses were imaged at 1/3 days.

Vesicle fusion events were identified as reported (Dent *et al.*, 2007; Gupton and Gertler, 2010; Hua *et al.*, 2011). First appearance of a vesicle is then followed by a bright increase in fluorescence intensity (>3-fold over background), and a rapid diffusion (<2 s). Since neurons generally are a mixed population of cells in different stages, we separated events occurring in the soma from events in neurites. Thus, comparison between area/number of events and time lapse duration could be done. All statistical tests were performed with the AnalyzeIT utility of Microsoft Office Excel, using ANOVA with a LDS post-hoc test to determine significance. All of the data indicate mean  $\pm$  SEM.

## Bioinformatics

Melting temperatures of PCR primers were calculated using DNA Calculator (<http://www.sigma-genosys.com/calc/DNACalc.asp>); possible occurrence of hairpins, self- and hetero-dimers was ruled out by Oligoanalyzer 3.1 (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx>) and the specificity was checked with MFEprimer (<http://biocompute.bmi.ac.cn/MFEprimer/>) (Qu *et al.*, 2009).



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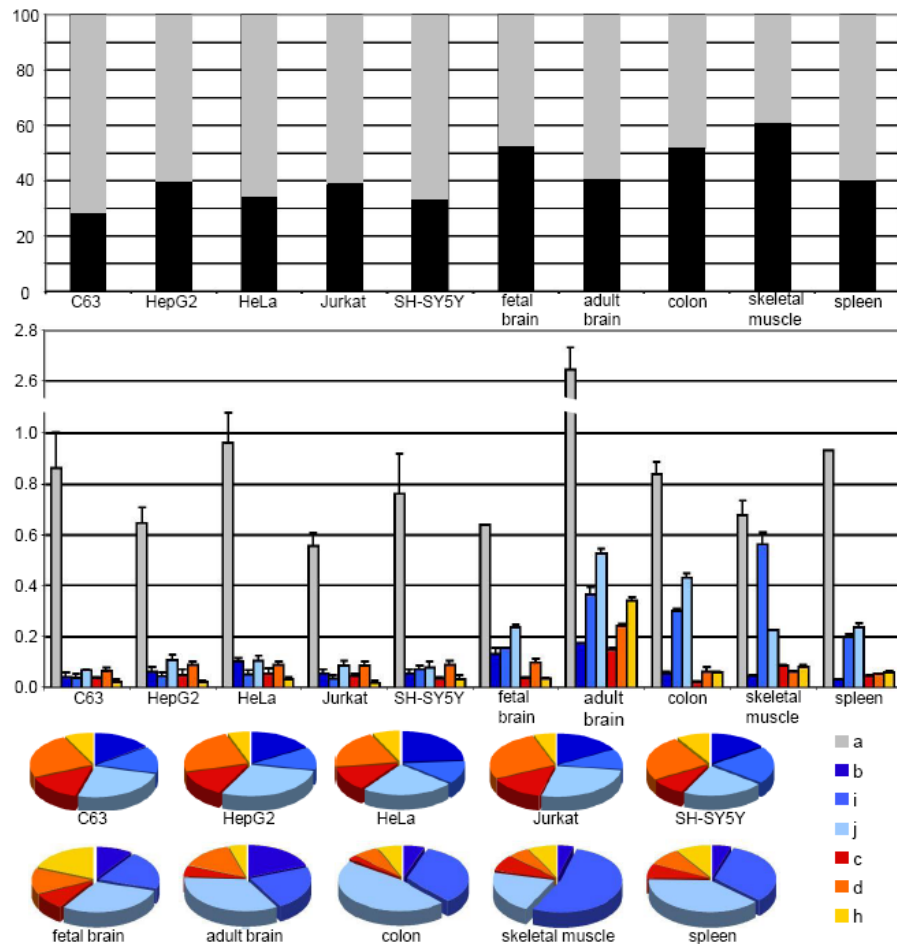
# RESULTS

## 1.1 Human VAMP7 isoforms: domain architecture, tissue-specificity and subcellular targeting

### 1.1.1 *Quantitative expression and tissue specific distribution of VAMP7 isoforms*

Since it was found that exon skipping at the SYBL1 locus can result in modulating VAMP7 isoform domain architecture (Martinez-Arca *et al.*, 2003), we further investigated alternative splicing mechanism at the SYBL1 locus (Vacca, Albania *et al.*, 2011). Therefore, once some putative variants were identified in the collaborating lab at IGB-CNR (see Figure 10, introductory chapter), real-time RT-PCR was performed on a panel of human cell lines and tissues, in order to verify their presence at physiologically meaningful mRNA levels. These analyses allowed to cut off most of multi-exon-skipping variants, the levels of which was found to be in the range of splicing errors, while results confirmed only a subset of SYBL1 mRNA variants (b, c, d, h, i, j), mainly resulting from skipping of a single exon or a couple of contiguous exons or exon fragments. Intriguingly, based on domain architecture, corresponding VAMP7 isoforms fall into two groups: non-SNARE longins (b, i, j) and  $\Delta$ -longin SNAREs (c, d, h). Quantitative expression data are shown in Figure 18. The upper graph illustrates relative contributions of VAMP7a (grey) and alternative splice variants (black) to the mRNA pool. VAMP7a amounts to 60-70% in cell lines (samples from C63 to SH-SY5Y) and 40-60% in tissues (other samples); in three out of five tissues, the sum of alternative variants (b, c, d, h, i, j) overpass 50% of the VAMP7 mRNA. The central graph illustrates the mRNA levels of each single isoform; splice variants range 5-12% of VAMP7a in cell lines, while their levels are much higher in tissues. For instance, in colon the aggregate level of VAMP7i+j mRNAs is comparable to VAMP7a and in skeletal muscle VAMP7i alone is comparable to the main isoform. In pie charts, 100% is the mRNA pool of alternative variants, excluding VAMP7a. Preponderance of LD<sup>+</sup>, non-SNARE isoforms (“b”, “i” and “j”) is clear in cell lines and it becomes more evident in tissues, where their aggregate level is comparable or even higher than that of VAMP7a. In particular, the non-SNARE/SNARE isoform ratio can shift from approximately 1:1 in cell lines to up 3:1 in tissues. In most tissues and cell lines, the second most expressed isoform (after VAMP7a) is VAMP7j, which is highly expressed in colon. The

level of alternative variants in neuroblastoma cells (SH-SY5Y) and fetal brain is lower than that found in adult brain, where the expression of VAMP7a is high (in agreement with literature - D'Esposito *et al.*, 1996).



**Figure 18. Quantitative analysis of VAMP7 isoform mRNA levels in human cell lines and tissues (from Vacca, Albania *et al.*, 2011).**

Color coding for all isoforms is reported; in the upper graph, 100% is the VAMP7 mRNA pool, of which the black part represents the pool of alternative isoforms (b+i+j+c+d+h) mRNA. In the middle, original data (mRNA levels of each isoform) are reported as % of the housekeeping reference gene (ribosomal S13) mRNA. Lower pie charts compare individual levels within the pool of alternative isoforms (excluding VAMP7a).

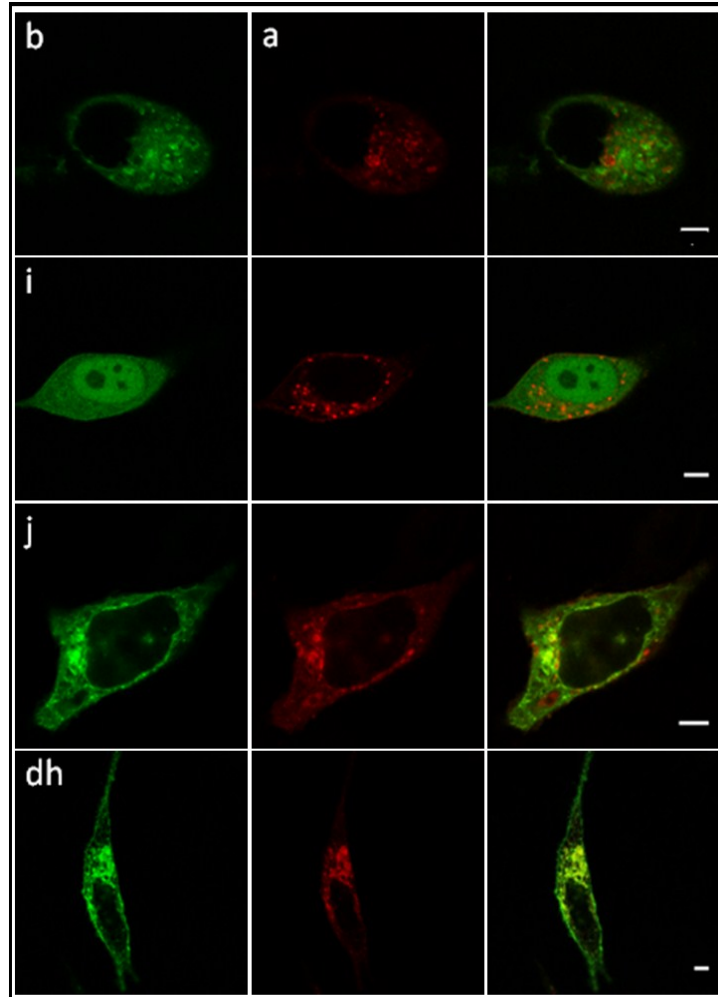
### 1.1.2 VAMP7 isoforms show different SCLs

The only VAMP7 splice variant characterized so far is VAMP7c: its truncated N-terminal region - unable to fold as a LD - is thus unable to bind  $\delta$ -AP3; this results in a different SCL from the main isoform (Martinez-Arca *et al.*, 2003). Since the newly identified isoforms (b, d/h, i, and j) also show variant domain architectures, their SCL was compared to the main isoform using fluorescent protein (FP) tagged chimeras. As explained in the introductory chapter and reported in Vacca, Albania *et al.*, 2011, the same VAMP7d/h protein variants is encoded by two distinct



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alternative mRNAs due to a Translation Reinitiation mechanism. For clarity and simplicity, hereafter VAMP7d/h will be just named VAMP7dh. Figure 19 shows confocal plane images from living HeLa cells that were co-transfected with enhanced green FP (EGFP)-tagged splice variant and red FP (RFP)-tagged main isoform.



**Figure 19. Variation of SC targeting among VAMP7 isoforms (from Vacca, Albania *et al.*, 2011).**

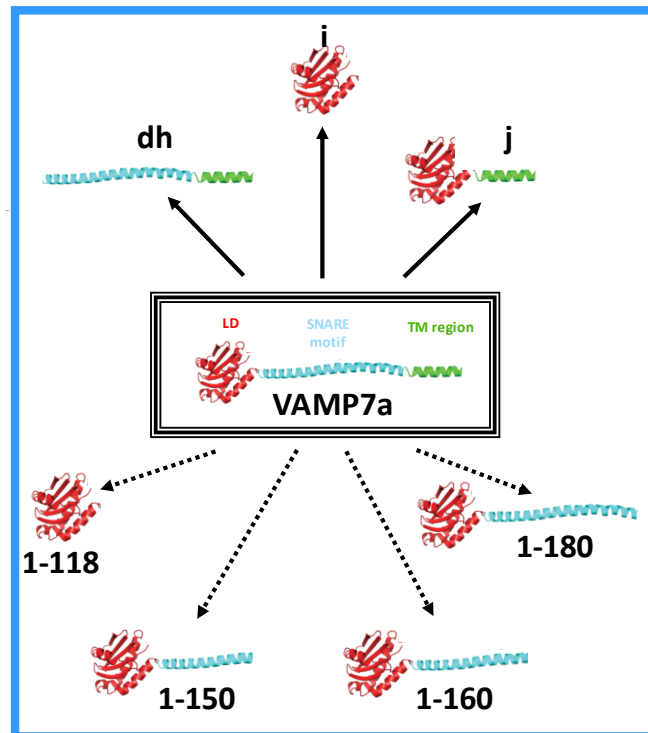
Confocal planes of HeLa cells, co-expressing an EGFP-tagged splice variant of VAMP7 (left column) and the RFP-tagged VAMP7a (central column). Merged images are presented (right column). Fluorescence was observed 24h after transfection. Scale bars correspond to 5  $\mu\text{m}$ .

VAMP7b shows a somewhat widespread distribution and the SCL of VAMP7j is also different from the punctuate localization of VAMP7a, as red areas are clearly apparent in merged images. However, partial co-localization with VAMP7a cannot be excluded. Intriguingly, VAMP7i is the only isoform that also shows a nuclear localization. However, highly widespread VAMP7i and punctuate, restricted-area VAMP7a indicate a quite different SCL also in the cytoplasm. On

the other hand, large yellow areas of co-localization are apparent in merged images for the non-LD variant VAMP7dh.

### 1.1.3 VAMP7 isoforms are a natural tool for dissecting domain roles in SC targeting

The LD is considered a key regulator of SC sorting in both human and plant VAMP7 (Martinez-Arca *et al.*, 2003; Uemura *et al.*, 2005; Mancias and Goldberg, 2007; Pryor *et al.*, 2008). Recent data have also revealed that the LD adopts a predominantly closed conformation requiring at least NT 160 residues, but it is fully stabilized in the closed state by additional 20 residues (Vivona *et al.*, 2010). Considering the different SCL displayed by each VAMP7 isoform and its relationship to the domain architecture, some of these splice variants were used for studying VAMP7 targeting determinants. In addition, a number of different fragments of the VAMP7a cytoplasmic region were cloned (Figure 20) for understanding the relative contribution of each different domain to sorting.

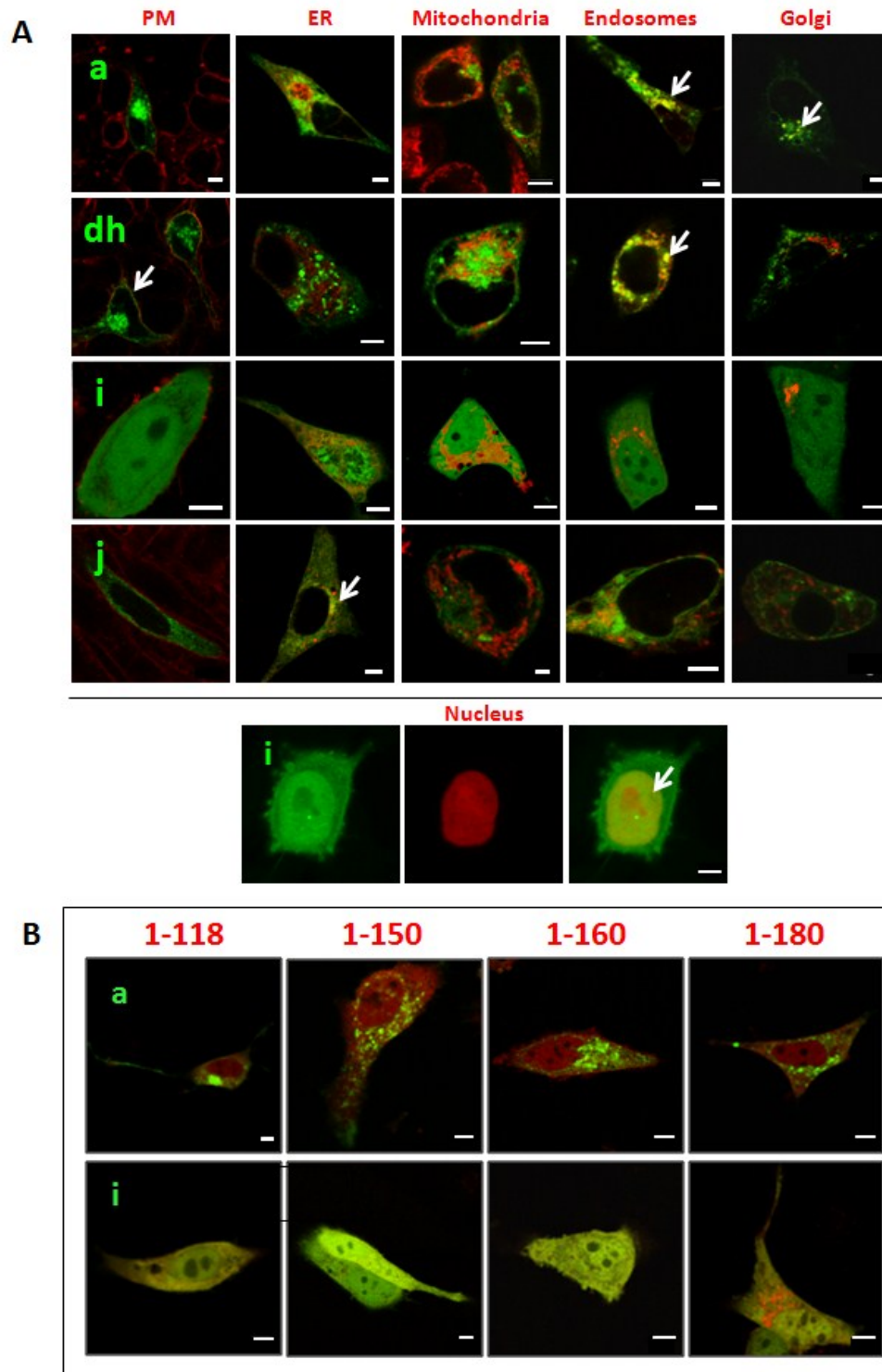


**Figure 20. VAMP7 domains as a tool for studying SC targeting.**

Top: combinatorial rearrangement of the three domains in VAMP7a (LD, SNARE motif and TM region) and alternative splice variants. The non-LD VAMP7dh retains SNARE+TM; the non-SNARE VAMP7i is the LD-only isoform and VAMP7j retains LD+TM. Bottom: recombinant fragments (1-118/150/160/180aa) of VAMP7a are identical to those structurally characterized (Vivona *et al.*, J 2010) except for fusion to the FP tag.

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In order to determine more precisely the SCL of each isoform, HeLa cells were co-transfected with FP-tagged VAMP7 splice variants and some organelle markers (ER: Calreticulin, EEs: Rab5), or labeled with SCL-specific dyes (PM: FM-464, Mitochondria: Mitotracker) after single isoform transfection (Figure 21A). The main isoform was used as a control; it only partially localizes with Golgi and EEs, according to its well-known secretory and recycling pathways from PM to late endosomes/lysosomes. The  $\Delta$ LD isoform dh displays PM localization as well as co-localization with Rab5, accordingly to previously observed co-localization with the main isoform. Intriguingly, an artificial  $\Delta$ LD construct shows the same early endosome localization (Martinez-Arca *et al.*, 2003). Among non-SNARE isoforms, VAMP7j shows partial co-localization with the ER marker; the nuclear fraction of VAMP7i co-localizes with HRB, according to data reported for another artificial construct (LD-only) (Martinez-Arca *et al.*, 2000). Nevertheless, a similar SCL between an artificial construct (LD) and a VAMP7 splice variant (i) is reached again. Intriguingly, literature data reported that these same constructs display physiological effects both developing (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Gupton and Gertler, 2010) and mature neurons (Hua *et al.*, 2011). Recombinant fragments (1-118/150/160/180 aa) of the VAMP7a cytoplasmic region - identical to those used in recent NMR analyses (Vivona *et al.* 2010) - were designed for SCL observation in HeLa cells (Figure 21B). All four recombinant fragments have a spread SCL in both cytosol and nucleus, different from VAMP7a (full length), which retains a non-nuclear and punctuate distribution. Furthermore, when comparing to VAMP7i (LD-only isoform), a clear co-localization with 1-118 (its artificial counterpart), 1-150 and 1-160 fragments is apparent. The 1-180 fragment represents the third two-domain combination (LD+SNARE), complementary to those already available among physiological splice variants (LD+TM and SNARE+TM); it also shows a different SCL. Considering that each combination of two out of the three domains of VAMP7 displays a specific SCL, these data strongly suggest that individual domains are unable to determine sorting by alone and that open/closed conformational switch is not relevant to SCL in the absence of TM region. Instead, it is known that the conformational switch is relevant to SCL for TM-endowed longin Sec22b (Mancias and Goldberg, 2007) as well as for the main isoform VAMP7a (Pryor *et al.*, 2008).



**Figure 21. SCL of physiological isoforms and recombinant fragments.**

A) HeLa cells transfected with VAMP7 isoforms were stained with different organelle markers (ER: Calreticulin, early endosomes: Rab5) or labeled with dyes (PM: FM-464, Mitochondria: Mitotracker) for live cell imaging. Merged images are presented: white arrows indicate co-localization areas. In the bottom, VAMP7i was also co-transfected with the nuclear marker H2B-RFP. B) Co-expression of recombinant fragments (1-118/150/160/180aa) of VAMP7a cytoplasmic region with either the full length protein or VAMP7i. Fluorescence was observed 24h after transfection. Scale bars correspond to 5  $\mu$ m.

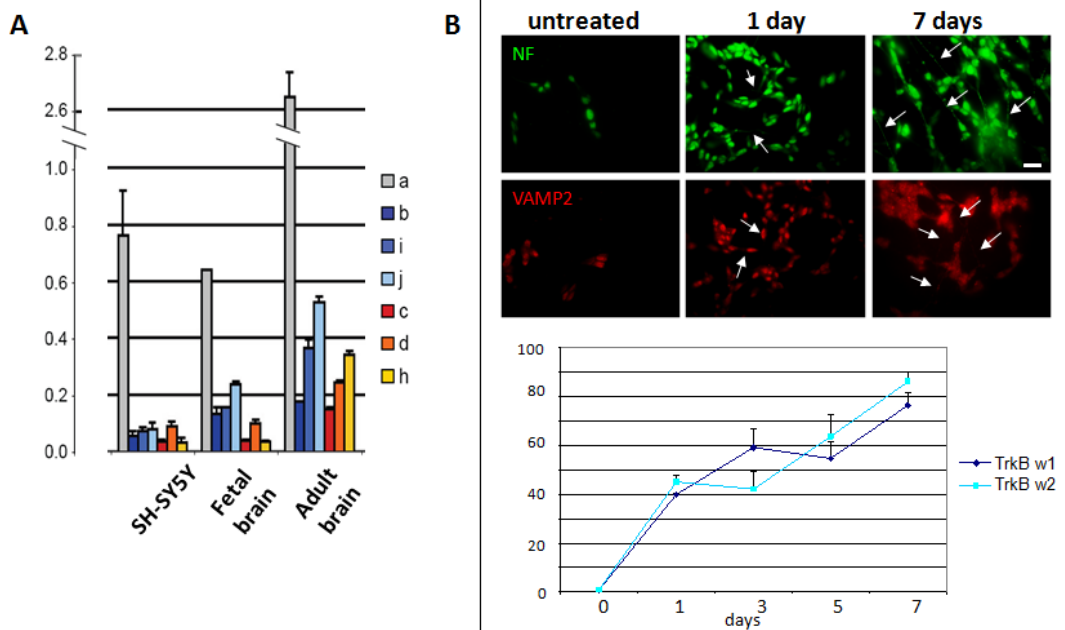
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## 1.2 VAMP7 and neuronal development

### 1.2.1 The human model: SH-SY5Y cell line

#### 1.2.1.1 Isoform modulation strongly increases during first days of neuronal differentiation

A consistent literature concerns the relationship between VAMP7 and neuritogenesis and artificial construct resembling VAMP7dh and VAMP7i showed peculiar roles in neurite outgrowth (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Chaineau *et al.*, 2009; Gupton and Gertler, 2010; Hua *et al.*, 2011; Sato *et al.*, 2011). Indeed, our previous real time RT-PCR data (Vacca, Albania *et al.*, 2011) suggest that also VAMP7 alternative isoforms are involved in this process (Figure 22A) therefore we chose the human dopaminergic SH-SY5Y cell line as a model for studying splicing modulation along neuronal differentiation. This latter was induced by all-*trans*-Retinoic acid (RA) and cells were harvested at day 1, 3, 5 and 7. Microscopy observation after first days from induction reveals neurite outgrowth and cell polarization followed by neurite elongation. Figure 22B displays further characterizations for confirming cell differentiation both at immunocytochemical and molecular levels. In the upper panel, staining for neuronal markers VAMP2 and neurofilaments is shown: white arrows indicate cell processes. In the lower graph, real time RT-PCR analysis reveals a large increase in TrkB (RA receptor) mRNA expression during 7 days of induction. Furthermore, the expression level of minor isoforms in undifferentiated SH-SY5Y cells fortifies previous results. Then, Figure 23 presents full quantitative analyses concerning all VAMP7 isoforms. The upper graph resumes the overall contribution of minor isoforms along neurite outgrowth: at time zero (undifferentiated cells) splice variants account for 30% mRNA; the major increase is observed in the first 1-2 days after RA addition, reaching 40% of mRNA pool. The central graph illustrates the mRNA levels of each isoform, following up modulation. The lower graph shows single isoform contributions with respect to VAMP7a: a progressive increase in LD<sup>+</sup>, non-SNARE isoforms b, i, j is clearly apparent, whereas VAMP7dh, a  $\Delta$ LD isoform, is reaching maximum level at day 3. In Figure S1, modulation of non-SNARE (b+i+j) and non-LD (c+dh) isoforms during differentiation is represented, highlighting the reverse modulation of the synaptobrevin-like isoforms (growth stimulation) and non-SNARE, longin ones (growth inhibition).

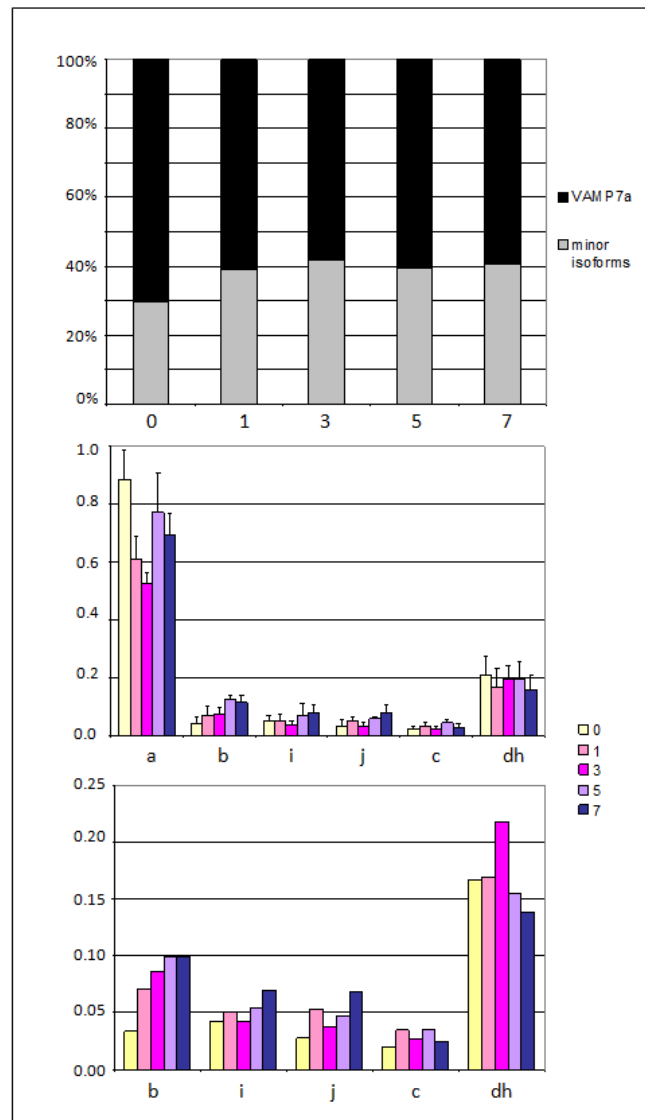


**Figure 22. Human VAMP7 and neuronal development.**

A) Quantitative analysis of VAMP7 isoform mRNA levels in the neuroblastoma cell line SH-SY5Y compared to fetal and adult brain tissues (adapted from Vacca, Albania *et al.*, 2011). B) Upper panel, immunocytochemistry of RA-treated SH-SY5Y cells stained for neuronal markers neurofilaments and VAMP2. White arrows indicate detection of these markers in axons of differentiated cells. Reference white bar corresponds to 20  $\mu$ m. Lower panel, quantitative analyses of TrkB (RA receptor, neuronal differentiation marker) during RA induction on SH-SY5Y cells: the graph shows data obtained using two different forward primers (TrkB w1, TrkB w2).

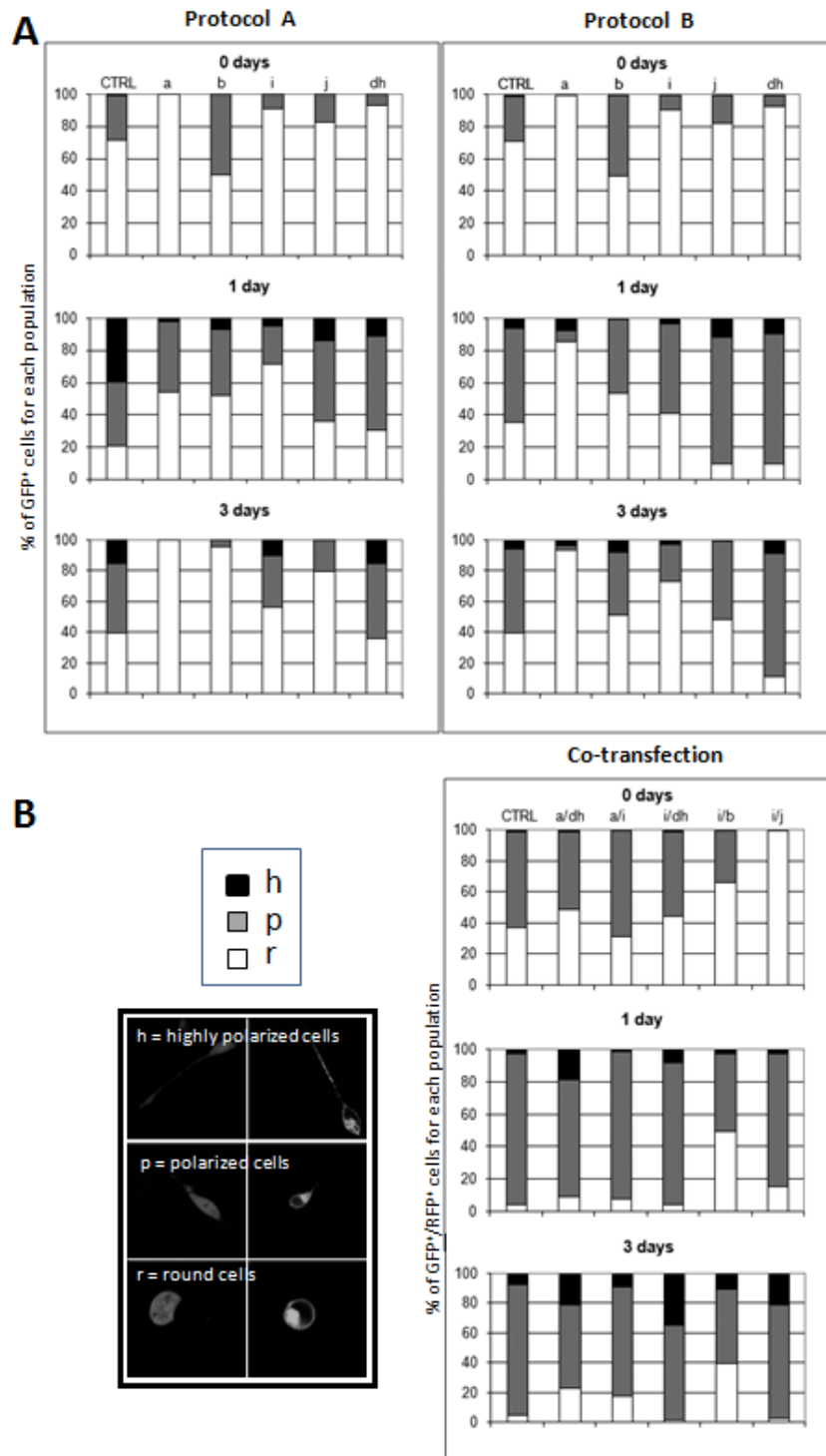
### 1.2.1.2 $LD^{-}/SNARE^{+}$ VAMP7dh variant seems to enhance cell differentiation also in the presence of VAMP7i

In order to understand the physiological role of VAMP7 isoforms in neuronal differentiation, SH-SY5Y cells were transiently transfected with these splice variants, checking both morphology and fluorescence parameters. Two different gain-of-function protocols were set up: A) transfection before cell differentiation; B) transfection 24h before each time point (1/3/5/7 days). Counts of different cell populations (r, round; p, polarized; h, highly polarized cells) were performed at each time point; most interesting results were observed during first days of differentiation (Figure 24). Literature data reported that a “synaptobrevin-like”  $\Delta$ LD fragment and an LD-only construct display opposite effects during early neuronal differentiation stages (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Gupton and Gertler, 2010).



**Figure 23. Quantitative analysis of the VAMP7 isoform mRNA levels in RA-treated SH-SY5Y cells.**  
 Upper graph: overall contribution of minor isoforms along neurite outgrowth. 100% is the VAMP7 mRNA pool, of which the black part represents the pool of alternative isoforms (b+i+j+c+dh) mRNA. Middle graph: original data (mRNA levels of each isoform) are reported as % of the housekeeping reference gene (ribosomal S13) mRNA. Lower graph: individual levels are compared within the pool of alternative isoforms (excluding VAMP7a).

Interestingly, splice variants VAMP7dh and VAMP7i, roughly correspond to  $\Delta$ LD and LD-only fragments. The development of neurons expressing GFP was indistinguishable from nontransfected cells, as normal axonal and dendritic outgrowth was observed. In Figure 24A, both protocol A and B highlight that VAMP7dh acts as an enhancer in axonal outgrowth, as an increase in number of polarized and highly polarized cells during first days is clearly apparent. On the contrary, overexpression of VAMP7i seems to reduce neurite outgrowth, when compared to the control and VAMP7dh.



**Figure 24. Contribution of VAMP7 isoform overexpression on neurite outgrowth in SH-SY5Y cells.**

A) Single transfection in the first stage of differentiation, using two gain-of-function protocols: Protocol A, (left panel), transfection before cell differentiation and Protocol B (right panel), transfection 24h before each time point (1/3 days) B) Co-transfection was performed with protocol B; cells were counted based on different morphology (h, highly polarized cells, black; p, polarized cells, gray; r, round cells, white). Relative contribution of each population is referred to 100% (total number of cells counted, n= 50). CTRL= empty EGFP vector.



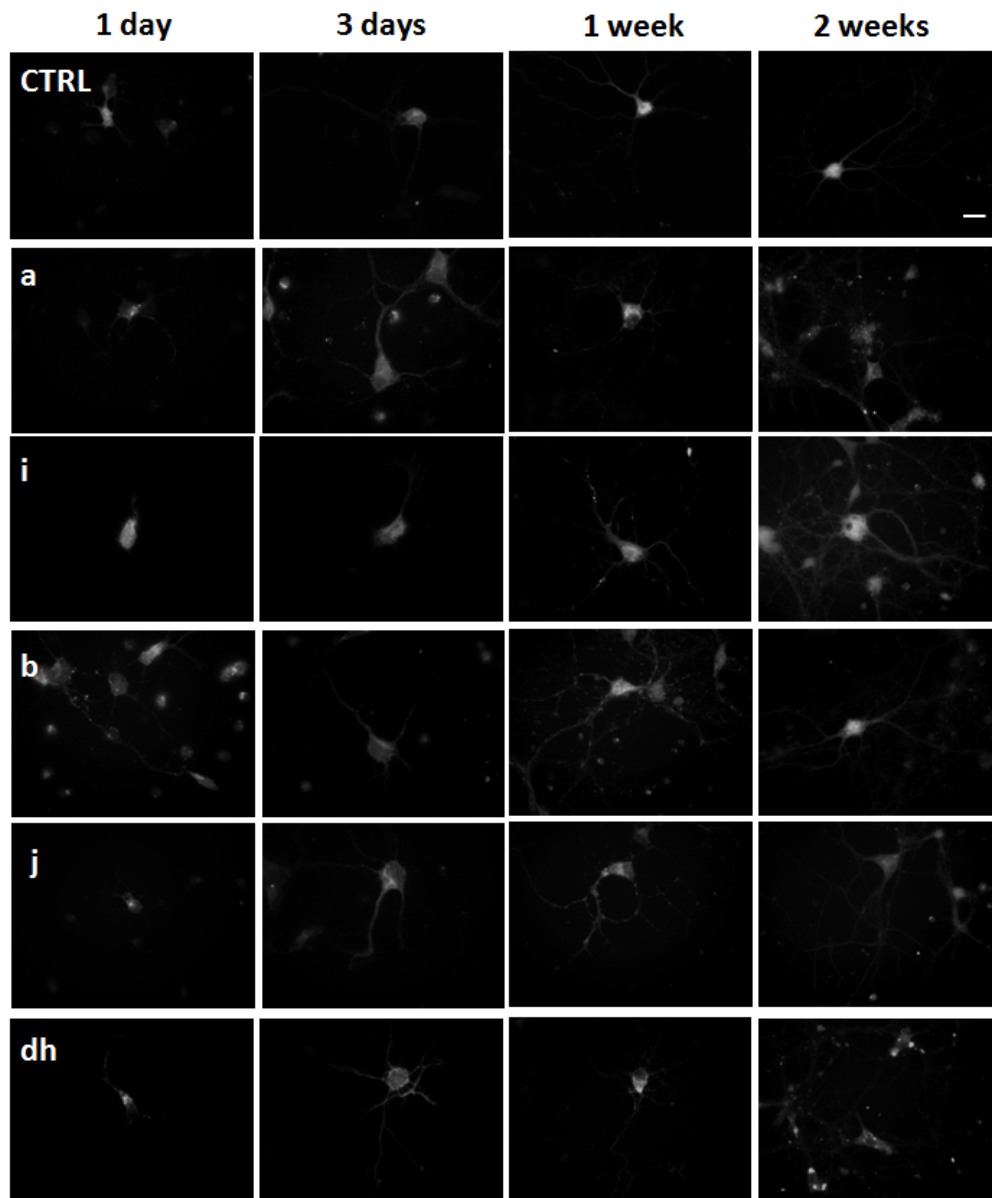
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In order to assess whether in nature VAMP7 modulation is developmentally regulated and isoforms may compensate each other, cells were co-transfected using different combinations of VAMP7 isoforms (Figure 24B). Co-transfection VAMP7i+VAMP7dh resulted in enhanced presence of “p” and “h” neurons after 3 days, suggesting that neurite outgrowth also occurs in presence of the inhibitory VAMP7i. Altogether these results opened a scenario that prompted us to further clarify effects of this modulation during neurite outgrowth.

## ***1.2.2 The mouse model: E16 cortical/hippocampal neurons***

### ***1.2.2.1 LD<sup>-</sup>/SNARE<sup>+</sup> VAMP7dh variant enhances neurite outgrowth on PDL coating***

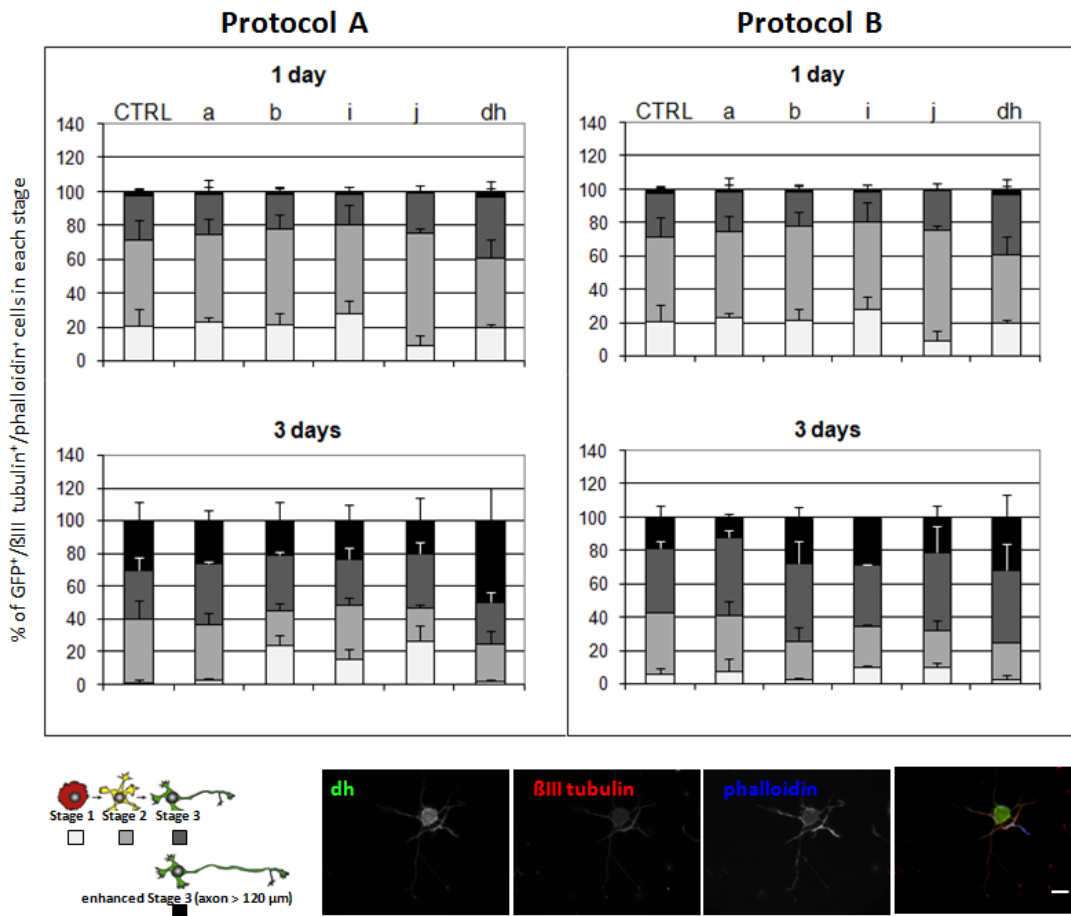
Recent works enforced the idea of a linkage between VAMP7 and VAMP2 during events that drive neuritogenesis (Gupton and Gertler, 2010). First, wild type neurons were used to assess the contribution of VAMP7 and its splice variants to neurogenesis. Neurons were grown using both PDL (unconditioned coating) and LN as coating agents. Morphology of transfected neurons - followed along 2 weeks (i.e. during the entire development) - is shown in Figure 25. Then, labeling for the neuronal marker  $\beta$ III tubulin allowed the distinction from other glial cells; in addition, the actin marker Phalloidin allowed cytoskeletal observations (ex. lamellipodia and filopodia), distinguishing neurons for counts at different morphological stages (Figure 26). Stage 1 neurons are characterized by the absence of neurites; stage 2 neurons show neurites of the same length; stage 3 neurons develop an axon twice longer than other neurites (Dotti *et al.*, 1988; Burgo *et al.*, 2009; Gupton and Gertler, 2010). Enhanced stage 3 neurons were separated from other groups, according to the enhanced length of some axons (>120  $\mu$ m). When neurons were transfected with VAMP7dh, both transfection protocols showed a strong increase in stage 3 and enhanced stage 3 populations. Such effect was clearly higher than that caused by transfection with VAMP7a, resulting in good levels of differentiated neurons, similar to the control. Such positive modulation was particularly evident using protocol A that was thus followed to perform next experiments. Conversely, transfection with non-SNARE isoforms (b, i, j) reduced the number of neurons in stage 2 and 3 (compared to other splice variants and control), keeping them in stage 1 also after 3 days in



**Figure 25. SCL of VAMP7 isoforms in cortical/hippocampal neurons.**

Primary neurons transfected with either the empty EGFP vector (CTRL) or VAMP7 isoforms were followed during the entire development, fixed at each time point and stained for the neuronal marker  $\beta$ III tubulin and the actin marker Phalloidin (not shown). Scale bar corresponds to 10  $\mu$ m.

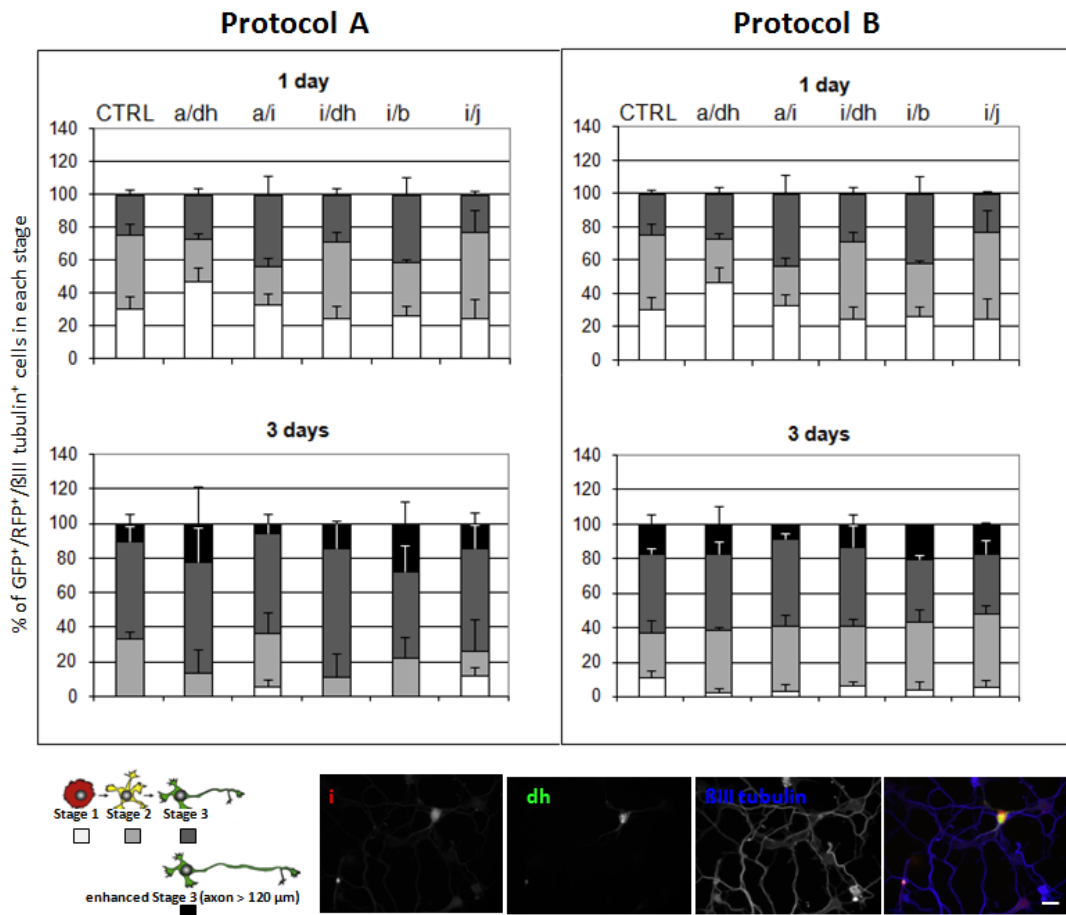
culture. Given that previous results on SH-SY5Y cells differentiated on PDL-coated coverslips were confirmed, the non-SNARE isoform VAMP7dh is likely a physiological enhancer of neuronal development. When VAMP7dh was co-transfected with VAMP7i or VAMP7b, neuritogenesis was found to occur even in co-presence of an inhibitory variant, suggesting that either VAMP7dh is able to overcome inhibition, the physiological role of non-SNARE variants is not just inhibition and/or it is more relevant at later differentiation stages (Figure 27).



**Figure 26. Contribution of VAMP7 isoform overexpression to neurite outgrowth in cortical/hippocampal neurons on PDL.**

Primary neurons were transfected with either the empty EGFP vector or VAMP7 isoforms, using protocol A or B and grown on PDL. Then, they were stained for the neuronal marker  $\beta$ III tubulin and the actin marker Phalloidin. Data from different counts were collected using morphological parameters based on different developmental stages (stage 1: cell without neurites, white; stage 2: cell with neurites, pale gray; stage 3: cell with an axon, gray; enhanced stage 3: cell with an axon longer than 120  $\mu$ m). Relative contribution of each population is referred to 100% (total number of cells counted,  $n \geq 50$ ; 4 different experiments; mean  $\pm$  SE). Scale bar corresponds to 10  $\mu$ m.

When neurons were co-transfected with two non-SNARE isoforms (the LD-only VAMP7i and the LD+TM VAMP7j), they were found more in stage 1 also after 3 days in culture, as shown for VAMP7a/i. Here, the “brake” effect (inhibition of differentiation) is more evident, confirming the idea that isoform modulation is likely involved in “fine tuning” of neuronal development.

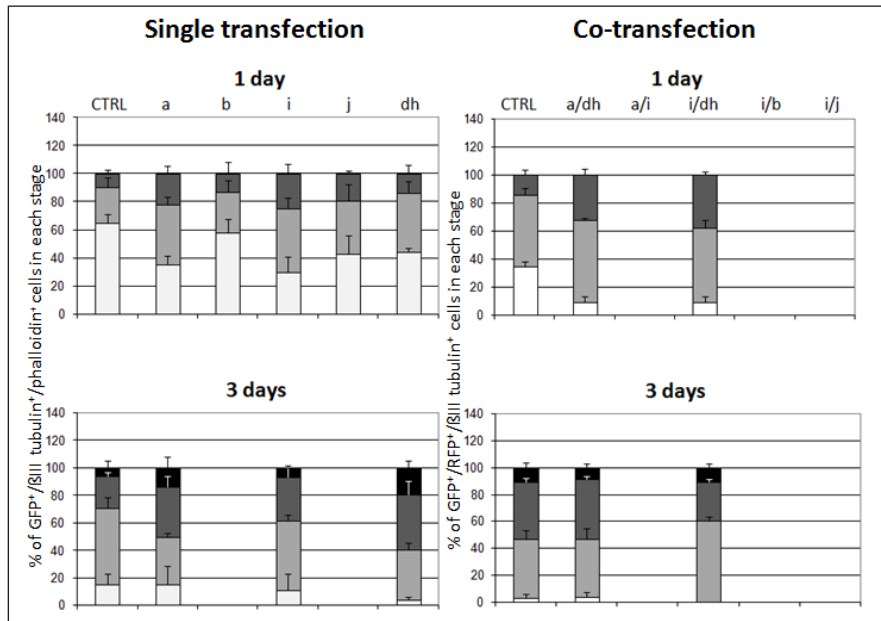


**Figure 27. Contribution of VAMP7 isoform co-overexpression to neurite outgrowth in cortical/hippocampal neurons on PDL.**

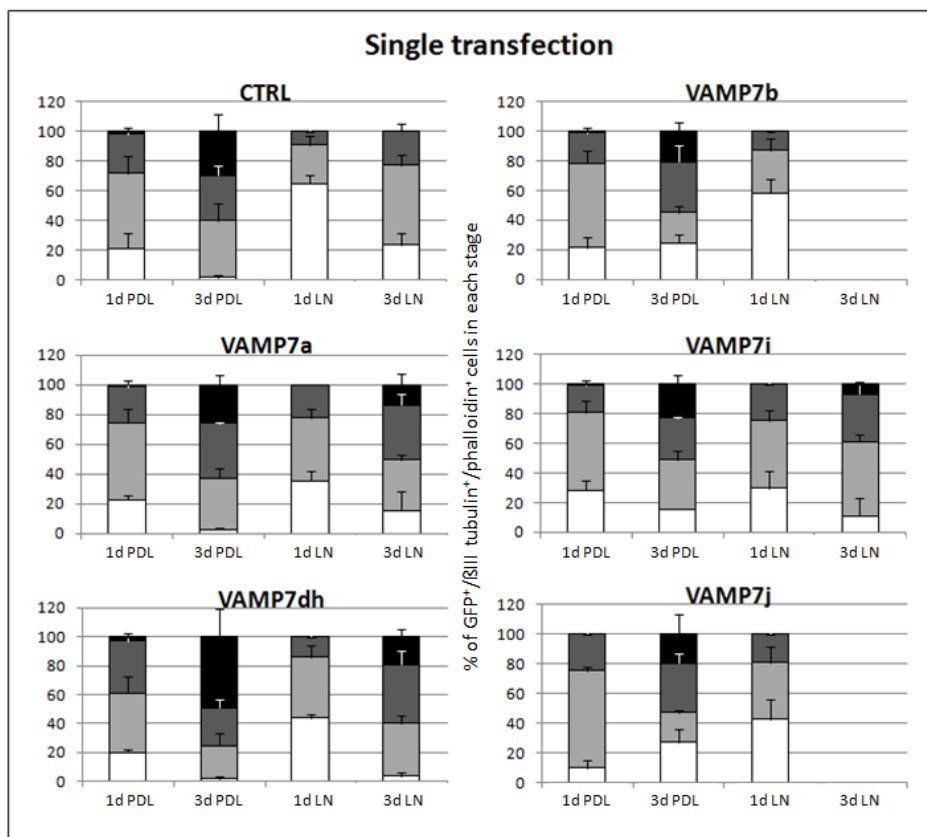
Primary neurons were co-transfected with VAMP7 isoforms, using protocol A or B and grown on PDL. Then, they were stained for the neuronal marker  $\beta$ III tubulin. Data from different counts were collected and are represented as reported in figure 9 legend. CTRL= empty EGFP vector; scale bar corresponds to 20  $\mu$ m.

### *1.2.2.2 Different effects of LN coating*

When neurons were grown on LN, events that differ from PDL coating occurred (Figure 28). In single transfections with VAMP7b or VAMP7j, fluorescence was not detectable at day 3 (lower left panel). In co-transfection experiments with the non-SNARE isoforms, fluorescence was detectable only in VAMP7i+VAMP7dh samples, during the entire neuritogenesis (1-3 days). The general evidence is that LN action results in a delay in neuritogenesis progress respect to neurons seeded on PDL (CTRL - Figure 29).



**Figure 28. Contribution of VAMP7 isoform overexpression on neurite outgrowth in cortical/hippocampal neurons on LN.**  
 Primary neurons were either transfected or co-transfected with VAMP7 isoforms, using protocol A and grown on LN. Then, they were stained for the neuronal marker  $\beta$ III tubulin. Data from different counts were collected and represented as reported in figure 9 legend. CTRL= empty EGFP vector.



**Figure 29. Influence of coating on immature neurons transfected with VAMP7 isoforms.**  
 Morphological comparison of neurons grown on either PDL or LN coating. Influence of each isoform in modulating neuron levels in different stages is also considered (see figure 9 legend for neuronal stages codes and calculations criteria). CTRL= empty EGFP vector.

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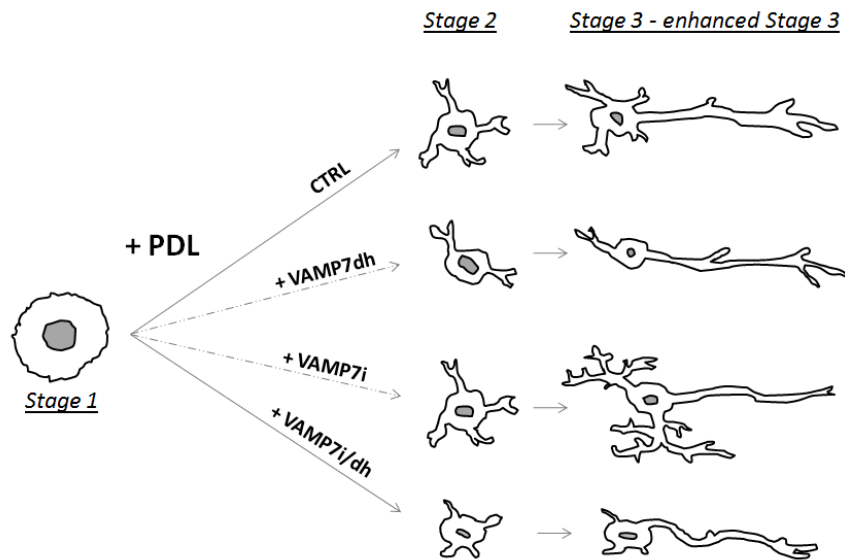
### 1.2.2.3 VAMP7 isoforms and extracellular substrates differentially influence neurite outgrowth

In order to answer at question concerning the relationship between VAMP7 isoforms and extracellular substrates, we studied neuronal development considering a number of different parameters: neurite number, neurite/axon length, number of neurite/axon branches, number of neurite/axon branches vs length. ANOVA statistical analyses were performed on data collected from neurons after 3 days in culture, considering differences due to all morphological stages. We focused our attention on the non-SNARE variant VAMP7i and the non-LD variant VAMP7dh, using data from other isoforms as internal comparisons and/or controls. Results concerning PDL coating are summarized in Table 1: panel A presents significant levels between CTRL (EGFP empty vector), VAMP7dh and i; panel B adds more information about relationship between these isoforms and the other ones. Starting from the well-known idea that LD has a inhibitory role in neurite outgrowth (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Gupton and Gertler, 2010), the only negative effect of the physiological isoform VAMP7i (LD-only) we found on PDL is on the number of axon branches; furthermore, neurons transfected with this isoform significantly increase their axon length respect to the control. During this committed, development status, VAMP7i is also involved in mechanisms forming and maintaining other neurites, compared to VAMP7dh. Conversely, at the same developmental stages, VAMP7dh shows a role in neurite elongation but not in their number or specification, as VAMP7i. The overexpression of both isoforms during these early steps shows typical effects due to VAMP7dh: control of neurite length but not neurite specification in branching. Considering the relationship with other splice variants (Table 1, panel B), in particular the main VAMP7a, we found the same results: VAMP7i does not inhibit axon elongation, whose level is also higher than transfection with VAMP7a; VAMP7dh again shows its involvement in neurite elongation. In addition, neuritogenesis occurs also when VAMP7i is co-expressed with VAMP7dh, highlighting a role of enhancer for the synaptobrevin-like isoform. This positive, strong effect is retained also when the two isoform VAMP7a/dh are co-expressed. On the contrary, the other two non-SNARE variants VAMP7b and VAMP7j display a similar, complete commitment for

PDL coating	cells in all stages	cells in stage 2	cells in stage 3	cells in enhanced stage 3	cells in all stages 3
VAMP7i	> CTRL in axon length; < CTRL in n° axon branches			< CTRL in n° axon branches vs length; > VAMP7dh in n° neurite branches vs length	> CTRL in axon length; < CTRL in n° axon branches ; > VAMP7dh in neurite number
VAMP7dh		< CTRL in neurite number	> CTRL in neurite length	< VAMP7i in n° neurite branches vs length	< VAMP7i in neurite number; > CTRL in neurite length
VAMP7i/dh	< CTRL in n° neurite branches and n° neurite branches vs length; > CTRL in neurite length	< CTRL in n° neurite branches; < CTRL in n° neurite branches vs length	< CTRL in n° neurite branches; < CTRL in n° neurite branches vs length		< CTRL in n° neurite branches and n° neurite branches vs length
PDL coating	cells in all stages	cells in stage 2	cells in stage 3	cells in enhanced stage 3	cells in all stages 3
VAMP7i	> VAMP7a in total axon length	> VAMP7j in neurite length			> VAMP7a in total axon length
VAMP7dh			> VAMP7a in neurite length		
VAMP7i/dh		> VAMP7i/j in neurite length	< VAMP7a/dh in n° neurite branches ; < VAMP7a/dh in n° neurite branches vs length	< VAMP7i/j in n° neurite branches vs length	< VAMP7a/dh in n° neurite branches and n° neurite branches vs length; < VAMP7i/b in neurite branches

**Table 1. Schematic summary of statistical analyses performed on morphological data from immature neurons grown on PDL.**

Statistical analyses were performed on neurons after 3 days in culture. The following morphological parameters were considered: neurite number, neurite/axon length, number of neurite/axon branches, number of neurite/axon branches vs length. Results concerning CTRL (empty EGFP vector), the non-LD VAMP7i, the non-SNARE VAMP7dh and VAMP7i/dh co-transfection are displayed with respect to different stages; gray columns refer to overall data (all stages or all stage 3), whereas white columns refer to single stages. A) Comparison of CTRL to VAMP7i, VAMP7dh, VAMP7i/dh transfections; B) Comparison also considering other isoforms.



**Figure 30. Morphological changes resulting from either VAMP7i, dh or i/dh overexpression.**

Influence of either VAMP7i or VAMP7dh transfection and of VAMP7i/dh co-transfection on cortical/hippocampal neurons during neurite outgrowth on PDL. Data represent results from statistical analyses. Dash arrows indicate opposite effect of VAMP7i and VAMP7dh.

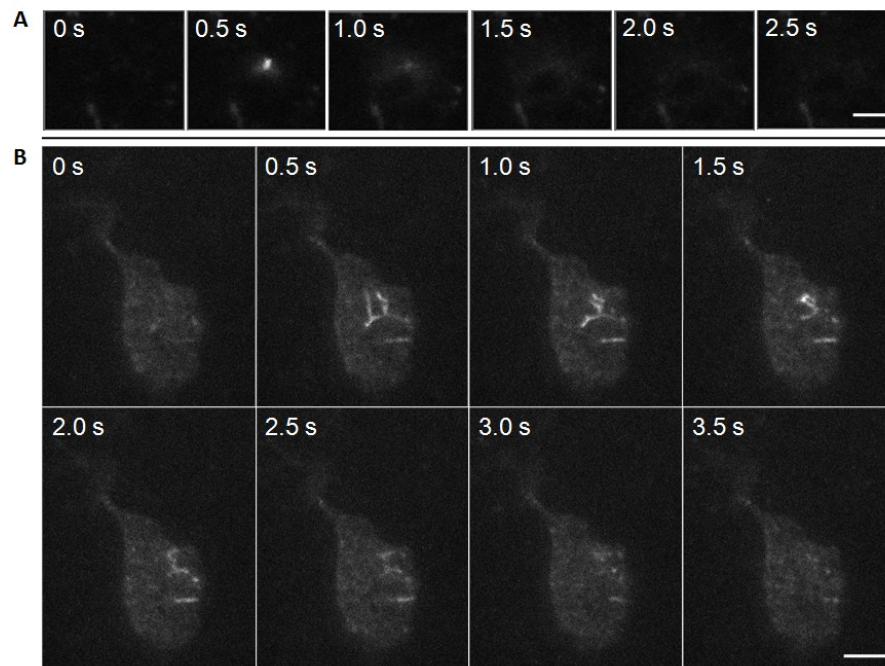
being involved in neurite branching. A schematic representation of morphological effects due to the LD-only VAMP7i and the synaptobrevin-like VAMP7dh overexpression is reported in Figure 30.

#### 1.2.2.4 VAMP7 isoforms have different roles in VAMP7-mediated exocytosis

In addition to morphological parameters, exocytosis has an important role in neuritogenesis establishment. Few works already studied VAMP7-mediated exocytosis, but they focused on different conditions: neurons in stage 1 (Gupton and Gertler, 2010) and mature neurons (synaptic boutons - Hua *et al.*, 2011). Nevertheless, further studies are needed to clarify the role of exocytosis during neuronal development; thus, we performed experiments on mixed population of neurons, after 1-3 days in culture. Analyses of exocytic vesicles were separated considering events occurring either at the soma or in the neurites/axon, because exocytosis is spatially and developmentally regulated (Tsaneva-Atanasova *et al.*, 2009; Chernyshova *et al.*, 2011). Neurons were co-transfected with both a pH-sensitive pHluorin-tagged VAMP7 and RFP-tagged VAMP7 splice variants a,dh and i; pHluorin VAMP7 alone was used as control. This labeling system let us to identify vesicle fluorescence of exocytic events, using live cell total internal



reflection fluorescence microscopy (TIR-FM) (Alberts *et al.*, 2006; Gupton and Gertler, 2010; Hua *et al.*, 2011) in time-lapse experiments. We observed transient puffs of light representing secretory vesicle fusion events (Figure 31A); in addition, tubules fusing with the plasma membrane were detected (Figure 31B) (Alberts *et al.*, 2006).

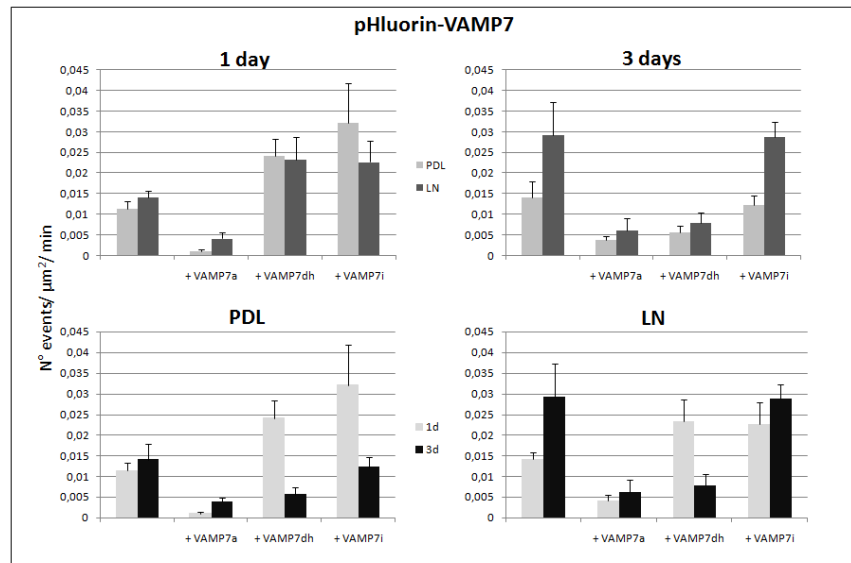


**Figure 31. Exocytic events at the PM of immature neurons.**

A) Time lapse, representative images of cortical/hippocampal neurons showing fusion events at the PM. Neurons transfected with a pH-sensitive VAMP7 or VAMP2 were imaged using TIRF microscopy. The fluorescence intensity of pHluorin is low within vesicle lumen ( $t=0$  sec). Upon fusion with the PM, a rapid increase in fluorescence intensity ( $t=0.5$  sec) is observed because of the higher pH of cytosol; then, rapid diffusion of fluorescence depends on molecules diffusion within the PM ( $t=1.0-2.0$  sec). B) Imaging of tubules fusing with the PM. Scale bars correspond to  $2\ \mu\text{m}$  in (A) and  $5\ \mu\text{m}$  in (B).

The most consistent data regard the soma; when considering neurite/axon events (data not shown), no significant differences between samples were found, probably due to the less amount of cells counted in stage 3/ enhanced stage 3. Considering the soma, we found that double overexpression of the same protein (pHluorin VAMP7+RFP-VAMP7a) strongly reduces their exocytosis, in particular on PDL (Figure 32, upper graphs). Intiguingly, early during the neurite outgrowth, the inhibitory role of LD is not apparent when VAMP7i is added to VAMP7 and the overall level of exocytic vesicles is comparable to the non-LD VAMP7dh that also increase VAMP7 fusion events, with no dependence from coating conditions. Figure S2 summarizes isoform effects. Taken together, these

results reveal that early during neuritogenesis process, VAMP7 is more subjected to isoform influence (both dh and i produce a similar increase).

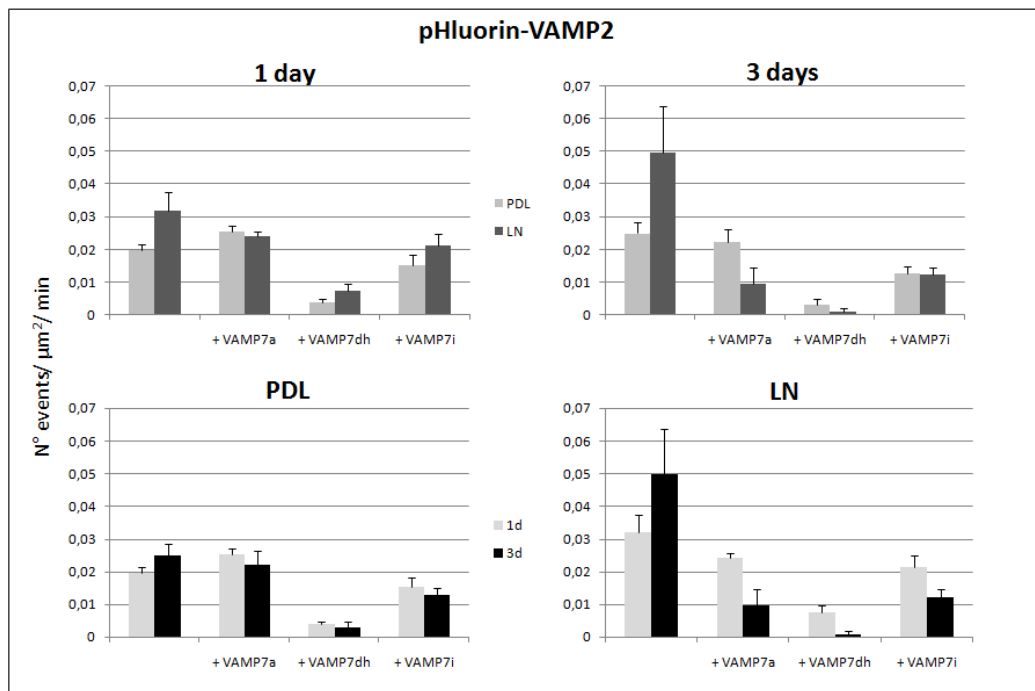


**Figure 32. Influence of VAMP7 isoforms on VAMP7-mediated exocytosis in the soma.**

Immature neurons were co-transfected with both pHluorin-VAMP7 and respectively RFP-tagged VAMP7a, VAMP7dh or VAMP7i, on either PDL or LN. Live cell imaging was performed after 1 and 3 days, acquiring frames every 0.5 sec for 5 min. Fusion events were counted and related to area and time. Graphs report statistical data collected from several cells ( $n \geq 10$ ; 2 different experiments; mean  $\pm$  SE).

#### 1.2.2.5 Cross-talk between VAMP7 and VAMP2 pathways: a modulation model driven by VAMP7 isoforms

VAMP2 is the v-SNARE for synaptic vesicle exocytosis, but VAMP7 also interacts with the t-SNAREs SNAP25 and Stx1 involved in transmitter release (Alberts *et al.*, 2003; Cotrufo *et al.*, 2011). Furthermore, recent evidence seems to suggest an involvement of synaptobrevin 2 in neurite outgrowth (Gupton and Gertler, 2010). In this complex scenario, we performed preliminary experiments to shed light on possible relationship between VAMP7 splice variants and VAMP2 in neurite outgrowth. Neurons were co-transfected with pHluorin-VAMP2 in combination to VAMP7 isoforms, as previous reported. VAMP2-mediated exocytosis was not particularly affected by isoform addition, rather than VAMP7dh, after 1 day (Figure 33, upper graphs). Probably, similar structure (SNARE+TM region) shared by synaptobrevin 2 and VAMP7dh might account for this. At day 3, expression of VAMP2 on LN shows a strong increase in exocytosis that is clearly reduced by VAMP7a and i addition; interestingly, the LD-only isoform display this inhibitory effect in both coating conditions.

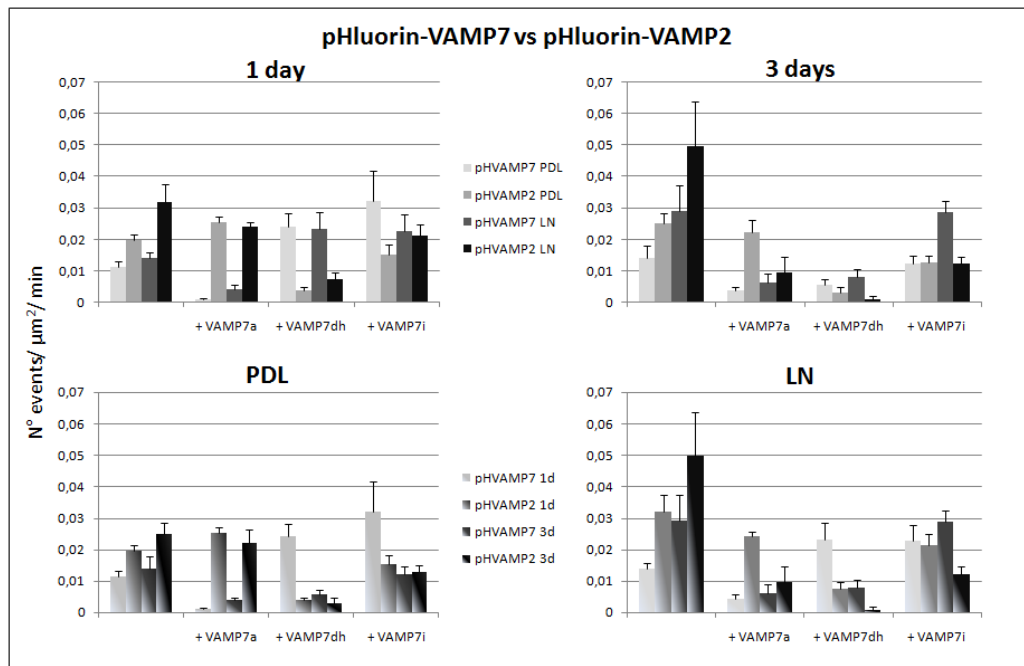


**Figure 33. Influence of VAMP7 isoforms on VAMP2-mediated exocytosis in the soma.**

Immature neurons were co-transfected, live cell imaging was performed, fusion events were counted and graphs report statistical data as indicated in Figure 16 legend.

More in general, it seems that when neurons are seeded on LN, all VAMP7 isoforms decrease VAMP2 exocytosis over time, with an opposite behavior respect to the control. Figure S3 summarizes these isoform effects. Thus, the most interesting data concern the strong effect mediated by LN on VAMP2 pathway that produce a relevant increase in fusion events and the inhibitory action displayed by VAMP7i. These two results are enforced during neuritogenesis progression.

Finally, comparing what happens in the soma of developing neurons transfected with either pHluorin-VAMP7 or VAMP2 (Figure 34), it was found that VAMP7a decreases VAMP2 levels at day 3. When considering VAMP7dh, it decreases exocytic events in presence of VAMP2 after 1 day from transfection; then, at day 3 both SNAREs are strongly inhibited with respect to controls. At day 1, VAMP7i does not have negative effect on VAMP7 on PDL, but it strongly decreases VAMP2-mediated exocytosis on LN, particularly after 3 days in culture; a sensitive increase is visible for other conditions. Interestingly, VAMP7a and VAMP7i seem to preferentially act on VAMP2 (Figure S4), whereas VAMP7dh acts on VAMP7, suggesting that in immature neurons, complex pathways and multiple players are involved in the control of neuritogenesis.



**Figure 34. Influence of VAMP7 isoforms on both VAMP7 and VAMP2-mediated exocytosis.**  
 Comparison between pHluorin-VAMP7 and pHluorin-VAMP2 experiments, considering influence of both VAMP7 isoforms, coating conditions (PDL, LN) and neuronal development.

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# DISCUSSION

## **1.1 Human VAMP7 isoforms: domain architecture, tissue-specificity and subcellular targeting**

Increased complexity in higher organisms is achieved by fine tuning of gene functions without needing a proportional increase in gene number. This tuning can be mediated by several mechanisms at different levels (ex. DNA methylation, phosphorylation, miRNAs); recent estimates indicate that nearly 60% of the human genome generates splice-variant mRNAs, of which 80% result in changes in the primary amino acid sequence (Modrek *et al.*, 2002). Humans have only one gene encoding for the SNARE VAMP7, whereas plants display more genes. When this work started, the existence of an AS mechanism governing SYBL1 locus and resulting in multiple variants production was just discovered; thus, the first aim was to characterize such splice variants that show varying domain architecture and probably function(s). Real time RT-PCR analyses revealed the subset of isoforms expressed at physiological level; interestingly, they retain a particular domain architecture: two non-SNARE isoforms consist of the LD alone (VAMP7i) or “membrane-anchored” LD (VAMP7j); two  $\Delta$ -longin isoforms (VAMP7dh and VAMP7c,- Martinez-Arca *et al.*, 2003-) share synaptobrevin-like architecture with different NT extensions. Finally, VAMP7b is only partially “non-SNARE” because it still keeps the NT part of the SNARE, which is crucial for intramolecular binding to the LD, closed conformation and targeting (Mancias and Goldberg, 2007). Moreover, tissue-specific expression of these isoforms suggests that AS at the SYBL1 locus is developmentally regulated along cell lineage/tissue differentiation. Alternative mRNAs encoding variants with different domain combinations are expressed at levels ranging 5-12% of VAMP7a in cell lines. In differentiated tissues the aggregate level of non-SNARE, inhibitory variants is comparable or even higher than VAMP7a. Moreover, interesting evidence correlates VAMP7 isoforms with a developmentally-regulated expression in brain, where VAMP7 is supposed to have a specific function (extensively reviewed in Chaineau *et al.*, 2009). More in general, each isoform might contribute to finely tune VAMP7-mediated pathways involved in different aspects of cell physiology, according to different cell types: indeed, several regulatory mechanisms do not require 1:1 interactions. This is particularly important when considering that SCL analysis also revealed a nuclear localization for the LD-only isoform VAMP7i. The difficulty in transfecting this isoform, the controversial point of the inhibitory

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role suggested for LD (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Martinez-Arca *et al.*, 2003) prompt us to further investigate on possible role of VAMP7i at the nucleus. Indeed, VAMP7i consists only of the LD, similar to some longin-like proteins (Rossi *et al.*, 2004) like sigma AP-3 (Collins *et al.*, 2002) and sedlin (Jang *et al.*, 2002). Intriguingly, sedlin has been recently reported to show nuclear localization and to interact with transcription factors (TF) and TF-binding proteins (Jeyabalan *et al.*, 2010; Liu *et al.*, 2010). Thus, next experiments will try to better characterize this intriguing VAMP7i feature. When considering other isoforms, VAMP7b and VAMP7a show different SCL, in agreement with evidence that other protein regions/domains can modulate SC targeting capacity of the LD (Pryor *et al.*, 2008; Chaineau *et al.*, 2009). This is evident when comparing VAMP7i and VAMP7j, both sharing the LD and missing the SNARE motif: the presence of the TM anchor in VAMP7j is able to prevent nuclear localization. Production of non-SNARE isoforms by AS further strengthens the concept that the LD can also play an important role in trafficking mechanisms independently on the SNARE motif. In agreement with such an idea, data on neurite outgrowth link VAMP7i to the regulation of vesicle pools during exocytosis.

Given the peculiar domain architecture, VAMP7 isoforms can represent a useful tool in understanding the function(s) of the LD and other regions/domains as SC sorting signals. Previous results from other groups only came from artificial constructs: the SCL of several chimeric VAMP7 proteins was compared in plants (Uemura *et al.*, 2005), whereas separated VAMP7 domains were used to investigate both SCL and effects on mammalian cell physiology. In particular, a synaptobrevin-like  $\Delta$ -longin fragment and an LD-only constructs were found to elicit somewhat opposite effects (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001). Indeed, such “artificial” fragments mime the “natural” domain architecture of isoforms VAMP7dh and VAMP7i, which are thus the physiological mediators of equal/similar effects. Indeed, both VAMP7dh and  $\Delta$ -longin fragment partially co-localize with VAMP7a, while both VAMP7i and LD-only do not, thus showing congruent SC sorting in two different cell types (neuronal and epithelial). Furthermore, labeling with several organelle markers highlighted that co-localization between VAMP7dh and the main isoform VAMP7a is in regions like PM and EEs. Additional investigation using different, recombinant VAMP7a cytosolic fragments showed that nuclear entrance is observed in the absence of a TM region. Preliminary results concerning another longin, Sec22b and its recombinant fragments without TM region show the same behavior (Rossi V, personal communication), therefore, a nuclear localization for the LD is not peculiar to VAMP7 and seems to be conserved among longins. Furthermore,

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differences in VAMP7 fragment length were used to study the relationship between VAMP7 open-closed conformation and SCL, recently investigated using NMR analyses (Vivona *et al.*, 2010). However, all fragments without the TM showed the same localization, suggesting that either closed or open, LD conformation is not relevant for nuclear targeting.

## 1.2 VAMP7 and neuronal development

When considering different SCL shown by the VAMP7 isoforms, it is evident that VAMP7a is spread over multiple locations along its trafficking routes, because of interactions mediated by all its domains, whereas alternative variants missing either the LD or the SNARE motif, or even showing new regions, cannot share all interactors with the main isoforms hence are likely to influence specific trafficking events. Early evidence suggesting a peculiar physiological role for VAMP7 splice variants came out from qPCR analyses on human cell lines and tissues, as modulation of their mRNA levels is clearly evident along different developmental stages, from SH-SY5Y cells to fetal and adult brain. This prompted us to further investigate the role of VAMP7 isoforms in neuronal development. The relevance of this work can be better appreciated when taking into account that in mammals - and in particular in humans - brain is devoted to accomplish multiple, very complex functions, because of the much higher behavioral complexity of these organisms and in particular of primates and humans. Neuronal connections, plasticity etc. depict an intricate system in which developmental and environmental conditions need to be continuously controlled and maintained. Cortical/hippocampal neurons are considered to be an elective model for studying mammalian neuron physiology (Pfenninger, 2009); therefore, environmental conditions and developmental stage of embryonic cultures *in vivo*, as well as time and modality for protein overexpression *in vitro* are frequently not comparable in different works or even in the same set of experiments. Neurons cultured with or without ECM components, prepared at different embryonic stages, transfected with diverse methods, at different points during development may result in difficulties to compare data. Most often, collected data and new results are poorly supported by a comparative discussion that was taking into account protocol and environmental differences, making it more and more difficult to properly and rigorously define and distinguish molecular pathways and protein roles/effects. Alberts and colleagues described differences in cellular response to LD transfection in dependence to embryonic age (apoptosis phenotype against cell survival) (Alberts *et al.*, 2003). In addition, the signaling cascade for

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neuritogenesis activation is sensitive to cell types and/or developmental stages (Chacòn and Fazzari, 2011). Other evidence highlights the influence of the cell substrate on cell motility and axon outgrowth (Geiger *et al.*, 2001; Tojima *et al.*, 2007; Murray *et al.*, 2009; Myers *et al.*, 2011); strikingly, this point is well-considered for designing of specific biomaterials in cell therapy. Thus, finding the best conditions should be the first aim of every study, in particular considering the brain complexity (Geiger *et al.*, 2001; Jang *et al.*, 2010; Pertz, 2011; Myers *et al.*, 2011).

This work took into account all considerations above to define best parameters for characterizing VAMP7 isoforms in neuronal developments. First, quantitative analyses of VAMP7 isoform mRNA expression were performed on differentiated neuroblastoma cells. It has to be stressed that, although primary neurons are an elective model system, obviously they cannot be obtained from human brains. Given that available primary neurons are derived from mice or rats brains, they are not suitable for investigating physiological, endogenous levels of human mRNAs hence human cells only should be used for this aim. In early differentiation steps (1-3 days), a progressive increase in minor isoforms (in particular, non SNARE ones) levels is observed, followed by a decrease for the non-LD variant VAMP7dh. Then, both human cells and mouse cortical/hippocampal neurons were used for checking isoform overexpression effects, following two different transfection protocols in order to better follow effects with respect to overexpression time. Effects in human SH-SY5Y cell line were more clearly evident when using Protocol B (transfection 24h before each time point for cell imaging), whereas Protocol A (transfection before differentiation) produced best results when using primary mouse neurons. Although it is well known that cell lines and primary cultures cannot be directly compared due to different behavior *in vitro*, choosing such best conditions was of great help in finding a common rationale for the results. Proper culture conditions are essential for avoiding non physiological responses; to this aim, both neuronal cell lines and rat/mouse neurons are usually cultured on either neutral substrate (PDL) or different feeder layer/coating for the most common brain extracellular environment (glial cells, gelatin, collagen, matrigel, LN, adhesion molecules) (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001; Alberts *et al.*, 2003/2006; Burgo *et al.*, 2009; Hua *et al.*, 2011; Cotrufo *et al.*, 2011). Anyway, during their differentiation, neurons reach different part of the developing brain and are subjected to specific environments in proximity or not of ECM components (Chen *et al.*, 2003; Yin *et al.*, 2003; Chen *et al.*, 2009). Although SH-SY5Y cells and primary neurons display morphological differences in neurite outgrowth (in terms of protrusion



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number, length, synaptogenesis etc.) (Meldolesi *et al.*, 2011), using the same coating conditions (PDL) made comparison more manageable. Finding that the non-LD isoform VAMP7dh acts as an enhancer and the LD-only isoform VAMP7i as a breaker provided a physiological rationale to preliminary observations with artificial constructs (Martinez-Arca *et al.*, 2000; Martinez-Arca *et al.*, 2001). Using also a primary culture model, it was found that such opposite effects are further influenced by coating conditions. Further relevant information was inferred by statistical analyses of peculiar, morphological parameters: VAMP7dh seems particularly involved in the control of neurite length, rather than of neurite branching; on the contrary, non SNARE isoforms show inhibitory effect on both neurite/axon elongation and branching. This is in agreement with current evidence on independent regulation of axon outgrowth and branching, during development (Hutchins and Kalil, 2008).

Neuritogenesis is based on several mechanisms and players: control of cytoskeletal remodelling, PM addition, receptor endo/exocytosis, extracellular signaling for growth cone direction. In such space-regulated molecular processes, SNARE involvement cannot be restricted to a single, simple role. At the same time, distinguishing between macroscopic, morphological observations that concern whole, cellular responses and single biological/molecular processes is essential. They are strictly related each other, but the latter is only part of a more complex phenotype. Since VAMP7 role in neuritogenesis is suggested to rely on SC sorting capacity of driving exocytosis routes (Alberts *et al.*, 2003), this pathway was also studied to clarify cross-talk with the deeply investigated synaptobrevin VAMP2. A surprising discovery was the implication of VAMP7 isoforms on VAMP2-mediated exocytosis, when using LN coating. Taken together, data from both morphological and exocytosis analyses reveal that the synaptobrevin-like isoform dh is a general neuritogenesis enhancer, whereas VAMP7i inhibits neurite outgrowth and this is even more evident when using LN coating. This is in agreement with the idea that multiple pathways are involved in the control of neuritogenesis in immature neurons: in particular, SNARE dependent exocytosis is finely tuned both spatially and temporally to properly challenge progressive changes in neuronal morphology and physiology. In such a general view, relevance of having a number of specific regulators, such as multiple VAMP7 isoforms, instead of just a few basic players, i.e. main isoforms only, can be easily argued. Results from co-expression experiments with different combinations of VAMP7 isoforms and VAMP7/VAMP2 are just in this track and provided us with a better representation of complex, physiological cross-talks within the differentiating cell. Both VAMP7 and VAMP2 are present in immature

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neurons and can undergo neuritogenesis; nevertheless cell exposure to particular environments seems to add selectivity for specific pathways (Gupton and Gertler, *Dev Cell* 2010). In a LN environment, coordinate action of VAMP7a and VAMP7i displays a strong, inhibitory effect on VAMP2-mediated exocytosis, in particular at day 3. This is likely to address neuritogenesis to VAMP7 pathway. An artificial interaction between VAMP7 LD and VAMP2 SNARE motif was previously tested in some experiments, showing reduced fusion capacity respect to VAMP2 alone (Martinez-Arca *et al.*, 2003). This results clearly remind the LD inhibitory role when VAMP7 is in closed-conformation and it is tempting to speculate about possible heterologous binding to other SNARE motifs, as confirmed by our results in neuronal culture. Thus, the LD can act as a competitor for other SNARE motifs, regulating membrane fusion in particular membrane area or in determinate moments of development. In the same conditions (LN coating), at day 1 the synaptobrevin-like isoform VAMP7dh directly acts on VAMP7 pathway, increasing vesicle fusion. In later stages (3 days), VAMP7 is the only SNARE able to mediate exocytosis, possibly because VAMP2 is inhibited by VAMP7i. It is important to remark that exocytosis analyses are referred to fusion events occurring in the soma, whereas morphological data clearly consider the shape of a whole, developing neuron. It is known that exocytosis usually takes place in a patchy manner along both neurites and in somata; nevertheless, events at the growth cone greatly promote neurite outgrowth, whereas along neurites they are more important for other functions, such as neurite branching or formation of nascent synapses (Sytnyk *et al.*, 2002; Chernyshova *et al.*, 2011).

The presence at the same time of two proteins with similar domain architecture (VAMP2 and VAMP7dh) but different SNARE motif (Rossi *et al.*, 2004) suggest that diverse vesicle pools could coexist and be driven by either VAMP7dh or VAMP2-mediated exocytosis. At early differentiation stages, VAMP2 vesicles are also secreted in absence of any formed synapse, but they do not directly contribute to membrane expansion (Matteoli *et al.*, 1992). In agreement with evidence from this work, recently Ramirez and coworkers set up a system for contemporary detecting VAMP2 and VAMP7-mediated exocytosis, highlighting independent action of the two SNAREs (Ramirez *et al.*, 2012). The overall exocytic pathway acting during neurite outgrowth is likely very complicate, hence not completely explainable by results from the “single” pH-sensitive system used in this work. It is well-known that neurons within the CNS offer one of the more striking examples in biology of the specialization of the SC structure–function relationship. Therefore it is quite unlikely that a single, main master would drive all neuritogenesis pathways (as extracellular cues), and a more balanced view is

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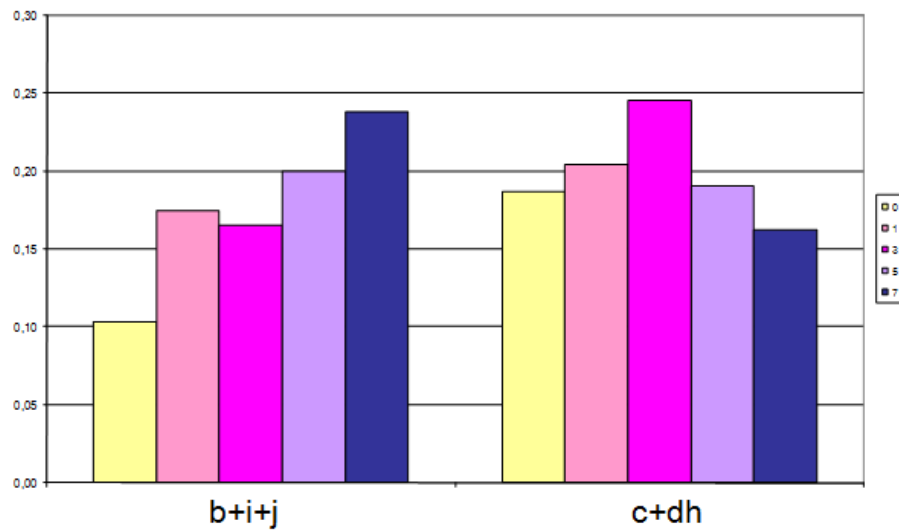
needed, where a straight collaboration between cytoskeletal rearrangement machinery (in particular actin accessory proteins) and SNAREs occurs. A peculiar relationship between Arf2/3 and VAMP7 or Ena/VASP and VAMP2-mediated exocytosis was reported (Gupton and Gertler, 2010); the Ena/VASP pathway seems to be linked also to chemotropic guidance by Netrin-1 (a LN-like protein) (Dent *et al.*, 2011). Further evidence (Lafuente *et al.*, 2004; Moore and Kennedy, 2006) suggests that VAMP2 exocytosis might be responsive to the same extracellular cues of VAMP7, in agreement with indications from this work on the inhibitory role of VAMP7i on VAMP2. Different stimuli are involved in cytoskeletal remodeling, using several mediator as Rac, Cdc42, src, FAK (Gupton and Gertler, 2010); L1-cam (Alberts *et al.*, 2006), Netrin-1 (Cotrufo *et al.*, 2011; Dent *et al.*, 2011), LN-integrin signaling; this clearly highlights the complexity of mechanisms involved in neurite outgrowth and the importance of a fine regulation, for example by VAMP7 AS. Indeed, new evidence indirectly and/or directly underlines the crescent importance of VAMP7 isoforms for the neuronal system. Another  $\Delta$ LD artificial construct (that does not completely match with the previous one and the VAMP7dh isoform) was recently used for performing similar exocytosis analyses at the synaptic boutons of mature neurons, showing an interesting behavior (Hua *et al.*, 2011). It localizes at the same membrane of the main VAMP7a and affects the behavior of both VAMP7 and VAMP2, but not VGLUT1, suggesting a specific role for a subset of synaptic vesicles. It can also contribute to evoked release (recycling pool) mediated by AP-1, even if VAMP7 is normally seen in the resting pool, pointing out the idea that the absence of LD is useful for changing the physiological role in particular cellular responses. In conclusion, current evidence is drawing a complex picture but it is still unable to clearly define and separate (whenever possible) all roles played by VAMP2 and VAMP7 in both immature and mature neurons. In such a mosaic, VAMP7 isoforms might account for some missing pieces. For instance, evidence that KO mice for VAMP7 protein shows no particular anomalies (Sato *et al.*, 2011) is difficult to be explained when considering only the main isoform, and could rely upon some isoform-dependent compensatory effects.

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## CONCLUDING REMARKS

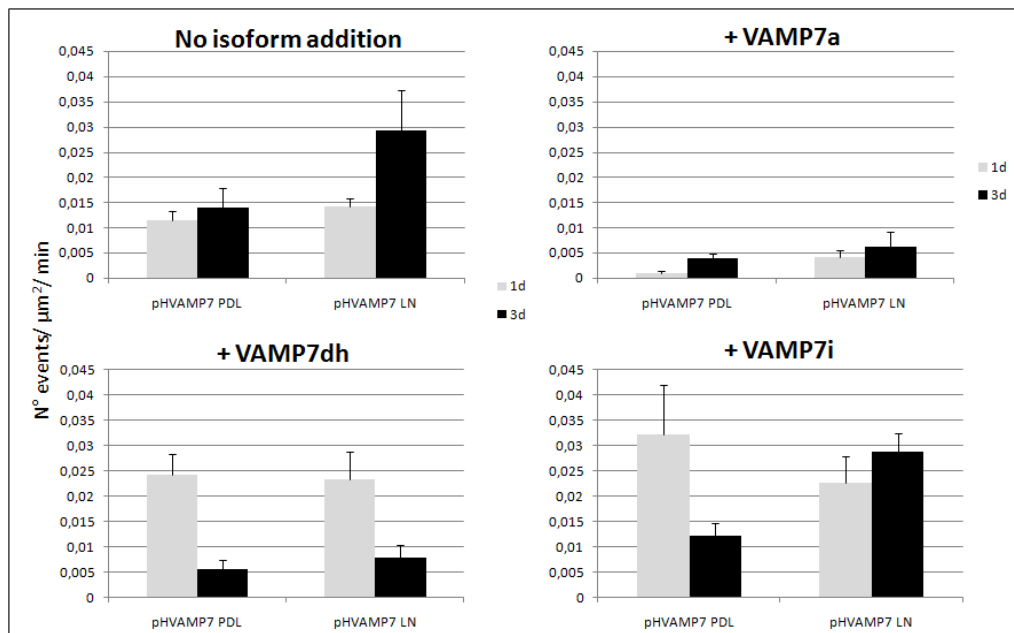
The existence of an AS mechanism for human gene SYBL1 and the involvement of VAMP7 protein isoforms in the fine modulation of neurite outgrowth is a further example of increased complexity of pathway regulation in higher organisms and, in particular, in the nervous system. Moreover, since the conservation of the LD among all Eukaryota, it is noteworthy its role in directing this fine system with effects on other isoforms and/or SNAREs. Cross-talk between VAMP7 and VAMP2-mediated exocytosis might depend on LD ability to inhibit SNARE complex formation and protein interactions for recycling from the PM. Intriguingly the only isoform able to bypass nuclear membrane seems to play an important role in neuronal regulation. Furthermore, evidence that VAMP7i shares its LD fold with longin-like proteins such as sedlin that is known to interact with TF and TF-binding proteins, suggests to perform further experiments aimed at identifying its nuclear interactors and check whether the inhibitory role can be mediated (also) by modulation of transcriptional regulation. Despite an important role for LD is confirmed, all three VAMP7 domains (LD, SNARE motif and TM region) were found to co-determine SC sorting, whereas a role for open/closed-conformational switch could not be found. Understanding the main sorting signals and thus, the contribution of each domain to SC sorting in key regulatory events is not only relevant for defining molecular mechanisms in cell physiology, but it is also an essential biotechnological goal for drug delivery (Rajendran *et al.*, 2010). This field needs to take advantage by cell biological strategies for avoiding frequent toxicity and therapy failure due to other used strategies. In this light, information carried out by cellular machineries is a real promise, in particular in the CNS (Miyashiro *et al.*, 2009). Another biotechnological implication of this work relies on the close relationship between extracellular cues, VAMP7 isoforms and neuronal development. Thus, VAMP7 splice variants can really represent a useful tool for studying novel stimuli in order to drive neuritogenesis and to improve ongoing efforts in regenerative medicine (Liu *et al.*, 2012). Indeed, the design of functionalized polymers that can elicit specific biological responses and the development of methods to fabricate new devices that incorporate biological cues are of great interest to the biomedical community.

## SUPPLEMENTARY MATERIALS



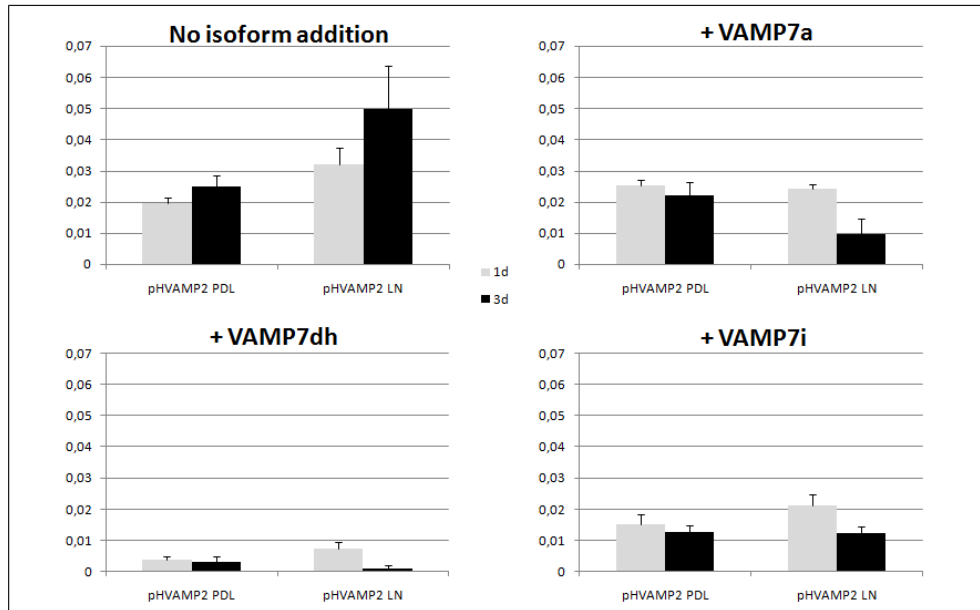
**Figure S1. Modulation of non-SNARE and non-LD isoforms during neuronal differentiation.**

Quantitative analysis of RA-treated SH-SY5Y are shown as total contribution of the two distinct VAMP7 subfamilies of non-LD (b+i+j) and non-SNARE(c+dh) splice variants respect to the main VAMP7a.

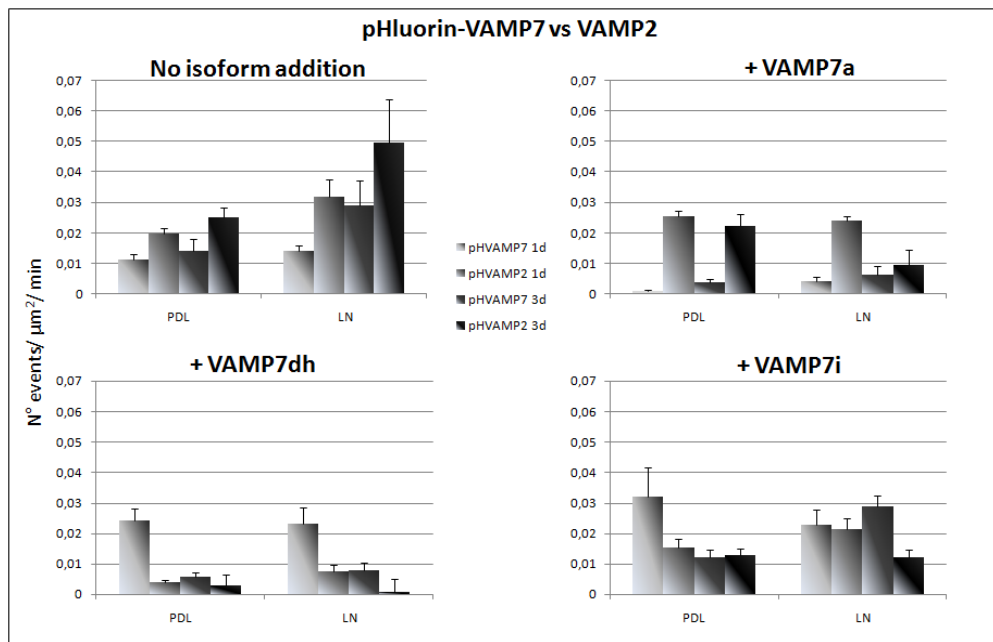


**Figure S2. Influence of VAMP7 isoforms on VAMP7-mediated exocytosis in the soma.**

Immature neurons were co-transfected, live cell imaging was performed, fusion events were counted and graphs report statistical data as indicated in Figure 16 legend. Data are shown considering VAMP7 isoform contribution to exocytic events.



**Figure S3. Influence of VAMP7 isoforms on VAMP2-mediated exocytosis in the soma.** Immature neurons were co-transfected, live cell imaging was performed, fusion events were counted and graphs report statistical data as indicated in Figure 16 legend, except for using pHuorin-VAMP2 instead of pHuorin-VAMP7. Data are reported considering VAMP7 isoform contribution to exocytic events.



**Figure S4. Influence of VAMP7 isoforms on both VAMP7 and VAMP2-mediated exocytosis.** Comparison between pHuorin-VAMP7 and pHuorin-VAMP2 experiments, considering influence of both VAMP7 isoforms, coating conditions (PDL, LN) and neuronal development. Data are reported considering VAMP7 isoform contribution to exocytic events.

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