



● INVITED REVIEW

SNARE complex in axonal guidance and neuroregeneration

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Abstract

Through complex mechanisms that guide axons to the appropriate routes towards their targets, axonal growth and guidance lead to neuronal system formation. These mechanisms establish the synaptic circuitry necessary for the optimal performance of the nervous system in all organisms. Damage to these networks can be repaired by neuroregenerative processes which in turn can re-establish synapses between injured axons and postsynaptic terminals. Both axonal growth and guidance and the neuroregenerative response rely on correct axonal growth and growth cone responses to guidance cues as well as correct synapses with appropriate targets. With this in mind, parallels can be drawn between axonal regeneration and processes occurring during embryonic nervous system development. However, when studying parallels between axonal development and regeneration many questions still arise; mainly, how do axons grow and synapse with their targets and how do they repair their membranes, grow and orchestrate regenerative responses after injury. Major players in the cellular and molecular processes that lead to growth cone development and movement during embryonic development are the Soluble N-ethylmaleimide Sensitive Factor (NSF) Attachment Protein Receptor (SNARE) proteins, which have been shown to be involved in axonal growth and guidance. Their involvement in axonal growth, guidance and neuroregeneration is of foremost importance, due to their roles in vesicle and membrane trafficking events. Here, we review the recent literature on the involvement of SNARE proteins in axonal growth and guidance during embryonic development and neuroregeneration.

Key Words: SNARE; vesicle associated membrane protein; synaptosomal associated protein; axon; guidance; neuroregeneration; nervous system; cell membrane

Soluble N-Ethylmaleimide Sensitive Factor (NSF) Attachment Protein Receptor (SNARE) Proteins and Neurite Outgrowth

Neuronal differentiation, axonal growth and guidance involve coordinated changes in the cellular cytoskeleton, protein and membrane trafficking processes. Early neurite outgrowth appears to involve protein trafficking machineries responsible for exocytosis to the plasma membrane utilizing many mechanisms that are also found in non-neuronal cells. These membrane trafficking events are usually directed towards several neurites, but can also be switched to concentrate on the growth of a single axon.

Membrane transport to the axonal growth cone is vital for the axon to grow, develop and move. Axonal membrane transport is mediated by the same machinery that governs vesicular trafficking in other parts of the cell. This machinery works in four steps: (1) Budding, in which coat proteins mediate the junction between a membrane donor compartment and motor proteins that direct the transport of vesicles through the cytoskeleton; (2) Movement, when the vesicle moves towards its destination along a cytoskeletal track *via*

molecular motors; (3) Tethering of the vesicle with its target membrane; and (4) Fusion of transmembrane SNAREs (on apposing membranes (Cai et al., 2007).

SNAREs are a large family of small membrane proteins (with more than 60 members in mammalian cells), characterized by the presence, in almost all of them, of the about 60 amino acid SNARE domain. SNARE proteins are related to three different neuronal protein families: vesicle associated membrane protein (VAMPs), Syntaxins, and synaptosomal associated proteins (SNAPs) (Ungar and Hughson, 2003). Mechanisms mediated by SNARE proteins are highly conserved and many of the SNARE proteins present in invertebrates are conserved in vertebrates (Teng et al., 2001). SNARE proteins were originally identified for their ability to regulate vesicle release at mature synapses (Chen and Scheller, 2001). In fact, the spontaneous and calcium guided interaction between members of the SNARE family allows for the quantal release of neurotransmitters at the synaptic cleft, which in turn guarantees the efficacy in synaptic transmission (Sudhof, 2013).

Membrane fusion is thought to occur by the formation of a SNARE complex through the association of specific

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SNARE proteins: a SNARE on a transport vesicle (v-SNARE) assembles with its/their cognate SNARE-binding partner on the appropriate target membrane (t-SNARE). Association between SNAREs is carried out through their SNARE domains and is highly regulated *in vivo* by several accessory proteins. Members of the Vesicle Associated Membrane Protein (VAMP) subfamily act as v-SNAREs whereas proteins from the Syntaxin and SNAP subfamilies act as t-SNAREs. The best characterized SNARE complex is the one that mediates the Ca²⁺ dependent neurotransmitter exocytosis at the synaptic cleft. It consists of a four-helical bundle formed by the v-SNARE Vamp2, and the t-SNAREs Stx1 and Snap25. However, other types of SNARE complexes can also be formed in different cells or for different functions. For example, spontaneous synaptic vesicle release seems to depend mostly on the v-SNARE Vamp7 instead of Vamp2 (Kavalali, 2015). Differentiating the evoked release of neurotransmitters from the spontaneous secretion of vesicles maybe necessary for the communication among neurons. Also, tonic exocytosis, occurring after repetitive stimulation of vesicle release, requires Vamp4 instead of Vamp2 and other types of proteins that allow for the recruiting of the recycling pool of synaptic vesicles and not of the readily releasable pool. In another example, exocytosis in glial cells needs mostly the interaction among Stx1, SNAP23, and VAMP3 (cellubrevin) (Schubert et al., 2011). In addition, diverse SNARE complex composition accounts for the differential sorting of AMPA (at excitatory synapses) and GABA (at inhibitory synapses): [SNAP25-STX1A/B-VAMP2] and [SNAP23-STX1A/B-VAMP2] respectively (Gu et al., 2016). And, regulated exocytosis of the AMPA receptor during long term potentiation involves a unique SNARE fusion machinery containing STX3 (Jurado et al., 2013). So, differential composition/configuration of SNARE complexes can mediate different functions both in the same cell and at distinct cell types (Kasai et al., 2012; van Keimpema and Kroon, 2015).

Neurite outgrowth involves the addition of new membrane, mainly at the tips of elongating axonal processes, coordinated with a dynamic cytoskeletal elongation. Exocytosis of diverse membrane vesicles of around 150 nm in diameter called plasmalemma precursor vesicles (PPVs) or growth cone particles (GCPs) is believed to contribute to the supply of new membrane in developing axons. In sympathetic neurons and PC12 cells, lysosomal and enlargeosome exocytosis, respectively, have been described to be also involved in neurite outgrowth (Arantes and Andrews, 2006; Colombo et al., 2014). Recently, a non-vesicular mechanism consisting on a lipid flow from the endoplasmic reticulum (ER) in close apposition to the plasma membrane has been proposed to account for bulk neurite outgrowth in cultured cells (Petkovic et al., 2014). This non-vesicular mechanism involved in membrane expansion requires SNARE proteins Sec22b and Stx1 to generate a SNARE bridge that contributed to plasma membrane expansion (Arantes and Andrews, 2006; Petkovic et al., 2014).

Evidence for the implication of SNAREs in neurite outgrowth date from around two decades ago, essentially from *in vitro* experiments. First, Snap25 requirement in axonal outgrowth was demonstrated by using inhibitory antisense oligonucleotides which prevented neurite elongation (Osen-Sand et al., 1993). Subsequent reports using different approaches, either employing botulinum toxins or protein overexpression confirmed the involvement of Snap25 in neurite outgrowth and sprouting (Moriyama et al., 1999; Shirasu et al., 2000). However, mice deficient for Snap25 did not display neural circuitry defects, suggesting the existence of compensatory mechanisms in action by other members of the SNARE family. Consistent with this idea Stx1a, Stx3, Stx6, Stx13, Snap23, Vamp2, Vamp4 and Vamp7 have also been implicated in neurite outgrowth in roles that vary from membrane trafficking to early endosomal and trans-Golgi network trafficking, exocytosis of endosomes and PPVs (Igarashi et al., 1996; Hirling et al., 2000; Shirasu et al., 2000; Zhou et al., 2000; Martinez-Arca et al., 2001; Darios and Davletov, 2006; Kabayama et al., 2008; Colombo et al., 2014; Grassi et al., 2015).

Evidence for differential SNARE protein expression and function, which can affect the neuronal growth in different cell types, is starting to appear. The best example constitutes the contribution of different isoforms of syntaxin1 protein during embryonic development. In mice, loss of function of STX1A and STX1B give rise to different phenotypes. Whereas STX1A mice have apparently just minor problems, STX1B ones die at early post-natal age. These observations have been attributed to isoform differences, expression pattern and function (Ruiz-Montasell et al., 1996; Kofuji et al., 2014; Mishima et al., 2014; Wu et al., 2015).

Taken together, all these results indicate that more experiments are needed to clarify the involvement of the SNARE complexes in exocytosis during neurite outgrowth.

SNARE Proteins and Endocytosis

As previously mentioned, SNARE proteins have been mainly known to be involved in vesicle release, both in the course of neurotransmission and during the elongation and guidance of the growth cone in development. However, recent findings indicate that SNARE proteins contribute also to the endocytic process, mainly after vesicle release at mature synapses. Endocytosis is needed to recover synaptic vesicles from places on the membrane in which they previously fused. In neurons, two types of endocytosis are generally accepted: a slow, clathrin-dependent endocytosis requiring the classical endocytic proteins as dynamin, AP2 and auxilin (Dittman and Ryan, 2009) and a fast endocytosis, necessary for a faster retrieval of vesicles and proteins as needed for kiss and stay or kiss and run mechanisms of release (Smith et al., 2008). During fast endocytosis synaptic vesicles would keep their identity and also localization. Slow or fast endocytosis are differently activated at synapses matching the activity of the specific synapse and its physiological requirements (Smith et al., 2008; Watanabe et al., 2013).

One of the first findings involving SNARE proteins in

endocytosis was found in *Saccharomyces cerevisiae*, where when abolishing the function of a t-SNARE, belonging to the syntaxin protein family, the early steps of the endocytic pathway were imbalanced (Seron et al., 1998). A more critical analysis on the different types of endocytosis in the nervous system showed that fast endocytosis was blocked knocking down Vamp2 in mouse hippocampal synapses (Deak et al., 2004), while abolishing Snap25 at cultured hippocampal neurons did not avoid sucrose induced dye uptake by endocytosis (Bronk et al., 2007). These results appeared to be in conflict with some others in which cleavage of synaptobrevin/Vamp2 with tetanus toxin at a giant nerve terminal, the calyx of Held, blocked slow endocytosis (Hosoi et al., 2009) whilst Snap25 was shown to mainly regulate slow-endocytosis in rat cultured hippocampal synapses (Zhang et al., 2013).

Another study examined all these findings and together with their experiments came to the conclusion that Vamp2 is needed for both slow and fast-endocytosis, as well as Snap25 and Stx1, suggesting the importance of all vesicular and membrane-targeted SNARE in this important process (Xu et al., 2013).

The next steps will be to understand which are the molecular interactions allowing SNARE proteins to differentially participate in exocytosis and endocytosis. At the moment, it has been suggested that the N-terminus of the SNARE motif of Vamp2 binds to the ANTH domain of endocytic adaptors AP180 and Clathrin Assembly Lymphoid Myeloid leukemia (CALM), both of which are involved in endocytosis (Koo et al., 2011; Miller et al., 2011). SNAP-25 is able to bind to intersectin, another endocytic protein, with the same strength it binds to Stx1 (Okamoto et al., 1999). Finally, Stx1 may interact with dynamin, a GTPase mediating vesicle fission after endocytosis (Galas et al., 2000). Overall, more experiments are needed to explore the role of SNARE proteins in endocytosis as it might be of great interest also in elucidating processes occurring during axonal regeneration.

The SNARE Complex in Axonal Guidance in Vertebrates and Invertebrates

Axonal growth cone navigation involves the coordination of cytoskeletal rearrangements with the regulation of adhesion components and membrane trafficking. The growth cone is a unique structure capable of guiding axons to their final destinations. Within the growth cone, extracellular guidance cues are interpreted and then transduced into physical changes and axonal movement. Growth cones are filled with vesicles and express most SNARE and exocyst proteins (Sabo and McAllister, 2003; Yuan et al., 2003; Condeelis et al., 2005; Chernyshova et al., 2011; Fujita et al., 2013). Many reports indicated that vertebrate axon guidance mechanisms require the participation of SNARE-mediated exocytosis for chemoattraction and endocytosis for repulsion (Cotrufo et al., 2011, 2012; Zylbersztejn et al., 2012; Tojima et al., 2014). For example, the vSNARE VAMP2 is required for L1-mediated chemoattraction and for Sema3A-induced chemorepulsion *in vivo* (Tojima et al., 2007; Zylbersztejn et al., 2012). Compatible with this, Vamp2 deficient mice show a

disorganized corpus callosum similar to the loss of Sema3A (Zylbersztejn et al., 2012). Furthermore, Stx1 and Vamp7 are required for Netrin-1-mediated attraction of axons and migrating neurons, whereas Vamp2 function is dispensable in this process in cultured mouse neurons (Cotrufo et al., 2011, 2012). However, Snap25 and Vamp2 deficient mice show virtually no neural circuitry defects but display a severe alteration of evoked synaptic activity (Schoch et al., 2001; Molnar et al., 2002; Washbourne et al., 2002). And TI-VAMP-deficient mice display behavioural defects but no alterations in gross brain morphology (Danglot et al., 2012). In addition, the knock-out (KO) mice currently available for Syntaxin-1A (Stx1a) isoform show only mild cognitive defects and a normal brain structure (Fujiwara et al., 2006). And mice KO for the other Stx1 isoform, Stx1b, revealed that STX1B is dispensable for the formation of the mouse neuromuscular junction (NMJ) but required to maintain the efficiency of neurotransmission (Wu et al., 2015). Accordingly, it was suggested that Stx1a and Stx1b are functionally redundant, leading to the need of creation of a double KO mouse by removing both isoforms. Recently, Vardar and colleagues achieved this and showed that Stx1 is essential for the maintenance of developing and mature neurons and also for vesicle docking and neurotransmission (Vardar et al., 2016). However, no analysis of axonal guidance phenotypes was performed in Stx1 double knockout mice. So, currently there is a clear need for more animal models that can clarify the different results obtained from *in vivo* genetic approaches versus *ex vivo* petri dish experiments.

Recently, this has been studied using chick and *Drosophila melanogaster* embryos, two models amenable for gene manipulation followed by *in vivo* analysis of axonal guidance. In both model systems, the participation of SNARE proteins in the development of the peripheral nervous system (PNS), in particular in the guidance of motor axons has been analysed (Barrecheguren et al., 2017). It was shown that loss-of-function of SNARE proteins leads to severe guidance phenotypes in motor axons highlighting a role for neurotransmitter-related SNARE proteins in motor axon guidance in both vertebrates and invertebrates (Barrecheguren et al., 2017).

Drosophila melanogaster presents neural expression of SNARE complex components homologous to the vertebrate complex (DiAntonio et al., 1993; Cerezo et al., 1995; Schulze et al., 1995; Risinger et al., 1997; Moussian et al., 2007). Mutations in components of the core SNARE complex give rise to synaptic transmission and neurotransmitter release phenotypes (Broadie et al., 1995; Schulze et al., 1995; Littleton et al., 1998). In addition, the *Drosophila* Syntaxin1 homologue, *Syntaxin1A* (*Syx1A*) has been reported to affect the properties of neuronal membranes (Schulze and Bellen, 1996). *Drosophila* embryos mutant for *Syx1A* presented defects in axonal navigation and fasciculation (Barrecheguren et al., 2017). These findings show that defects linked to SNARE protein downregulation are clearly connected to axonal guidance mechanisms. Previous *in vitro* studies indicated that various SNARE proteins are required for axonal guidance mechanisms linked to Netrin1/DCC and Class III Semaphorins/Plexins (Tojima et al., 2007; Cotrufo et al.,

Table 1 GeneBank nomenclature for the Sensitive Factor (NSF) Attachment Protein Receptor (SNARE) complex proteins appearing in this article

| Homo sapiens | Rattus norvegicus | Mus musculus | Gallus gallus | Drosophila melanogaster |
|---|-------------------|--------------|---------------|--------------------------------|
| Syntaxins | | | | |
| STX1A | Stx1a | Stx1a | STX1A | Syx1A |
| STX1B | Stx1b | Stx1b | STX1B | - |
| STX2 | Stx2 | Stx3 | STX2 | - |
| STX3 | Stx3 | Stx3 | STX3 | - |
| Vamp (vesicle associated membrane protein) | | | | |
| VAMP1 | Vamp1 | Vamp1 | VAMP1 | Syb (Synaptobrevin) |
| VAMP2 | Vamp2 | Vamp2 | VAMP2 | n-Syb (neuronal Synaptobrevin) |
| VAMP7 | Vamp7 | Vamp7 | VAMP7 | Vamp7 |
| Snap (synaptosome associated protein) | | | | |
| SNAP25 | Snap25 | Snap25 | SNAP25 | Snap25 |

We kept the human nomenclature for generic mentions to SNARE proteins and each species nomenclature when referring to specific model organisms. Stx: Syntaxin; Syx1A: Syntaxin1A.

2011, 2012), thereby suggesting that the coupling of guidance receptors to the cell machinery regulating exocytosis is a common mechanism in axonal guidance. Results in fly embryos are in accordance with an interaction of *Drosophila Syx1A* with *frazzled* (*fra*, the fly DCC homolog). However, *Syx1A* motor axon phenotypes are stronger than the *beat-la* or *unc-5* compound guidance mutants (Fambrough and Goodman, 1996; Labrador et al., 2005; Zarin et al., 2014). This suggests that *Syx1A* may collaborate with axonal guidance pathways other than Netrin/Frazzled. Accordingly, in *Drosophila* embryos a genetic interaction was detected between *Syx1A* and Robo pathway components (Barrecheguren et al., 2017).

Taking together the knowledge generated from using these many different model systems and approaches and taking advantage of the conservation of SNARE protein function between vertebrates and invertebrates, two main models have arisen to incorporate the involvement of SNARE proteins in axonal growth and guidance. These try to incorporate how guidance signals coordinate spatio-temporally the new membrane addition with the cytoskeletal rearrangements despite many of the details not being well understood yet.

One model proposes that SNARE proteins participate in signalling receptor trafficking (Figure 1A). In this model, the blockade of a particular SNARE protein will affect the exocytic delivery and/or the endocytic receptor turnover and, consequently, affect the chemotropic response. This model has been postulated to account for Vamp2 action during Sema3A chemorepulsion (Zylbersztein et al., 2012) and for the regulation of Robo1 surface expression on commissural axons in vertebrates (Philipp et al., 2012). However, in other systems alternative mechanisms may operate. Another model proposes that SNARE proteins participate in the clustering of receptors onto a specific part of the growing growth cone (Figure 1B). This model accounts for the Netrin-1/DCC-mediated attraction of axons depending on Stx1 and Ti-Vamp/Vamp7 (Cotrufo et al., 2011). In this system,

the blockade of SNARE proteins does not affect the delivery of the receptor DCC to the plasma membrane. As Stx1 physically associates with DCC and this association is enhanced by the binding of the ligand, Netrin-1 activation of DCC receptors results in ligand dependent clustering of DCC/Stx1 complexes in activated membrane domains. It has been postulated that the membrane expansion at these domains will be produced by the fusion of exocytic vesicles mediated by Stx1-Ti-Vamp/Vamp7 association (Cotrufo et al., 2011). The molecular details explaining why in this scenario Stx1 recruits Ti-Vamp vesicles and not Vamp2 ones, as occurs during neurotransmission, are still not known. There is the possibility that both models can be in play to control axon guidance, the SNARE complex acting in receptor trafficking/turnover but also being responsible for receptor clustering onto specific parts of the growing growth cone.

Overall, studies on SNARE proteins in axonal guidance, have presented conflicting data regarding the implication of these proteins directly in axonal guidance at the midline, but seem to suggest that motor axon guidance depends on a functional SNARE complex, which is conserved from invertebrates to vertebrates (Barrecheguren et al., 2017). Current models are missing more studies on the involvement of the SNARE complex in axonal guidance in the midline of both vertebrates and invertebrates.

The SNARE Complex in Neuroregeneration

Injury to the nervous system damages axons, causing their retraction, a widespread loss of synaptic connections, and consequently a deficit of function that can be devastating to the overall organism. Axonal regeneration after damage requires the axon to repair its damaged membrane, redistribute or manufacture what it needs in order to survive, and grow and form new synapses within a more mature, complex environment. For nerve repair to work and regeneration to occur, distinct events have to occur in a coordinate manner. First, anterogradely transported vesicles accumulate at the axons, while others are generated at the severed end to restore a selective barrier to the cut axon. Then, retrograde transport of vesicles along microtubules informs

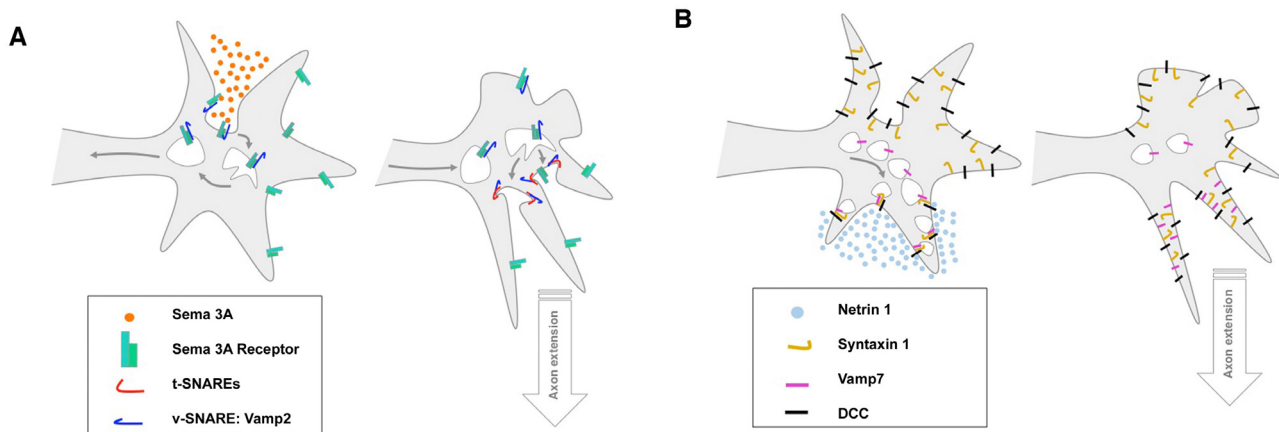


Figure 1 Schematic representation of the different models implicating the Sensitive Factor (NSF) Attachment Protein Receptor (SNARE) complex in axonal guidance.

(A) SNARE proteins participate in signalling receptor trafficking. (B) SNARE proteins participate in the clustering of receptors onto a specific part of the growing growth cone.

the cell body that damage has occurred in the distal axon. Finally, membrane addition to a newly formed growth cone, or to the axonal membrane is required to promote axonal re-growth and elongation (Tuck and Cavalli, 2010).

The molecular mechanisms that support axon repair and growth clearly parallel the mechanisms that mediate synaptic vesicle trafficking and neurotransmitter release within uninjured axons, especially during embryonic development. Specifically, both require calcium, SNARE proteins and their effectors and cytoskeletal remodelling. Members of the SNARE machinery appear to regulate not only vesicle fusion to promote axon resealing but also axonal membrane extension and regrowth (Bloom and Morgan, 2011). For instance, syntaxin13 (Stx13) expression was increased by injury of mouse sciatic nerves *in vivo* and knockdown of Stx13 in cultured DRG neurons prevented axonal growth and regeneration (Cho et al., 2014).

Most of what we know about SNARE-mediated vesicle exocytosis comes from studies of synapses within uninjured axons (Augustine et al., 1999; Lin and Scheller, 2000; Pang and Sudhof, 2010). Here, calcium entering at the synapse binds to its sensor, synaptotagmin-1. Synaptotagmin then interacts with the SNARE complex, comprising the plasma membrane proteins syntaxin and SNAP-25 and the vesicle-associated membrane protein VAMP-2/ synaptobrevin-2, and in doing so triggers vesicle fusion and neurotransmitter release. Similarly, after injury to squid and crayfish axons, membrane sealing also requires the functions of Syntaxin and Synaptotagmin (Detrait et al., 2000). And regenerating photoreceptors can regulate the expression of a proper set of synaptic vesicle proteins with VAMP being present in all stages of regenerative growth (Yang et al., 2002). Therefore, a model emerges indicating that the critical requirements for ensuring proper membrane sealing and axon extension after injury include iterative bouts of SNARE mediated exocytosis, endocytosis, and functional links between vesicles and the actin cytoskeleton, similar to the mechanisms utilized during synaptic transmission.

Insights into the role of SNAREs in neuroregeneration

may come from regenerating model organisms. In geckos (*Gekko japonicus*), SNAP25 has been shown to be involved in spinal cord regeneration by promoting outgrowth and elongation of neurites (Wang et al., 2012). In tiger salamanders (*Ambystoma tigrinum*), regenerating adult photoreceptors require autonomous VAMP expression, whereas SNAP-25 is undetectable (Yang et al., 2002). In worms (*Caenorhabditis elegans*), axonal regeneration is promoted by alternative splicing of Syntaxins (Chen et al., 2016). More studies are needed in these model organisms to bring on a clearer picture of the role of SNAREs in neuroregeneration (Table 1).

Future Directions

Despite the evidence indicating the implication of several SNARE members in neurite outgrowth and axon guidance, their precise role in these processes is far from being well understood. The main challenges for future research in this field are: i) to characterize in detail the precise composition and function of SNARE complexes participating in neurite outgrowth/axon guidance. This could be achieved using imaging techniques such as Fluorescence Resonance Energy Transfer (FRET) or single molecule detection in living cells together with genetic and biochemical strategies; ii) to understand how different SNARE members are selectively regulated and how their action is coordinated with other events relevant in neurite outgrowth/axon guidance; and, iii) to confirm whether and how the nature of cargo molecules, whose transport/sorting is mediated by SNARE complexes, is relevant during the neurite/axon guidance processes. This can be achieved using different model systems, from cell culture to *in vivo* whole organism analysis. Studies using genetically amenable model organisms such as *Drosophila melanogaster* in parallel to vertebrate models are starting to prove to be extremely useful in dissecting the function of SNARE proteins in nervous system development (Barrecheuren et al., 2017).

Last but not least, we believe that SNARE function to be extremely relevant in axonal regeneration. Therefore, strategies

modulating the activity of SNARE proteins in lesioned axons can potentially be useful in the establishment of new therapies to enhance membrane insertion at the cut axonal edges, thereby potentiating neuronal repair and regeneration.

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Reviewer: Rong-Kung Tsai, Buddhist Tzu Chi General Hospital, China.

Comments to authors: This review article tries to explain the importance of SNARE complex in axonal guidance and neuroregeneration. It is one of the important issue for neurite regeneration after nerve injury.

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