1. COMMUNICATION WITHIN THE NERVOUS SYSTEM

The human brain is a 1.5 kg tissue mass, key in the regulation of many processes in the body, such as learning and memory, motor control, perception and body homeostasis. This requires a complex network of cellular entities - neurons - communicating with each other. It has been estimated that a human brain consists of 86 billion neurons and 10 times more supporting glial cells (Herculano-Houzel, 2009). Neurons communicate with each other over long distances. To facilitate this, neurons have a long axonal projection conducting electrical signals away from the cell body. Highly branched dendritic projections receive (electro)chemical signals from other cells. Electrical activity of neuronal cells is governed by their membrane potential. The resting potential is generated by a net negative charge in the interior of the cell. The plasma membrane separating the inside of the cell from the exterior environment contains voltage gated ion channels that can be temporarily opened upon depolarization, causing positively charged sodium ions to flow into the cell. A large depolarization can generate an action potential in an all-or-none fashion. During this action potential, voltage gated calcium channels in the axonal nerve terminal are opened. The calcium ion influx stimulates the release of chemical neurotransmitters. This axon terminal, the presynapse, is usually located in very close proximity to a dendritic spine from a signal receiving cell, the postsynapse. Neurotransmitter molecules released into the synaptic cleft activate receptors on the postsynaptic membrane. This leads to the opening of ligand-gated postsynaptic ion channels and subsequent depolarization (or hyperpolarization) of the postsynaptic cell (Figure 1). A special type of synapse is the neuromuscular junction (NMJ), formed by the axon of a motor neuron on a postsynaptic muscle fiber, regulating muscle contraction.

2. THE SYNAPTIC VESICLE CYCLE

2.1 Vesicle secretion is mediated by SNARE proteins

Neurotransmitters are stored in vesicles and can be released upon fusion of the vesicular membrane with the plasma membrane, a process called exocytosis. After full collapse fusion (Figure 1A), membrane reuptake is accomplished via endocytosis (Figure 1B). These newly formed vesicles - that could be formed via an endosomal intermediate (Figure 1C) - need to be reacidified before being reloaded with neurotransmitters (Figure 1D). The vesicles from the reserve pool of vesicles (Figure 1E) must then dock close to release sites (Figure 1F), and next be further primed (Figure 1G) to become fusion-ready upon entry of an action potential (Figure 1A; reviewed in Südhof, 2004).
Figure 1. The synaptic vesicle cycle. Schematic view of a presynaptic nerve terminal connecting to a postsynaptic cell. Dark arrows depict events leading to exocytosis, while the endocytosis pathways are shown with light arrows. A synaptic vesicle (black lined circles) filled with neurotransmitter (NT, dark grey dots) can (A) fuse with the plasma membrane upon depolarization and subsequent influx of Ca\(^{2+}\). After neurotransmitter release into the synaptic cleft, synaptic vesicles are (B) endocytosed and reused either directly or (C) via an endosomal intermediate. (D) A proton pump acidifies the vesicle interior, after which it can be refilled with neurotransmitters. From the (E) generated reserve pool of vesicles, a vesicle can (F) dock at the active zone and (G) be primed for a next fusion event using ATP. PSD: postsynaptic density. Modified from Südhof, 2004.
Vesicle docking, priming and fusion requires the formation of a complex of SNAP (Soluble NSF Attachment Protein) REceptor (SNARE) proteins (Söllner et al., 1993b). These proteins, synaptobrevin (VAMP) anchored in the vesicular membrane and syntaxin and SNAP25 on the synaptic membrane, contribute in total four a-helices to the complex (Figure 2; Sutton et al., 1998; Weber et al., 1998). The coiled coil structures are thought to zipper up in an N- to C-terminal fashion to form a tight complex bringing the vesicular and synaptic membranes closer together (Hanson et al., 1997a, 1997b; Sørensen et al., 2006). The energy released upon SNARE complex formation could supply the energy required to overcome the repulsive forces between the membranes, such that their lipids can mix during vesicle fusion (Cohen and Melikyan, 2004).

Figure 2. SNARE complex formation. Current working model for SNARE complex formation during docking, priming and fusion of synaptic vesicles. Syntaxin can be present in a ‘closed’ confirmation in which its three a-helical Habc domain (red cylinders) bind to its SNARE domain (grey cylinder). (A) Upon opening, plasma membrane-bound SNAP25 (green) can bind to (B) form a t-SNARE complex. (C) Vesicular SNARE protein VAMP2 (blue) provides the last of four coiled coil structures in a trans-SNARE complex that (D) zippers up to (E) fuse the vesicular membrane (white) with the target membrane (grey). (F) The cis-SNARE complex on the target membrane can be subsequently be decomposed, such that the proteins are reused in another round of fusion. Modified from Rodkey and McNew, 2013.
2.2 Regulatory proteins affect SNARE complex formation and function

SNARE complex-mediated vesicle docking, priming and fusion are tightly regulated in space and time by various proteins. Munc18 binds to a closed conformation of syntaxin and could thereby function in vesicle tethering to the membrane. It is indispensable for downstream steps in exocytosis. Munc13 might function in priming of the vesicles by opening up syntaxin. Calcium sensors such as synaptotagmin and Doc2 support vesicle fusion upon calcium elevations in the presynapse, possibly by interacting with membrane phospholipids and bending of the target membrane. Synaptotagmin additionally mediates vesicle docking. Complexin binds to a partly zippered SNARE complex and has been suggested both to stimulate and inhibit the progression of the process. Disassembly of SNARE proteins after vesicle fusion is mediated by N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-vesicular membrane and attachment proteins (SNAPs), so that the SNARE proteins can be reused in another round of fusion (All reviewed in Jahn and Fasshauer, 2012). Homologous proteins function in the fusion of other vesicular organelles with their target membranes, such as hormone secretion from endocrine cells or Golgi trafficking. Most proteins regulating SNARE assembly play a positive overall role in neurotransmitter secretion, as evidenced by hampered synaptic release upon genetic ablation (Geppert et al., 1994; Augustin et al., 1999; Verhage et al., 2000; Reim et al., 2001; Groffen et al., 2010). Tomosyn however is thought to be an inhibitor of vesicle priming.

BOX 1: METHODS TO STUDY SYNAPTIC PROTEIN FUNCTION

To study synaptic protein function, various techniques can be exploited. This box describes the main approaches used in this thesis.

Knockout animals

To analyze the function and significance of a single component in a complex network, one could study what happens to this system upon removal of the component. In knockout mice, this principle is employed by inactivating a gene encoding a protein of interest. This powerful tool allows the analysis of protein function on different levels. Skewed Mendelian population ratios indicate that the protein is crucial for embryonic development or postnatal survival. More subtle phenotypes, such as defects in anxiety, motor coordination, learning and memory, can be assessed by in vivo behavioral experiments. Even more detailed evaluation of protein function can be done on a cellular or molecular level in vitro in different kind of cells. In vitro approaches used in this thesis are electrophysiology, imaging and biochemistry methods.
Electrophysiology in autaptic neurons

Neurotransmission can be analyzed using electrophysiological methods. In vivo, neurons form an ingenious web of interconnected cells. Synaptic output of a single cell within this network is determined by the number, nature and location of input signals that it receives from other cells. In this thesis we employed primary cultures of autaptic hippocampal neurons, generated by culturing them individually on microislands of supportive glial cells, resulting in innervation of their own dendritic tree (Figure 3; Bekkers and Stevens, 1991). This simple model system lacks network regulation and can therefore be used to study the unfiltered transmitter release from synaptic vesicles by a single cell. It allows stimulation and recording from the same cell, ensuring the direct and stable output of a constant set of activated synapses. To record ion currents through the postsynaptic membrane, the whole-cell voltage clamp method was used. Generated excitatory postsynaptic currents (EPSCs) reflect a chain of events, starting with the release of neurotransmitters into the synaptic cleft upon depolarization of the cell and ending with the opening of ligand-gated ion channels on the postsynaptic membrane. Spontaneous release events occurring at the resting potential represent single vesicle fusion events. Brief depolarization of the cell can be administered to trigger the fusion of multiple vesicles. These evoked currents can be used to assess the number of release-ready vesicles, their fusion kinetics and the rate of vesicle replenishment. High frequency stimulation eventually ensures the fusion of the total pool of readily releasable vesicles. This readily releasable pool size can alternatively be estimated by the charge released upon application of a high osmotic sucrose solution.

Other methods used

Localization of a protein within a cell or relative to other proteins provide additional hints towards the function of this protein. Immunocytochemistry and live imaging of (overexpressed fluorescently tagged) proteins are useful techniques allowing the study of protein localization. Protein interactions can additionally be studied on a molecular level using biochemistry methods, such as yeast two-hybrid and immunoprecipitation experiments.

Figure 3. Patch clamp of an autaptic neuron. (A) Schematic representation of an autaptic glial microisland with a single, self-innervating neuron on top. A micropipette is attached to the somatic membrane and contains an electrode that is part of a voltage clamp circuit for recording and stimulation of the cell. (B) Picture of a voltage clamped autaptic neuron.
3. TOMOSYN: AN INHIBITOR OF SECRETION IN ENDOCRINE CELLS

The soluble 130 kDa tomosyn-1 (syntaxin-binding protein-5; STXBP5) protein was first isolated from rat brain extracts in a syntaxin pull-down experiment by Fujita et al. in 1998 and named after the Japanese word for friend, tomo, of syntaxin, syn. Since then it has become apparent that tomosyn functions as a negative regulator of secretion in endocrine cells as well as neurons. Here the role of tomosyn in endocrine secretion is described in detail, for its function in neurons: see section 4.

3.1 Overexpression of tomosyn in chromaffin cells reduces priming of vesicles

Up to 13 times overexpression of tomosyn in adrenal bovine chromaffin cells inhibits vesicle secretion by >50% in a short dual-depolarization protocol. Since vesicle docking and fusion kinetics are not affected, this secretory inhibition might be caused by attenuated priming into the readily releasable pool (RRP; Rosenmund and Stevens, 1996), leading to a decreased probability of release (Yizhar et al., 2004). In the same cell type, priming of vesicles has been associated with a reduction in vesicle mobility (Nofal et al., 2007). Indeed, with total internal reflection fluorescence microscopy in chromaffin cells it is shown that, at rest, rat tomosyn-1 overexpression hampers the immobilization of newly arriving vesicles at the plasma membrane. Additionally, tomosyn-1 is suggested to reduce the release from already immobile vesicles upon stimulation (Yizhar and Ashery, 2008). It is not yet clear whether this is a general mechanism of vesicle fusion inhibition or a property of chromaffin cells specifically, since it has not been reported for other cells.

3.2 Stimulus length may affect the magnitude of tomosyn-mediated exocytosis inhibition in PC12 cells

Similarly, rat tomosyn-1 overexpression in the adrenal medulla PC12 cell line (natively expressing tomosyn) inhibits high potassium-stimulated exocytosis, as measured by amperometry (Hatsuzawa et al., 2003) and secretion of co-transfected growth hormone (Fujita et al., 1998; Gladycheva et al., 2007). Interestingly, a reduction of almost 80% is seen in the number of amperometric spikes upon tomosyn overexpression, while a more modest reduction is seen on growth hormone secretion (about 26% in Fujita et al., 1998; about 20% in Gladycheva et al., 2007). This could simply reflect differences in tomosyn overexpression levels, but might also be caused by the duration of high potassium stimulation, which was ten times as long in the growth hormone secretion studies. It is therefore conceivable that tomosyn inhibits secretion more strongly during rest than upon prolonged stimulation.
3.3 Tomosyn secretory inhibition could be relieved by increased calcium
Relief of tomosyn inhibition upon prolonged demand could be mediated by increased levels of calcium. In agreement with this, tomosyn overexpression in mouse pancreatic beta-cells decreases calcium sensitivity in depolarization-induced exocytosis, measured by membrane capacitance (Zhang et al., 2006). Moreover, under conditions of elevated calcium, inhibition of secretion by rat tomosyn-1 overexpression in chromaffin cells is partially relieved, measured by membrane capacitance and amperometry. Furthermore, an enhanced sustained phase of secretion is observed in these cells (Yizhar et al., 2004). The sustained phase of secretion is enhanced when at least the tomosyn-1 C-terminus is expressed. This indicates that the C-terminal part of tomosyn-1 reflects the stimulatory function of the protein in sustained release (Yizhar et al., 2007).

3.4 Enhanced inhibition by chromaffin cell acetylcholine receptor stimulation
Contradicting with the proposed activity-induced relief of tomosyn inhibition, the amount of tomosyn interacting with plasma membrane SNARE proteins increases with stimulation of chromaffin cell acetylcholine receptors with the nicotinic acetylcholine receptor agonist DMPP. Phosphorylation of syntaxin via the Rho-GTPase/ROCK pathway leads to a similar increase in tomosyn membrane targeting. This might increase secretory inhibition (Gladycheva et al., 2007) and could represent a mechanism specific for the activation of nicotinic acetylcholine receptor.

3.5 Debated function of tomosyn as a negative regulator of insulin secretion
Results conflicting with an overall negative role of tomosyn in secretion were reported for insulin release. Membrane capacitance measurements in pancreatic beta-cells upon tomosyn overexpression and knockdown are in line with a negative regulatory function. This was confirmed by glucose-induced secretion of transfected growth hormone (hGH) from insulin-secreting insulinoma INS-1E cells (Zhang et al., 2006), that also express tomosyn endogenously (Cheviet et al., 2006). In a separate study however, overexpression of rat or mouse tomosyn-1 in INS-1E cells does not affect secretion of hGH upon stimulation and knockdown reduces exocytosis, measured by hGH release and capacitance measurements. The number of docked secretory granules does not decline upon prolonged stimulation, which is another sign of reduced release upon tomosyn knockdown (Cheviet et al., 2006). Importantly, cells in this study were generally stimulated with a combination of glucose, K⁺, forskolin and the insulin secretagogue IBMX. Forskolin specifically could affect tomosyn’s function (see section 9) and thereby mask the effects of tomosyn manipulations. Additional overexpression and siRNA (control) experiments are required to validate that tomosyn is a positive regulator of insulin release.
Thus, evidence suggests that tomosyn generally functions as an inhibitor of endocrine secretion in chromaffin cells, although a permissive role in certain cells or circumstances, such as insulin secretion, cannot be excluded. Possibly, this inhibition ensures a reserve pool of vesicles that can be calcium-dependently primed and fused upon higher demand.

4. NEUROSECRETORY VESICLE FUSION IS INHIBITED BY TOMOSYN IN VITRO AND IN VIVO

4.1 Tomosyn overexpression inhibits presynaptic release in vitro
Similar to endocrine systems, rat tomosyn-1 overexpression inhibits secretion from synaptic vesicles in neurons, as shown by an increased EPSP (excitatory postsynaptic potential) failure and decreased EPSP amplitude in cultured superior cervical ganglion (SCG) neurons. This can be ascribed to a presynaptic function of tomosyn, since action potentials were generated in neurons overexpressing tomosyn, while postsynaptic EPSPs were recorded from non-transfected neurons (Baba et al., 2005).

4.2 C. elegans neuromuscular junction readily releasable pool size is regulated by tomosyn
In a forward genetic screen for negative regulators of acetylcholine release, a C. elegans tom-1 deletion mutant was identified. This mutant is hypersensitive to the cholinesterase inhibitor aldicarb, which is an indication of increased acetylcholine secretion in this mutant (Dybbs et al., 2005). Indeed, electrophysiology in neuromuscular junction (NMJ) synapses of tom-1 mutants indicates that evoked currents are prolonged without an increase in current amplitude, leading to increased charge transfer. An explanation of prolonged responses may be found in postsynaptic receptor saturation. However, responses were still significantly prolonged when measured in low calcium (0.5 mM instead of 5 mM). Additionally, in lower calcium concentration, evoked response amplitudes seemed somewhat enhanced in the tomosyn deletion mutants. In contrast, overexpression of tomosyn-1A in C. elegans NMJ decreases neurotransmitter release. This could not be attributed to postsynaptic changes, since kinetics of spontaneous release events from single vesicles was unaffected. Increased secretion in the deletion mutants correlated with increased hyperosmotic response indicating a larger primed vesicle pool. Electron microscopy confirms the accumulation of vesicles contacting the plasma membrane. These vesicles are more dispersed throughout the terminal, potentially explaining the prolonged EPSC (excitatory postsynaptic current) response. Ectopically primed vesicles might experience delayed calcium sensing by increased distance to calcium channels. Thus, in C. elegans NMJ, presynaptic tomosyn might inhibit priming and
release from distal synaptic vesicles specifically (Gracheva et al., 2006) and therefore functions as a spatial regulator of vesicular transmitter release. McEwen et al. (2006) found similar results; the same tom-1 deletion mutant nematodes in this study exhibit a prominent prolonged evoked current and modestly increased current amplitude. Possibly, the lower concentration of extracellular calcium (1 mM) allows elevation of postsynaptic current amplitude, while in the higher calcium concentrations used in Gracheva et al. (2006), a ceiling effect on EPSC amplitude is reached. All in all, it can be concluded that in C. elegans NMJ tomosyn inhibits evoked release by regulating the size of the readily releasable pool. These presynaptic tomosyn protein levels are enhanced by a retrograde synaptic signal that is regulated by neurexin and neuroligin in C. elegans NMJs (Hu et al., 2012).

4.3 Tomosyn regulates release probability in the Drosophila neuromuscular junction
In Drosophila NMJs, tomosyn knockdown also leads to enhanced evoked release with a slight increase in EPSC amplitude and a prolonged response. A modest increase in spontaneous release decay time was also found, without a concomitant significant increase in spontaneous release charge transfer. Presynaptic vesicle loading therefore seems to be unaffected, but a minor postsynaptic component in Drosophila tomosyn knockdown NMJ transmission might be involved. Paired pulse ratio is decreased with tomosyn knockdown, indicating increased release probability (Chen et al., 2011).

4.4 Synaptic plasticity is affected in tomosyn-1 deficient mice
Consistently, in tomosyn-1-deficient mice synaptic transmission is enhanced in hippocampal mossy fiber synapses. Unlike C. elegans and Drosophila NMJ, generated EPSP responses are not prolonged. Possibly, within these synapses spatial vesicle distribution is not tomosyn-mediated and/or postsynaptic receptor saturation is not an issue. In line with previous evidence about tomosyn’s function, the probability of neurotransmitter release in tomosyn-1 deficient hippocampal mossy fibers is increased, shown by decreased paired-pulse facilitation. Mossy fiber long-term potentiation is initially reduced in tomosyn-1 deficient mice, while the late phase of LTP is unaffected (Sakisaka et al., 2008). This suggests that tomosyn inhibition serves to decrease initial release probability in order to uphold levels of sustained release during long-term potentiation, similar as was suggested for endocrine cells (see section 3). The late phase of LTP could be mediated by tomosyn-independent, slower mechanisms.
4.5 Tomosyn knockdown in superior cervical ganglion neurons may reduce release

Like in endocrine cells, there have been observations in support of a positive role of tomosyn in synaptic secretion. EPSP amplitude is reduced upon expression of tomosyn siRNA in rat SCG neurons. Rescue experiments re-expressing tomosyn should be performed to confirm the absence of off-target effects (Baba et al., 2005). Generally, tomosyn is thought to function as a negative regulator of vesicular neurotransmitter release.

4.6 Neuronal dense-core vesicle fusion is mediated by tomosyn in C. elegans

In addition to transmitter release from synaptic vesicles, the role of tomosyn in neuropeptide secretion from large dense-core vesicles (LDCVs) is studied in C. elegans. Tom-1 mutants show a reduction in the number of presynaptic LDCVs, corresponding to enhanced release, while overexpression of tomosyn leads to an accumulation of LDCVs as seen with electron microscopy, indicating that tomosyn negatively regulates LDCV exocytosis in vivo. This idea is confirmed by measuring neuropeptide release from cholinergic neurons, which is increased in tom-1 mutant nematodes (Gracheva et al., 2007a).

4.7 Direction of neurite outgrowth by tomosyn-induced local restriction of vesicle fusion

In extending neurites of cultured rat hippocampal neurons, tomosyn localizes at the palm of growth cones, where it could locally inhibit membrane supplying vesicle fusion. This seems to be important for controlling neurite outgrowth, by channelling vesicle fusion to the growth cone tip. In LPA-induced retracting neurites, tomosyn localizes all over the edges of the growth cone. Indeed, hippocampal neurons and differentiated NG108 neuroblastoma cells transfected with HA-tagged full-length tomosyn form fewer and shorter neurites when compared to control cells. Tomosyn knockdown with siRNA in NG108 cells results in more sprouting neurites that are shorter than those of the control cells (Sakisaka et al., 2004). Additionally, in the Drosophila third instar larvae neuromuscular system, overexpression of the last two exons of tomosyn leads to pathfinding defects and reduced or abnormal synapses (Kraut et al., 2001). However, tomosyn knockdown in another Drosophila study does not affect NMJ synapse morphology and synapse number (Chen et al., 2011). Furthermore, in nematodes with a null mutation in tom-1, axon morphology and synapse number are normal (Gracheva et al., 2006; McEwen et al., 2006). Thus, in spite of strong indications that tomosyn is involved in neurite outgrowth, further studies are needed to confirm this hypothesis unequivocally.
In summary, tomosyn functions as a negative regulator of vesicle priming in neurons in space and/or time, both for synaptic and dense-core vesicles, as well as membrane supplying vesicles in neurite outgrowth.

5. REGULATION OF SNARE COMPLEX FORMATION BY TOMOSYN

5.1 Synaptobrevin and tomosyn compete for SNARE protein binding
Tomosyn is thought to exert its function in vesicle priming by inhibition of SNARE complex formation through substitution of the synaptobrevin coiled coil domain in the four-helical SNARE complex (Masuda et al., 1998). Tomosyn-1 binds not only to syntaxin-1A/SNAP25 (Fujita et al., 1998), but forms a complex in vitro with syntaxin-4 and SNAP23 as well (Widberg et al., 2003). The original paper did not report binding to syntaxin-4 (Fujita et al., 1998), presumably because syntaxin-1A affinity is considerably higher (Widberg et al., 2003). Binding of tomosyn-1 to a preassembled syntaxin-1 and SNAP25 complex (Cheviet et al., 2006) seems not weaker than that of synaptobrevin (Yersin et al., 2003) and both complexes form with similar rates in vitro. Additionally, rat tomosyn-1 can be released from membrane-bound syntaxin by NSF and α-SNAP (Hatsuzawa et al., 2003). Although the tomosyn and synaptobrevin containing complexes are thus similar, complexin does not bind to the tomosyn-1/SNAP25/syntaxin complex (Pobbati et al., 2004) and the tomosyn-1 containing SNARE complex is, unlike the synaptobrevin containing complex, not resistant to SDS (Pobbati et al., 2004).

Tomosyn-1 is not able to remove synaptobrevin from a preformed SNARE complex in vitro, and vice versa, estimated by SDS resistance and fluorescence anisotropy. Of note, the in vitro complexes in this study were generated from protein fragments (Pobbati et al., 2004). Conceivably, full-length proteins and/or lipid binding are required for protein behavior more representative to the in vivo situation. A more recent paper suggests that full-length synaptobrevin can replace tomosyn-1 on preformed SNARE complexes. While different tomosyn fragments and full-length tomosyn bind equally well to full-length t-SNAREs on liposomes, subsequent v-SNARE incubation enhances liposome fusion to a different extent, indicating that synaptobrevin replaces tomosyn fragments to various degrees (Yamamoto et al., 2010a). It is important to further elucidate this mechanism in vivo, since it is unlikely that tomosyn acts as a fast regulator of SNARE protein availability if it constitutes a dead-end complex that has to go through time-consuming NSF disassembly.

5.2 Tomosyn cooperates with other SNARE protein regulators
Additional proteins may be required to relieve tomosyn inhibition and facilitate synaptobrevin binding to t-SNAREs. Functional links exist between tomosyn and several regulatory proteins: CAPS, Munc13, Munc18 and synaptotagmin.
5.2.1 Munc13 and tomosyn act to regulate open syntaxin levels
From studies in C. elegans, tomosyn and the Munc13 homolog UNC-13 are thought to function in an antagonistic manner. UNC-13 stabilizes the open form of syntaxin and tomosyn might preferentially interact with this open syntaxin conformation, in which syntaxins SNARE motif is available. Thereby, tomosyn could act as a buffer for the availability of open syntaxin and affect Munc13-mediated priming of synaptic vesicles. This is confirmed by both overexpression of constitutively open syntaxin and knockout of tomosyn, either of which rescue the abolished hyperosmotic response in unc-13 deletion animals. Also, synaptic UNC-13 levels increase upon knockout of tomosyn-1. Since a combination of the three mutations has an additive effect, tomosyn seems to inhibit priming also via another mechanism, such as competition with synaptobrevin. Furthermore, stimulus evoked secretion and locomotor defects are not fully rescued in unc-13 mutants, indicative of a tomosyn independent post-priming role of UNC-13 (Gracheva et al., 2006; McEwen et al., 2006).

5.2.2 CAPS and tomosyn have opposite effects on dense-core vesicle fusion
UNC-31, a homolog of calcium-dependent activator protein for secretion (CAPS), mediates peptide release from LDCVs in C. elegans. It may function similar to the synaptic vesicle priming factor UNC-13 to promote LDCV fusion. Hampered body movement is seen in unc-31 knockout animals. This can be partially rescued by genetic deletion of tomosyn, indicating antagonistic functions in LDCV fusion with the plasma membrane. In line with this, tomosyn deletion rescues the increase in synaptic LDCVs in unc-31 mutants. Additionally, reduced evoked release in these animals is compensated for by tom-1 deletion (Gracheva et al., 2007b).

5.2.3 Munc18 and tomosyn compete for syntaxin binding and vesicle tethering
Munc18 is important for the distribution of syntaxin to the plasma membrane and stabilizes syntaxin in a closed conformation. In vitro, purified Munc18a and tomosyn-1 compete with each other for syntaxin-1A binding (Fujita et al., 1998). This was confirmed with Fluorescence Resonance Energy Transfer (FRET) in HEK293 and bovine adrenal chromaffin cells (Gladycheva et al., 2007). Syntaxin-1A localization to the HEK293 plasma membrane is facilitated by Munc18-1, but not tomosyn-1 expression. In the absence of tomosyn, FRET experiments indicate that Munc18-1 inhibits formation of plasma membrane-targeted syntaxin-1A/SNAP25 complexes, probably by stabilizing inactive syntaxin-1 in a closed conformation. Co-expression of tomosyn results in efficient localization of the cytosolic form of SNAP25 to the plasma membrane, also in the presence of Munc18-1. Tomosyn might thus allow formation of SNARE complexes at the plasma membrane, downstream of membrane targeting of syntaxin by Munc18 (Gladycheva et al., 2007).
Also in *C. elegans*, UNC-18 and tomosyn-1 compete for syntaxin-dependent plasma membrane localization. *Unc-18* mutants have reduced NMJ synaptic transmission. Knockout of *tom-1* in *unc-18* mutants partly rescues priming assessed by hyperosmotic responses as well as stimulus evoked currents. Furthermore, both vesicle tethering and docking are increased in these double knockout animals (Gracheva et al., 2010). Tethering is induced by UNC-18/closed syntaxin complexes, after which UNC-13/open syntaxin may further reduce vesicular distance from the plasma membrane. Possibly, increased UNC-18-mediated tethering in *tom-1* mutants increases the number of downstream docked vesicles, which could rescue priming defects in *unc-13* mutants by such an indirect effect instead of direct competition of UNC-13 and tomosyn.

Tomosyn-1 is able to bind to syntaxin-4/Munc18c dimers *in vitro*. Syntaxin-4 functions together with SNAP23 in trafficking of the glucose transporter GLUT4 in fat and muscle cells. In adipocytes, overexpression of either tomosyn-1 or Munc18c inhibits insulin-stimulated exocytic GLUT4 translocation to the plasma membrane, indicating they act in concert in this particular model system (Widberg et al., 2003).

**5.2.4 Tomosyn binds to synaptotagmin in a calcium-stimulated fashion**

Tomosyn-1 co-purifies in the same complexes as the calcium sensor synaptotagmin (Fujita et al., 1998). It binds directly to transmembrane domain lacking synaptotagmin-1 via its N-terminal WD40 domain in a Ca\(^{2+}\)-enhanced manner *in vitro*, with a concomitant increase in tomosyn/syntaxin/SNAP25 complex formation. This was suggested to reduce synaptotagmin-mediated membrane curvature induction in a liposome fusion assay. Of note, reduction of available soluble synaptotagmin by binding with free tomosyn might cause this effect. This may also affect EPSP amplitude measurements in cultured superior cervical ganglia (SCG) neurons. Injection of either transmembrane domain lacking synaptotagmin, full-length tomosyn or the N-terminal fragment of tomosyn reduces release, which is relieved by the synaptotagmin/tomosyn interaction. Furthermore, the correlation between tomosyn-SNARE complex formation and the amount of EPSP amplitude inhibition is yet uncertain, since tomosyn-SNARE complex formation with the tomosyn N-terminal fragment is modest, while EPSP amplitude is clearly inhibited. Also, synaptotagmin-enhanced tomosyn-SNARE complex formation seems not to further inhibit release, but rather – if anything – relieves inhibition (Yamamoto et al., 2010b). In line with this, tomosyn-1 inhibition of secretion can be overcome by increased calcium concentration in chromaffin cells and pancreatic beta-cells (Yizhar et al., 2004; Zhang et al., 2006). All in all, tomosyn binds to synaptotagmin with increased affinity in the presence of calcium, but the significance of this interaction for tomosyn-mediated secretory inhibition remains unclear.
Thus, tomosyn competes with Munc18 for syntaxin binding (Fujita et al., 1998; Gladycheva et al., 2007). Therefore, competition for plasma membrane targeting of UNC-18 and TOM-1 in C. elegans is likely to be syntaxin-mediated. At the plasma membrane, such competition could be involved in vesicle tethering (Gracheva et al., 2010). Downstream, tomosyn could compete with Munc13 to regulate the transition from Munc18/closed syntaxin dimers to a SNAP25 containing complex (Gracheva et al., 2006; McEwen et al., 2006). Also, tomosyn restricts binding of the fourth coiled coil motif from the vesicular SNARE syntaptobrevin (Masuda et al., 1998; Pobbati et al., 2004; Yamamoto et al., 2010a). Upon high demand, elevated calcium might induce tomosyn binding to synaptotagmin (Yamamoto et al., 2010b), for example to bridge t-SNAREs with vesicular synaptotagmin for targeted replenishment of vesicles near release sites. Mechanisms similar to syntaptobrevin C-terminal fragment stabilization of syntaxin/SNAP25 complexes and rescue of docking in the absence of Munc18 could be involved (Pobbati et al., 2006; de Wit et al., 2009). All in all, tomosyn could regulate SNARE complex formation in concert with other proteins at different moments in the vesicle cycle. This may ensure controlled release from vesicles and efficient supply of additional vesicles and SNARE complexes in an activity-dependent manner.

6. HOMOLOGOUS TOMOSYN PROTEINS

Proteins homologous to tomosyn have been found in various species. Amino acid sequence based evolutionary development and conservation between species can be assessed in a phylogenetic tree (Figure 4).

6.1 Yeast Sro7p and Sro77p are homologous to tomosyn

Yeast expresses two tomosyn homologs, Sro7p and Sro77p, showing 55% amino acid similarity to each other (Kagami et al., 1998). N-terminal WD40 and tail domains (Figure 5) are conserved in tomosyn and Sro7p/Sro77p. Both yeast proteins also contain a C-terminal region that is predicted to form an a-helix and could function similar to tomosyn’s C-terminal coiled coil domain (Pobbati et al., 2004; Hattendorf et al., 2007; see also section 8). Interestingly, only helical layers +1 through +8 are conserved in Sro7p/Sro77p (Pobbati et al., 2004), while tomosyn layers -7 to -1 may additionally contribute to coiled coil function. Functionally, no clear difference between the two yeast proteins has been observed, although it is striking that yeast also contains two homologous proteins for both syntaxin (Sso1p/Sso2p) and syntaptobrevin (Snc1p/Snc2p). Different combinations of SNARE proteins might for example be required to regulate the release from different kind of vesicles.
6.2 Vesicle secretion is stimulated by Sro7p/Sro77p in yeast

Both Sro7p and Sro77p bind to the yeast SNAP25 homolog Sec9p and have redundant functions in polarized promotion (!) of exocytosis in budding cells. Defects upon loss of both Sro7p and Sro77p present as a cold-sensitive growth defect with accumulated cytoplasmic vesicles, caused by reduced Golgi-to-cell surface transport (Lehman et al., 1999; Hattendorf et al., 2007). Up to 16 times Sro7p overexpression also results in vesicle clustering, possibly reflecting an overexpression artifact by early, unregulated activation of the vesicle fusion machinery before a vesicle is able to reach the plasma membrane (Rossi and Brennwald, 2011).

Disruption of sodium pumping ATPase Ena1p cell surface delivery causes salt sensitivity in sro7p mutants. Sro7p aids targeting of this pump to the cell surface upon sodium exposure, in order to remove excess intracellular salt. Post-Golgi vesicle accumulation is seen upon subjection of sro7p mutants to high salt concentrations. Since the accumulated vesicles in sro7p mutants lack Ena1p, Sro7p seems to mediate both early sorting of Ena1p into post-Golgi vesicles and downstream vesicle fusion with the plasma membrane (Wadskog et al., 2006).

Disruption of the actin cytoskeleton polarity in sro7p/sro77p mutants is a late, secondary effect by prolonged impairment of cell surface delivery (Lehman et al., 1999). However, disruption of the interaction of Sro7p with cytoskeletal type V myosin does result in cold-sensitive secretory defects. This interaction recruits Sro7p to post-Golgi vesicles and seems required for the localization of Sro7p to sites of polarized growth (Rossi and Brennwald, 2011).
Sro7p and Sro77p are thus positive mediators of vesicle fusion, needed in Golgi protein sorting and polarized growth in budding yeast.

6.3 Sro7p/Sro77p regulate SNARE complex formation

Similar to tomosyn, Sro7p and Sro77p proteins are suggested to exert their function in vesicle fusion through regulation of SNARE complex formation. Since Sro7p lacks conservation of helical layers -7 to -1 (Pobbati et al., 2004), which could be crucial in initiation of coiled coil formation enabling rapid binding to syntaxin/SNAP25 dimers (Wiederhold et al., 2010), formation of this complex may be slow. This might also facilitate t-SNARE stabilization and replacement by synaptobrevin homologs, as was suggested for liposome fusion in the presence of the very N-terminal part of tomosyn’s VAMP-like domain as well as a C-terminal synaptobrevin fragment (Pobbati et al., 2006; Yamamoto et al., 2010a). Through such a mechanism it could function as a positive regulator of SNARE complex formation.

Direct binding of full-length Sro7p to yeast SNAP25 homolog Sec9p (Hattendorf et al., 2007) and to yeast syntaxin homologs Sso1p/Sso2p has not been shown (Lehman et al., 1999). A N-terminal Sro7p WD40 fragment does interact with Sec9p Qbc-SNARE domain, while it binds only weakly to Sso1p (Hattendorf et al., 2007). Since this fragment does not bind to a pre-assembled SNARE complex, Sro7p binding to Sec9p might compete for Sro7p-enhanced monomeric SNARE complex formation (Williams and Novick, 2009).

The involvement of SNARE protein complexes in secretion phenotypes remains unclear. Reduced Sec9p/Sso1p dimerization in the presence of the Sro7p fragment in GST pull-down assays has been correlated with restricted growth and reduced post-Golgi vesicle secretion. Yeast lacking Sro7p/Sro77p shows a similar phenotype, although in the absence of Sro7p in vitro, Sso1p/Sec9p interaction is higher (Hattendorf et al., 2007). Moreover, Sso1p binding to Sec9p is similar in the absence of Sro7p and in the presence of a Sro7p mutant lacking the C-terminal end. This Sro7p mutant is capable of a (partial) rescue of the growth and vesicle secretion deficits in sro7p/sro77p mutant yeast, further indicating an involved mechanism independent of tomosyn’s role in Sso1p/Sec9p complex formation (Hattendorf et al., 2007).

SNARE complex formation may not be the limiting factor in other mutants as well. Sec9-4 has reduced affinity for Sso1p/Sso2p and synaptobrevin yeast homologs Snc1p/Snc2p. Increased SNARE complex formation by Sro7p overexpression does not suppress growth inhibition in sec9-4 mutants (Williams and Novick, 2009) and sro7p deletion in sec9-4 mutants further slowed down growth (Grosshans et al., 2006), further indicating that exocytosis restriction in sro7p/sro77p mutants might be (partially) independent of SNARE complex formation. Yeast mutants for Sso1p/
Sso2p (Aalto et al., 1993) and Snc1p/Snc2p (Protopopov et al., 1993) also accumulate vesicles, but have not been reported in relation to Sro7p. All in all, cell surface localized Sro7p/Sro77p in the bud tip could facilitate SNARE complex formation. This is likely not to be the (single) mechanism causing polarized vesicle fusion deficits upon deletion of Sro7p/Sro77p in yeast.

6.4 Exocyst proteins bind to Sro7p and Sro77p
Besides SNARE complex interactions, Sro7p and Sro77p interact both in vitro and in vivo with Exo84p, a protein functioning in the exocyst complex. Expression of a Sro7p-binding defective Exo84p mutant results in accumulation of vesicles, indicating that this interaction is required for secretion of post-Golgi vesicles (Zhang et al., 2005). Also, Sro7p binds to the active form of the Rab GTPase Sec4p, a vesicular protein required for exocyst complex formation. Overexpression of Sro7p partially rescues growth defects of exocyst subunit mutants (Grosshans et al., 2006). Whether Sro7p and Sro77p affect exocyst complex organization and/or targeting has not yet been investigated. Interestingly, tomosyn has been shown not to co-localize with the Sec6/8 exocyst complex in neuronal growth cones (Sakisaka et al., 2004). Perhaps, the interaction of Sro7p/Sro77p with the exocyst complex in yeast facilitates its permissive function in vesicle fusion, for example by enhancing assembly, stability or recruitment of the complex.

6.5 Lethal (2) giant larvae (Lgl) is functionally related to Sro7p/Sro77p and modulates neuromuscular junction morphology
Tumor suppressor protein lethal (2) giant larvae (Lgl) is highly conserved across species and is a cytoskeletal regulator associated with apico-basal transport in polarized cells (Bilder et al., 2000). Loss of cell polarity has been associated with development and progression of cancer. Overproliferation in lgl mutant Drosophila larvae leads to malignant neuroblastomas, imaginal disc and blood cell neoplasms (Gateff, 1978). Lgl is also involved in asymmetric neuroblast cell division in Drosophila (Albertson and Doe, 2003). In Lgl1 deficient mouse embryos, loss of cell polarity leads to hyperproliferation of neural progenitor cells that do not differentiate by asymmetric cell division (Klezovitch et al., 2004). Compared to tomosyn, Lgl lacks the C-terminal tail domain and VAMP-like α-helix, but the N-terminal β-propeller structure is conserved (Betschinger et al., 2005). Both Drosophila Lgl and mammalian Mlgl-1 are able to bind to syntaxin-4 in pull-down and immunoprecipitation assays. This is retained upon removal of a large part of the N-terminal β-propeller domain and could be involved in syntaxin-4/SNAP23/Mlgl-1 complex formation. Also, Mlgl-1 binds to both myosin-V and myosin-II in immunoprecipitation experiments (Müsch et al., 2002; Gangar et al., 2005).
Mlgl-1 is unable to rescue cold-sensitive growth and secretion defects in a yeast sro7p/sro77p mutant, indicating that it has a different function (Gangar et al., 2005). In another study however, using a slightly higher temperature, growth was less restricted upon expression of Mlgl-1 in sro7p/sro77p mutants (Kim et al., 2005). Also, a partial rescue of salt sensitivity was seen (Larsson et al., 1998; Kim et al., 2005). Thus, Sro7p/Sro77p and Lgl functions seem to partially overlap. Rescue of yeast phenotypes by Lgl depends on intact WD40 domains (Kim et al., 2005).

Interestingly, Drosophila Lgl is expressed at the NMJ and may function both pre- and postsynaptically. Presynaptic Lgl is required for axonal branching, synaptic bouton formation and active zone organisation. Also, Lgl positively mediates presynaptic vesicle cycling measured by FM1-43 dye (un)loading. Postsynaptic Lgl restricts glutamate receptor expression, specifically GluR11B containing receptors. Pre- and postsynaptic Lgl mechanisms are both necessary to rescue reduced NMJ transmission in lgl mutants (Staples and Broadie, 2013).

6.6 Amisyn is a small tomosyn homolog inhibiting exocytosis
Amisyn is a small, 210 amino acid, brain specific protein homologous to tomosyn’s coiled coil domain. It is expressed in higher organisms, such as zebrafish, frogs, mice, rats, rabbits, chimpanzees and humans. Like tomosyn, it forms a complex with syntaxin and SNAP25 and inhibits exocytosis in PC12 and chromaffin cells (Scales et al., 2002; Constable et al., 2005).

All in all, both yeast Sro7p/Sro77p and tumor suppressor protein Lgl function in cellular polarity. They might exert their function by binding to SNARE and/or exocyst complexes. Also the small tomosyn homolog amisyn is associated with SNARE proteins.

7. TOMOSYN MOUSE ISOFORMS
In mice, two genes expressing tomosyn are identified: STXBP5 (tomosyn-1) on chromosome 16 and STXBP5L (tomosyn-2) on chromosome 10 (Groffen et al., 2005). Alternative splicing generates in total seven tomosyn isoforms that primarily vary within a hypervariable region located in a loop structure extending from the β-propeller backbone (Figure 5). Removal of this particular loop structure from the protein does not hamper its inhibitory function on secretion (Williams et al., 2011). It might therefore possess mainly modulatory properties causing subtle differences between tomosyn-1 and -2.
7.1 Mammalian tomosyn-1 and -2 are differently expressed in the (developing) nervous system

Mouse tomosyn-1 and -2 show distinct expression patterns in the central nervous system. Tomosyn-1 is ubiquitously expressed in the adult brain, with prominent expression in structures of the limbic system, while tomosyn-2 expression is restricted to certain areas, including the hippocampus (CA2 region), medial habenular nucleus and the internal granular layer of the cerebellar cortex (Groffen et al., 2005). Both isoforms express in the spinal cord (Lein et al., 2007). In peripheral tissues, tomosyn-1 is expressed in endocrine systems, in line with its function in endocrine release, such as the adrenal paraganglion, Clara cells of the bronchial epithelium (Groffen et al., 2005) and pancreatic beta-cells (Cheviet et al., 2006; Zhang et al., 2006). Out of the three tomosyn-1 splice variants, the biggest variant is expressed peripherally, the other two are brain specific (Yokoyama et al., 1999). The big tomosyn-1 splice variant accordingly plays a role in exocytosis in non-neuronal cell-types, such as the insulin-stimulated glucose transporter GLUT4 translocation in adipocytes (Widberg et al., 2003). According to the UniGene EST-based expression profile for mouse tomosyn-2 (Mm.80170), expression is seen mainly in the brain, but also in some peripheral tissues, such as the eye, inner ear, kidney, ovary, pituitary gland and sympathetic ganglion. Interestingly, tomosyn-2 may be expressed in dendrites emerging from the pyramidal cells in the stratum radiatum of hippocampal CA2 (Barak et al., 2010).

During development, a stable level of tomosyn-1 expression is seen, while tomosyn-2 expression increases with development, along with syntaxin-1 and Munc18-1 (Groffen et al., 2005). This is confirmed with UniGene EST-based expression profiles, which show that tomosyn-1 (Mm.331751) is stably expressed in various developmental stages. Tomosyn-2 expression (Mm.80170) is detected in the unfertilized oocyte and later fetus, neonate, juvenile and adult stages, but is absent during zygote to blastocyst stages, as well as early embryonic development. These differences in expression pattern might reflect the functional significance of each of the isoforms during neuronal development and exocytosis in various tissues. Tomosyn-1 may be important during early oocyte development or later by regulating targeted membrane supply for neurite outgrowth. Furthermore, it could function to establish/fine-tune synaptic connections by regulating synaptic activity in neural network formation. It might still exert these functions in the adult brain, in addition to the regulation of synaptic transmission and endocrine release. Tomosyn-2 could be a specific regulator of synaptic transmission in a subset of cells, requiring distinctive regulation of neurotransmitter release, for example because of a different firing pattern, or secreting specific types of vesicles.
7.2 Tomosyn-1 and -2 are both inhibitors of secretion

Despite the clear differences in expression pattern of the two mammalian tomosyn isoforms, the vast majority of data so far relates to tomosyn-1. So far, no animal model exists in which the function of endogenous tomosyn-2 can be studied. Recently, an overexpression study did focus on the functional difference between mammalian tomosyn-1 and -2. Overexpression of either tomosyn-1 or tomosyn-2 in PC12 cells inhibits potassium evoked growth hormone secretion to a similar extent. Surprisingly, basal secretion in these cells seems upregulated upon expression of the medium isoform of tomosyn-2 specifically, but not for the tomosyn-2 extra big isoform. Somewhat lower protein levels are found for the medium tomosyn-2 isoform in particular. It cannot be excluded though that these differences are caused by enhanced basal growth hormone secretion due to increased cell death (Williams et al., 2011). Overexpression of tomosyn-2 in pancreatic beta-cells inhibits insulin secretion (Bhatnagar et al., 2011), which was also shown for tomosyn-1 (Zhang et al., 2006). Furthermore, syntaxin interaction of the different isoforms is similar (Bhatnagar et al., 2011; Williams et al., 2011). All in all, no clear functional differences were found yet for tomosyn-1 and -2.

Figure 5. Functional domains in mouse tomosyn isoforms. (A) Model representing the predicted structure of tomosyn, based on the yeast homolog Sro7, showing the β-propeller structures (grey and blue) and three loop structures extending from it (yellow, red, cyan). The tail domain, predicted to form a coiled coil structure, is depicted in green. The VAMP-like domain is not shown (Modified from Williams et al., 2011). (B; next page) An amino acid sequence based alignment of mouse tomosyn-1b and tomosyn-2xb (the largest splice variants) was generated using Clustal Omega. Fully conserved residues (*), conservation between groups of strongly similar properties (|) and conservation between groups of weakly similar properties (.) are shown. The colours correspond to the structures depicted in (A). Additionally, splice variation is shown in olive. This domain also contains a known phosphorylation (P) and SUMOylation site (S). The synaptobrevin-like coiled coil domain is represented in pink.
Figure 5 (legend on previous page).
Thus, based on the available tomosyn-2 data from overexpression in PC12 and pancreatic beta-cells, both tomosyn-1 and -2 seem to be inhibitors of secretion. The main differences between the mammalian tomosyn isoforms may be found in their differential tissue expression and relative expression during development, which could determine their specific functions. Tomosyn-1 might be a general regulator of neural development and neurosecretion, while tomosyn-2 may function to regulate synaptic transmission in a subset of cells specifically. Small structural differences in the hypervariable region could fine-tune their actions by for example intramolecular interactions or post-translational modifications (see sections 8 and 9).

8. FUNCTIONAL DOMAINS AND STRUCTURE OF THE TOMOSYN PROTEIN

8.1 Tomosyn contains multiple conserved domains
Several functional tomosyn domains have been identified, of which the specific roles in and necessity for proper protein functioning is debated (Figure 5). Complexity in this research question is highly increased by the fact that studies cannot be directly compared because of the use of different model organisms, experimental designs and tomosyn homologous proteins. It is clear that tomosyn contains a large N-terminal domain that is homologous to the Lgl and Sro7p/Sro77p family proteins. This region is enriched with conserved WD40 repeats: stretches of about 40 amino acids that end with Tryptophan (W) and Aspartic Acid (D) residues, predicted to fold into a propeller-like structure in yeast Sro7p (Hattendorf et al., 2007). The basic β-propeller structure is conserved in tomosyn, in which 3 extra loop structures extend from the propeller backbone (Figure 5A; Williams et al., 2011). WD40 repeats generally function as a docking platform for interacting proteins. A so-called tail domain that is conserved in tomosyn and Sro7p/Sro77p can be found downstream. In addition, tomosyn contains a C-terminal domain that is homologous to synaptobrevin (Masuda et al., 1998). Yeast Sro7p and Sro77p contain a 40 amino acid C-terminal sequence that could also organize into an α-helical structure, but which is not a conserved SNARE motif. Lgl proteins on the other hand do not harbour tail or coiled coil domains at their C-terminal end (Hattendorf et al., 2007). The proposed functions of the individual tomosyn domains are discussed below.

8.2 Tomosyn is able to bind SNARE proteins via its WD40 and SNARE domains
Tomosyn's C-terminal domain is homologous to the VAMP2 (synaptobrevin) SNARE domain (Figure 5B), is able to bind to syntaxin-1 and SNAP25 α-helices and is thought to inhibit monomeric SNARE complex formation by competition with VAMP2 (Masuda et al., 1998; Hatsuizawa et al., 2003; Pobbati et al., 2004; Sakisaka et al., 2008). Most likely, the absence of a membrane anchor prevents the tomosyn-SNARE complexes from acting as fusogenic SNARE complex analogues.
The tomosyn SNARE motif is sufficient for interaction with full-length syntaxin in *in vitro* pull-down assays (Yizhar et al., 2007) as well as binding to t-SNARE containing liposomes (Yamamoto et al., 2010a). Also in *in vitro* circular dichroism spectroscopy experiments, tomosyn’s SNARE motif is able to form a complex with syntaxin-1 and SNAP25 (Hatsuzawa et al., 2003). In line with an important role for the C-terminus in SNARE protein binding, mutations in this domain strongly reduce syntaxin binding *in vitro* (Constable et al., 2005). Notably though, the SNARE motif of rat tomosyn-1 binds to syntaxin only with relatively low affinity *in vitro* (Yokoyama et al., 1999). Furthermore, Lgl homologs lacking both a SNARE motif and upstream tail domain have retained SNARE binding (Gangar et al., 2005). This indicates that the WD40 domain could be sufficient for SNARE binding as well.

Yeast Sro7p and Sro77p contain a C-terminal sequence that is predicted to form a coiled coil structure. Like full-length Sro7p, a mutant lacking this domain is not capable of Sec9p Qbc-SNARE domain binding *in vitro*. Upon additional cleavage of the tail domain, Sec9p Qbc-SNARE domain binding is induced (Hattendorf et al., 2007). Possibly, intramolecular tail binding to the N-terminal WD40 β-propeller reduces SNARE interaction. Similarly, reduced SNARE protein binding of a tomosyn mutant lacking the SNARE domain (Yizhar et al., 2007) can be explained by the tail domain still being present. Tail binding to the C-terminal VAMP-like domain does not affect binding to t-SNARE containing liposomes, but might affect the strength of this interaction (Yamamoto et al., 2010a). Thus, tomosyn’s C-terminal coiled coil is sufficient for SNARE binding and N-terminal WD40 domains can be sufficient as well, but may be regulated by tail domain interaction.

### 8.3 Tomosyn induces SNARE complex oligomerization *in vitro*

Tomosyn enhances oligomerization of VAMP2/syntaxin-1/SNAP25 complexes *in vitro* (Sakisaka et al., 2008). Its WD40 domain interacts with SNAP25 and syntaxin-1 and regulates such oligomerization. It is yet unclear whether the oligomeric complexes are functional and how they affect secretion. Tomosyn-mediated SNARE complex oligomerization may inhibit neurotransmitter release, but inhibition of EPSP amplitude upon expression of tomosyn N-terminus in SCG cells could also be due to inhibited monomeric SNARE complex formation (Sakisaka et al., 2008). On the other hand, increased stoichiometry of the fusion complex has been suggested to enhance fusion efficiency (Mohrmann et al., 2010). Thus, tomosyn mediates SNARE complex oligomerization *in vitro*, but the *in vivo* significance and mechanism behind this remain elusive. Of note, complexin is also known to induce oligomerization of preassembled SNARE complex *in vitro*. Unlike tomosyn, it requires the presence of SNARE protein transmembrane regions (Hu et al., 2002). It is therefore unlikely that tomosyn-induced oligomerization is mediated by complexin.
8.4 Intramolecular tail binding might regulate tomosyn’s inhibitory function

Although the amount of t-SNARE binding is unaffected, binding of tomosyn’s tail domain (Figure 5) to its C-terminal coiled coil represses its inhibitory activity on monomeric SNARE complex formation and lifts the EPSP amplitude reduction in SCG neurons (Yamamoto et al., 2009). Possibly this induces oligomerization of the SNARE complex (Sakisaka et al., 2008) and in liposome fusion assays it induces displacement of tomosyn by synaptobrevin (Yamamoto et al., 2010a). Binding of the tail domain to the N-terminal WD40 repeats restores the inhibitory function of the C-terminus on monomeric SNARE complex formation and EPSP amplitude reduction in SCG cells. The affinity of the tail domain for the N-terminal region is higher than for the C-terminal region (Yamamoto et al., 2009). Thus, tail binding may balance different functional modes of tomosyn. N-terminal WD40 domain-mediated inhibition could be stronger than inhibition of monomeric SNARE complex formation by the C-terminal domain, as suggested by faster restoration of reduced EPSCs in SCG neurons (Sakisaka et al., 2008). Also tomosyn-mediated inhibition of calcium-induced growth hormone secretion from PC12 cells is more prominent with a SNARE motif mutant, although the implications for SNARE complex formation are unclear (Constable et al., 2005).

8.5 Tomosyn’s inhibitory function could involve binding to myosin-V

Yeast tomosyn homolog Sro7p binds to the IQ motif within the neck domain of type V myosin, an interaction for which its very N-terminal amino acids, even upstream of WD40 repeats, are crucial (Rossi and Brennwald, 2011). For Drosophila Lgl, the N-terminal WD40 repeats are necessary for membrane binding, but association with the cytoskeletal myosin-II occurs via its C-terminus (Betschinger et al., 2005). Rat syntaxin-1A and the neck domain of myosin-Va interact in a Ca^{2+}-dependent manner. Upon blockade of this interaction with an injected anti-myosin-V neck antibody, reduced chromaffin cell catecholamine secretion upon stimulation with high potassium is observed, measured by amperometry (Watanabe et al., 2005). Since syntaxin and tomosyn bind to the same myosin domain, tomosyn might bridge myosin-V by N-terminal binding and syntaxin by binding with its C-terminal SNARE domain. As such, this interaction could be required for plasma membrane targeting of a myosin-V containing vesicle. Since they are now in close proximity, initiation of vesicle fusion by the calcium-dependent syntaxin/myosin interaction can be rapidly induced upon calcium elevation.
8.6 Full-length protein is required for tomosyn function

The tomosyn-1 C-terminal SNARE domain is unessential and not sufficient by itself for the inhibition of vesicle fusion, measured by capacitance changes in chromaffin cells upon flash photolysis of caged calcium (Yizhar et al., 2007). High K⁺ evoked human growth hormone secretion from PC12 cells is inhibited to a similar extent upon expression of either full-length or C-terminal truncated tomosyn (Gladycheva et al., 2007). Furthermore, EPSP amplitudes in SCG cells are reduced upon expression of a C-terminal truncated tomosyn mutant lacking tail and coiled coil domains (Yamamoto et al., 2010b). Also oligomerization of the SNARE complex in vitro occurs in the absence of these domains (Sakisaka et al., 2008). The tomosyn VAMP-like domain is sufficient however to potently induce inhibition of SNARE-mediated liposome fusion (Yamamoto et al., 2010a).

Yeast Sro7p/Sro77p C-terminal coiled coil is also dispensable for its function, since growth defects in Sro7p/Sro77p deletion mutants can be rescued with mutant Sro7p, albeit to lesser extent than full-length Sro7p. Also post-Golgi vesicle secretion seems slightly reduced upon rescue with the C-terminal mutant, compared to wildtype Sro7p re-expression. This indicates that the Sro7p α-helical C-terminus is not required, but does contribute to its function. Expression of a C-terminal deletion mutant additionally lacking the tail domain in sro7p/sro77p mutants is unable to rescue growth inhibition. Also, enhanced internal Bgl2 enzyme remaining inside spheroplasted cells, reflecting post-Golgi vesicles, is seen, further confirming the necessity of the C-terminal tail domain for the permissive yeast Sro7p function (Hattendorf et al., 2007).

The entire N-terminal region of tomosyn-1 is required for tomosyn's inhibitory effect on capacitance increase upon calcium uncaging in chromaffin cells (Yizhar et al., 2007). In C. elegans, both the N- and C-terminal domains of an intact tomosyn protein are indispensable for proper NMJ vesicle fusion inhibition, despite correct synaptic localization of mutant tomosyn fragments (Burdina et al., 2011). In a liposome fusion assay, expression of the WD40 domains alone is insufficient for inhibition of liposome fusion (Yamamoto et al., 2010a, 2010b).

Loop 1 and 3 extending from the β-propeller backbone structure are essential for inhibition of potassium-induced growth hormone secretion from PC12 cells. Loop 2, which contains the hypervariable region and various regulatory elements (Figure 5), seems not crucial for this (Williams et al., 2011).

Thus, it is likely that the full-length tomosyn sequence is required for its full inhibitory potential. Both the N-terminal WD40 and C-terminal coiled coil domain are able to bind SNARE proteins, mediated by the tail domain. Possibly, the tail domain thereby balances several tomosyn regulated mechanisms: SNARE complex oligomerization and/or vesicular myosin-V binding by the N-terminal β-propeller
upon C-terminal binding of the tail domain and inhibition of monomeric SNARE complex formation upon tail binding to the N-terminus. These mechanisms are not necessarily all inhibitory, or only temporarily. SNARE complex oligomerization and myosin-V binding could function to increase fusion efficiency by increasing the number of SNARE complexes available for fusion or providing a local supply of vesicles near active SNARE complexes respectively. Binding to monomeric SNARE complexes may function to provide a steady supply of stabilized SNARE proteins that can be activated for fusion upon tomosyn replacement by synaptobrevin. Net inhibition by full-length tomosyn may prevent premature vesicle fusion.

9. POST-TRANSLATIONAL MODIFICATIONS OF TOMOSYN

9.1 Disinhibition of synaptic release by PKA phosphorylation of tomosyn

In addition to splice variation, tomosyn’s function is modulated by post-translational modifications. Tomosyn-1 is phosphorylated by protein kinase A (PKA) on tomosyn-m1 serine S724 (Figure 5B). Phosphorylation of this residue reduces tomosyn’s interaction with syntaxin and lifts its inhibitory role on monomeric SNARE complex formation. By this mechanism, neurotransmitter release from SCG neurons is upregulated by a PKA-induced increase of the RRP size (Baba et al., 2005). Also, tomosyn phosphorylation increases oligomerization of SNARE complexes in vitro (Sakisaka et al., 2008). Possibly, tomosyn phosphorylation switches intramolecular tail-binding preference from N-terminal binding to C-terminal binding. Since activation of the PKA pathway is involved in synaptic plasticity (Nguyen and Woo, 2003), such regulation of tomosyn function could be important during prolonged activity. PKA activity depends on levels of cyclic AMP (cAMP), which can be raised by forskolin treatment. The stimulation of synaptic release by forskolin in Drosophila NMJ is tomosyn-dependent, further confirming a role for PKA phosphorylation of tomosyn in regulation of vesicular transmitter release (Chen et al., 2011).

In insulin-secreting mouse beta-cells and growth hormone co-transfected INS-1E cells however, forskolin-mediated exocytosis increase seems to be (partially) independent of tomosyn, assessed by both overexpression and knockdown experiments. Additional or different pathways might be responsible for the forskolin effect in this type of cells. Also, in overexpression experiments forskolin enhancement of release is not significant and aspecific RNAi effects cannot be excluded. Tomosyn overexpression might require a higher concentration of forskolin to phosphorylate all tomosyn molecules (Zhang et al., 2006). INS-1E cells stimulated with forskolin (and glucose, potassium, IBMX) did show a reduction in exocytosis enhancement upon knockdown of tomosyn. This is in line with forskolin relieving tomosyn inhibition, but additional controls are desired (Cheviet et al., 2006).
Most likely, activity-dependent PKA phosphorylation of tomosyn reduces its affinity for syntaxin, a mechanism by which secretory inhibition could be lifted upon prolonged activity.

9.2 Possible concerted actions of tomosyn SUMOylation and phosphorylation

Only six amino acids downstream of the PKA phosphorylation site, tomosyn-1 can be SUMOylated on lysine K730 (Figure 5B). Functionally, SUMOylation reduces the inhibitory activity of tomosyn-1, as suggested by enhanced inhibition of growth hormone secretion in PC12 cells that overexpress a tomosyn SUMOylation mutant (Williams et al., 2011). It is not yet known via what mechanism such disinhibition is established, although its syntaxin binding is not affected. Interestingly, long term potentiation is correlated with increased protein SUMOylation (Craig and Henley, 2012) and protein phosphorylation has been suggested to regulate SUMOylation (Yang et al., 2003; Hietakangas et al., 2006; Chamberlain et al., 2012). Thus, concerted actions of tomosyn phosphorylation and SUMOylation could reduce tomosyn-mediated secretory inhibition and affect synaptic strength. It is not clear though whether the tomosyn phosphorylation and SUMOylation events happening in such close proximity are actually influenced by each other.

These two post-translational modification sites are present in the big splice variant of mouse and rat tomosyn-1, but absent in the smallest variant (Figure 5B). In humans, the sites are present in both of the tomosyn isoforms listed in GenBank. The residues are not conserved in any tomosyn-2 species. Moreover, the sites are not present in any isoforms of either Lgl or tomosyn in C. elegans or Drosophila, neither is the sequence conserved in yeast Sro7p and Sro77p homologs. This suggests phosphorylation and SUMOylation of tomosyn is not an intrinsic property, necessary for its basal function in exocytosis, but could serve as an additional layer of regulation in mammals.

All in all, it seems likely that presynaptic plasticity in mammals is mediated by disinhibition of mechanisms involving tomosyn-1, regulated by both PKA-induced phosphorylation and SUMOylation.

9.3 Spatial regulation of tomosyn's inhibitory strength by syntaxin phosphorylation

Post-translational modifications of tomosyn-interacting proteins could further affect its function. An example of this is phosphorylation of syntaxin by the Rho-GTPase/ROCK pathway. Axon outgrowth and growth cone dynamics are regulated by the actomyosin-based cytoskeleton, which is mediated by Rho-associated serine/threonine kinases (ROCK) and its activator Rho small G protein. Activation of Rho/ROCK induces growth cone collapse and neurite retraction (Bito et al., 2000; Da
This system has been linked to the inhibitory effect of tomosyn on neurite outgrowth. Activated ROCK phosphorylates syntaxin-1 at serine residue 14, thereby increasing the affinity of syntaxin-1 for tomosyn and reducing the availability of functional SNARE complexes for plasmalemmal vesicle fusion at the tip of the growth cone (Sakisaka et al., 2004). Also in chromaffin cells, activation of the Rho-GTPase/ROCK signalling pathway increases tomosyn association with plasma membrane SNARE complexes. RhoA/ROCK-induced phosphorylation enhances cycling of tomosyn-SNARE complexes, since inhibition of SNARE complex recycling by NEM treatment increases the amount of membrane targeted tomosyn only when phosphorylation is induced. Enhanced interaction of tomosyn with syntaxin is mediated by the VAMP-like domain, since growth hormone secretion from high K\(^+\)-stimulated PC12 cells upon expression of a C-terminal truncated mutant is not further reduced by activation of the RhoA/ROCK pathway (Gladycheva et al., 2007). Thus, syntaxin phosphorylation by the RhoA/ROCK pathway could be involved in the spatial regulation of tomosyn-mediated inhibition of vesicle fusion during neurite outgrowth.

9.4 PKC phosphorylation is involved in autoinhibition of Lgl specifically
The tomosyn homolog Lgl is phosphorylated by atypical protein kinase C on three conserved serine residues in a positively charged, presumably unstructured region just downstream of the WD40 domain \textit{in vitro} (Drosophila Lgl residues 656, 660 and 664). The negatively charged phosphate groups interact with the positively charged amino acids, leading to a less flexible conformation in which the N-terminus binds to the C-terminus. Such autoinhibition of the protein releases its association with the membrane and cytoskeletal myosin-II heavy chain (Betschinger et al., 2003; Betschinger et al., 2005). The serine residues that are subject to aPKC phosphorylation in Lgl are not directly conserved in tomosyn homologs. Therefore, a similar autoinhibitory mechanism probably does not exist for tomosyn.

Thus, PKA phosphorylation and SUMOylation of tomosyn as well as phosphorylation of syntaxin mediate tomosyn’s inhibitory strength. To fully understand signalling processes that govern tomosyn-dependent regulation of secretion, it is essential to understand the full functional consequence of these modifications and find novel modifications. Other modification events may also occur within the hypervariable region of tomosyn, making it a unique regulatory element within an otherwise well conserved protein.
10. DISEASES ASSOCIATED WITH TOMOSYN DYSFUNCTION

Tight spatial and temporal regulation of SNARE complex formation and maturation is key to dictate secretion events in an ordered manner, for example during neurite outgrowth and synaptic plasticity. These mechanisms thereby ensure the correct development and adult functioning of the brain. Mutations in tomosyn affecting its function or altered expression levels could disturb this balance and cause disease. Deregulated tomosyn protein could be involved in autism. A 260 kb deletion in the tomosyn-1 gene is found in a boy diagnosed with autism, mental retardation and seizures. His mother, affected with bipolar disorder, is carrying the same deletion. The deletion is not present in a brother with mild autism and in an unaffected sister (Davis et al., 2009). In a separate study, a patient with autism and a coloboma of the eye is found to carry a partly silenced amisyn gene on chromosome 14 by formation of a mosaic ring chromosome by genetic translocation (Castermans et al., 2008). This haploinsufficiency could lead to altered secretion of large dense-core vesicles. IBMX/forskolin-induced secretion of the neuropeptide FLAG-AGRP in the pancreatic beta-cell line β-TC3 upon amisyn overexpression and knockdown is reduced and enhanced respectively. Moreover, dense-core granules in blood platelets from patients with an affected amisyn gene seem more irregular (Castermans et al., 2010). This is in line with elevated blood platelet serotonin levels in many autism patients, possibly involved in early brain development (Janusonis, 2008). Thus, reduced large dense-core vesicle secretion might give rise to the autism disease phenotype.

Outside of the brain, tomosyn-1 may regulate von Willebrand factor (VWF) concentration. VWF is a thrombogenic molecule released from endothelial cells and blood platelets. Cardiovascular disease and the bleeding disorder von Willebrand disease (VWD) are associated respectively with elevated and reduced VWF plasma levels (Reviewed in Lenting et al., 2012). Human SNPs in the gene encoding for tomosyn-1 are correlated with lower VWF plasma levels and increased bleeding score. Two of these SNPs, rs1039084 and rs9399599, are in high linkage disequilibrium. The first might be the causal variant, since it constitutes a mild amino acid change from the polar asparagine to the polar serine at tomosyn-1 residue 436 in one of the WD40 domains, not conserved in mammalian tomosyn-2 (Figure 5), while the latter is intronic. Additionally, rs9390459 is a silent SNP in mammalian tomosyn-1b lysine residue 816 (Figure 5; van Loon et al., 2010; Antoni et al., 2011; Smith et al., 2011; van Loon et al., 2012). Further in line with an involvement in thrombosis, secretion from tomosyn-1 deficient mouse blood platelets is reduced, giving rise to a bleeding phenotype in these animals (Ye, 2012). Tomosyn-1 could thus be involved in cardiovascular risk and VWD by regulation of VWF secretion.
Furthermore, tomosyn-1 (Zhang et al., 2006) and tomosyn-2 (Bhatnagar et al., 2011) are both implicated in insulin secretion from pancreatic beta-cells. A SNP increasing the stability of tomosyn-2 protein seems to confer the susceptibility to type 2 diabetes in obese mice of the BTBR strain. This G>A SNP (rs4173885), found on the tomosyn-2 complementary strand and associated with a non-synonymous polar to hydrophobic tomosyn-xb2 S937L mutation in a WD40 motif (Figure 5), occurs in multiple strains. The serine residue is not conserved in mammalian tomosyn-1. Overexpression of tomosyn-b2 inhibits insulin secretion in INS1 pancreatic beta-cells upon high glucose stimulation, supporting causality between increased protein stability and mRNA levels and reduced insulin secretion in these mice. It is not yet clear whether this SNP is correlated with susceptibility to type 2 diabetes in other strains, as well as humans. This does give an indication however of tomosyn-2 function specifically in insulin secretion and diabetes (Bhatnagar et al., 2011).

Thus, genetic mutations in tomosyn have been suggested to be involved in altered secretion of serotonin, von Willebrand factor and insulin, contributing to respectively autism, thrombosis and diabetes. Because of the clear role of tomosyn in regulation of vesicular neurotransmitter release, tomosyn mutations are likely to contribute also to defective neurotransmission in neurological diseases. Besides secretion, tomosyn (homolog) mutations might affect neuronal development. For example, Lgl is suggested to regulate transport of fragile X mental retardation 1 (Fmr1) protein during neural development in flies and mice. Since fragile X mental retardation is caused by loss of function of this gene, loss of a functional interaction between Lgl and Fmr1 could contribute to this disease (Zarnescu et al., 2005). No Lgl mutations have yet been found however in Fragile X patients. Yet unidentified SNPs in proteins indirectly affecting tomosyn function, such as the regulatory proteins suggested above to balance and regulate tomosyn function - Munc18, Munc13, synaptotagmin, myosin-V, PKA, SUMOylation proteins - might additionally contribute importantly to disease. Therefore, in order to fully understand the diseased and the healthy functioning of the human body, it is crucial to know more about tomosyn’s function in the various secretory pathways, the mechanisms regulating its activity in time and space and how the disease mutations affect these pathways specifically.

11. THESIS AIMS

As outlined above, tomosyn is an important regulator of vesicular secretion in endocrine cells, as well as neurons. In general, tomosyn is suggested to be a negative regulator of release. It may balance the level of SNARE complex formation and maturation induced by positive regulators of neurotransmitter release, such as Munc18, Munc13 and synaptotagmin. Spatial and temporal regulation of the amount of inhibition by tomosyn is likely to be important for key processes in the
brain, such as neurite outgrowth and synaptic plasticity. Such regulation of tomosyn may occur in various (activity-dependent) ways. First, tomosyn inhibitory strength is mediated by post-translational modification of tomosyn, such as phosphorylation and SUMOylation. Furthermore, intramolecular tail binding might balance several tomosyn functions, such as SNARE complex formation and oligomerization. So far most of our knowledge about tomosyn function comes from in vitro studies and in vivo experiments in the nematode C. elegans. To enhance our understanding of the mammalian brain it is essential to assess the function of both isoforms in a mammalian nervous system such as that of the mouse.

This thesis aims to further unravel tomosyn's function in mammalian neural development and secretion by in vivo behavior experiments and in vitro studies of synaptic function, protein localization and cellular morphology. For this purpose, an existing knockout mouse model of tomosyn-1 and a newly generated model of the scarcely examined isoform tomosyn-2 were used. In addition, a novel protein-protein interaction was analyzed to acquire more clues about the role of tomosyn within the overall network of proteins functioning together in a cell.

Tomosyn-1 is studied in chapter 2. Tomosyn-1 deficient mice showed reduced survival, already prenatally, indicative for a crucial role of the protein in early development. We found that in autaptic hippocampal neurons tomosyn-1 was not required for neurite outgrowth, possibly because of tomosyn-2 redundancy. Interestingly however, dendritic and synaptic Munc18 levels were decreased, suggesting that tomosyn-1 stabilizes Munc18 levels. This might be mediated by competition of Munc18 and tomosyn for syntaxin binding. Electrophysiological analysis of these cells indicate a positive function of tomosyn-1 in sustained release. Reduced Munc18 levels may attribute to this phenotype. Tomosyn-1 and Munc18 in these cells could function together to uphold a pool of vesicles for fusion upon activity-induced tomosyn disinhibition.

Chapter 3 focuses on the tomosyn-2 isoform. We generated tomosyn-2 knockout mice and validated tomosyn-2 deficiency on both mRNA and protein level. These animals exhibited a sublethal phenotype that was accompanied by decreased blood clotting activity and degeneration of the nervous system. Surviving adult tomosyn-2 knockout mice developed a normal body weight, but showed decreased sensorimotor gating, motor coordination and muscle strength. This could not be attributed to neural development defects, since morphology of cultured neurons was normal. Hippocampal autaptic neuron electrophysiology did not indicate general synaptic transmission impairments. However, spontaneous neurotransmitter release in the NMJ was enhanced in tomosyn-2 deficient mice. During prolonged simulation, depression of NMJ transmission was more prominent in these animals. Possibly, initial release inhibition by tomosyn-2 functions to ensure sufficient, fast
supply of additional vesicles required for sustained release. Tomosyn-2 inhibition may be relieved upon high demand.

Tomosyn overexpression localization is described in chapter 4, showing that it localized not only to cytosol of wildtype hippocampal neurons, but also targeted into puncta. These puncta moved and co-localized with various presynaptic and vesicular markers. Also tomosyn on moving puncta often overlapped with the synaptic vesicle marker synapsin and the large dense-core vesicle marker NPY, suggesting that tomosyn migrates with both types of vesicles. Since tomosyn does not contain a membrane-binding motif by itself, it may bind to vesicles through interaction with vesicular proteins. Synaptotagmin-1 was excluded for this. Vesicular tomosyn targeting may function to efficiently localize tomosyn to sites of vesicular release and/or affect vesicular release characteristics.

A protein can dynamically diversify and fine-tune its function by post-translational modifications, such as the conjugation of a SUMO peptide. In chapter 5 we show that tomosyn-1 is subject to SUMOylation by SUMO-2/3 specifically. In order to find interactors that could mediate this post-translational modification, we performed yeast two-hybrid screens and identified the SUMO E3 ligase PIASγ as a novel interactor. The interaction was validated with immunoprecipitation experiments. The significance of PIASγ-mediated SUMOylation of tomosyn-1 for the regulation of synaptic plasticity remains to be elucidated.

The main findings in this thesis are summarized in chapter 6. Additionally, the results are discussed and placed in the context of the existing literature to provide an updated model of tomosyn function.